Proceedings of The National Conference
On Undergraduate Research (NCUR) 2016
University of North Carolina Asheville
Asheville, North Carolina
April 7-9, 2016

D-limonene Increases Efficacy of Rifampicin as an Inhibitor of In Vitro Growth of Opportunistic *Staphylococcus epidermidis* RP62A

Natcha Rummaneethorn and Charlene Mae Caoili
Department of Biology
Manhattanville College
2900 Purchase Street
Purchase, New York 10577 USA

Faculty Advisors: Dr. Anna K. Yeung-Cheung, Dr. Christopher J. Pappas

Abstract

An increase in nosocomial infections due to *Staphylococcus epidermidis* has been a recent focus in the medical field. At the molecular level, the *ica* gene locus is responsible for biofilm formation in *S. epidermidis*, which contributes to its pathogenicity. Current strategies in treating biofilm-forming pathogens include antibiotics. To reduce antibiotic resistant bacteria populations, focus has shifted to naturally derived antibiotic alternatives. D-limonene is a terpenoid-based essential oil, found in citrus fruits, that has previously been shown as an effective antimicrobial agent against various related bacteria, such as *S. aureus*. Yet, its antimicrobial effectiveness has not been thoroughly studied in the biofilm-forming *S. epidermidis* strain ATCC 35894 (RP62A). Thus, the objective of this research was to study the antimicrobial effectiveness of D-limonene on *S. epidermidis* RP62A in comparison to the parental strain ATCC 12228. Growth studies of *S. epidermidis* strains ATCC 12228 and RP62A treated with various concentrations of D-limonene, rifampicin control, and a D-limonene-rifampicin drug cocktail were performed. Preliminary results indicate the inhibition of *S. epidermidis* ATCC 12228 when exposed to 305.6 mg/L of D-limonene. Interestingly, no effect was observed in *S. epidermidis* RP62A growth for studied concentrations of D-limonene. Inhibition of *S. epidermidis* ATCC 12228 was observed when exposed to the rifampicin control. However, *S. epidermidis* RP62A was observed to resume its normal growth after 16 hours of exposure to the rifampicin control. Inhibition of both strains was observed when exposed to the D-limonene-rifampicin-drug cocktail. With these results, it is hoped that the D-limonene-rifampicin drug cocktail will be further evaluated for its value in clinical settings to prevent initial nosocomial *Staphylococcus* infections. Future studies include obtaining the minimum inhibitory concentration (MIC) of the drug cocktail in both strains, as well as investigating the role of the *ica* locus in *S. epidermidis* RP62A's D-limonene resistance. The significance of this research is the potential clinical use of D-limonene as a naturally derived antimicrobial agent against hospital-acquired *S. epidermidis* infections.

Keywords: antibiotic susceptibility, D-limonene, *Staphylococcus epidermidis,* biofilm formation

1. Introduction

Staphylococcus epidermidis is a Gram-positive bacterium that belongs to the family *Staphylococcaceae*[37]. It is commonly found on human skin and classified as coagulase-negative *Staphylococci* because of its inability to clot blood plasma[37,58]. Furthermore, it is considered as the most common coagulase-negative *Staphylococci* species responsible for major nosocomial infections[37]. It was originally considered non-pathogenic due to its commensal behavior[37]. However, its pathogenicity is attributed to being the primary agent in causing infection in immunocompromised patients and individuals that maintain indwelling medical devices for a

prolonged period of time[40,53]. The ability that *S. epidermidis* is able to produce a slime-like substance, or biofilm, has led to greater adherence to biomaterial and difficulty with treatment of these infections[37]. Due to biofilm, the enclosed bacteria are protected from detrimental external conditions, such as antibiotics[5,37]. This enables the bacteria to survive and the recurrence of infection when cells detach[37]. Under chronic conditions, these infections can result in sepsis and death[10,47].

A biofilm is a population of bacterial cells that is enclosed in a self-produced extracellular polymeric substance (EPS)[37]. It is composed of 85% extracellular matrix components such as polysaccharides, proteins, enzymes, DNA, bacterial glycolipids, and water, and 15% of bacterial cell aggregates[67]. The five-step process involved in biofilm production requires conditioning film, reversible bacterial adhesion, irreversible adhesion and matrix formation, biofilm formation, and bacterial cell detachment[37]. Biofilm production is attributed to the *icaADBC* locus in *S. epidermidis*, as well as other *Staphylococcus* species[33]. The *icaADBC* locus[11] is located between nucleotides (NT) 2334220 and 2337647[26]. The locus consists of four intercellular adhesion (*ica*) genes - *icaA*, *icaD*, *icaB*, and *icaC* genes, in order of gene location from upstream to downstream[37]. The *icaA* gene (SERP2293) is located at NT2334220_2335458[26]. The *icaD* gene (SERP2294) is located at NT2335422_2335727[26]. The *icaB* gene (SERP2295) is located at NT2335724_2336593[26]. The *icaC* gene (SERP2296) is located at NT2336580_2337647[26]. Researchers believe the expression of the *icaA* gene is partly responsible for the biofilm production in many biofilm-producing bacteria, including *Staphylococcus epidermidis*[6].

In regards with the relationship between biofilm production and the presence of the *icaADBC* locus, co-transcription of the *icaA* and *icaD* genes are known to be responsible for an enhanced biofilm production[37]. It is unknown whether the *icaB* and *icaC* genes are also co-transcribed along with the *icaA* and *icaD* genes, however, the *icaB* and *icaC* are believed to be involved with regulating and working together with the *icaA* and *icaD* genes[37]. Although co-transcription of the *icaA* and *icaD* genes can increase biofilm production, *S. epidermidis* can still create a biofilm with expression of the *icaA* gene alone[37]. The protein product of the *icaA* gene is N-glycosyltransferase, which is homologous to the transmembrane protein N-acetyl-glucosaminyltransferase that is responsible for creating glycosidic linkages between cell wall components[67]. The function of the *icaD* gene is likely a link between the *icaA* and *icaC* genes[19], which is more similar to a supporter gene. While the functions of the *icaA* and *icaD* genes are more or less defined, the functions of the *icaB* and *icaC* genes are unknown[37]. However, it is hypothesized that the product of the *icaB* gene may perform as a transporter of the *icaA* gene product whereas an expression of the *icaC* gene might produce an integral membrane protein that functions in exporting the *icaA* gene product out of the cell for biofilm construction[37]. The expression of the *icaADBC* locus is regulated by a repressor, which is encoded from the transcription of the *icaR* gene[19]. Although the *icaR* gene is responsible for the regulation of the *icaADBC* locus, the loci's expression is mainly controlled by the global stress response factor known as sigma factor B, *rpoB* (SERP0183, NT185929_189480), because this sigma factor positively regulates the expression of the *icaADBC* locus by negatively regulating the expression of the *icaR* gene[27]. Staphylococcal accessory regulator A, *sarA*, and regulator of *rpoB*, *rsbU*, is also partly responsible for indirectly regulating the expression of the *icaADBC* locus with a similar mechanism[27].

Biofilm formation is dependent on the production of polysaccharide intercellular adhesion (PIA) polymers[11]. The synthesis of PIA recruits proteins that are encoded by the *icaADBC* gene locus[11]. Thus, expression of the *ica* locus is attributed as the major constituent for the conversion of *S. epidermidis* strains from commensalism to pathogenicity[32,65]. A majority of genes that encode for adhesion are found to be intact in the wild type non-biofilm forming and non-infectious strain, *S. epidermidis* ATCC 12228; however, the *ica* operon is not present in the wild-type strain[6,11]. In contrast**,** *S. epidermidis* ATCC 35984 (RP62A), a biofilm-forming strain, contains the *icaADBC* operon[6,33].

Previous studies have caused commensal *S. epidermidis* strains to become virulent in in vivo animal models**,** with the transformation of the *ica*-absent strain into the *ica*-present strain by inserting the *ica* locus into the commensal strain[32,49-52]. In a rat central venous catheter (CVC)-associated infection model[49], CVC-associated infections with metastasis of the disease were statistically different amongst the population of the *ica*-locus expressed subjects than those exposed to the parental wild-type strains[32]. This enclosed EPS barrier increases the bacteria's resistance to antimicrobial agents by at least 10 to 1000 times, leading to greater difficulty of removal from surfaces[27,36,39].

Furthermore, previous studies have found the formation of biofilms is not restricted to forming on moist/wet hard surfaces, but as well as on dry and fabric surfaces[36,39]. *S. aureus* and *S. epidermidis* are part of the *Staphylococcus* species of Gram-positive cocci bacteria[2]. Both have been identified to possess the *ica* locus, as well as agents in causing nosocomial infections[2,11,56]. *S. aureus*-associated infections, however, are treated

with greater emphasis in the clinical setting due to a higher morbidity and mortality than compared to *S. epidermidis*[11]. The viability of multiple-drug resistant *S. aureus* was examined from five common hospital surfaces: smooth 100% cotton, 100% cotton-terry, 60% cotton/40% polyester, 100% polyester, and 100% polypropylene plastic[36]. These microorganisms were found to linger on surfaces, such as scrub suits and stretchers, even after drying; this emphasizes the importance of thorough contact procedures and disinfection that are needed in the hospital setting[36].

Antibiotics are the current method of treatment against *Staphylococcus* infections. Previous studies have shown that biofilm-producing bacteria can be resistant to synthetic antibacterial agents such as tetracycline and gentamycin[10]. One of the most common antibiotics used to treat hospital-acquired infections caused by *S. epidermidis* is rifampicin[16]. It is a well-established treatment against various other diseases, such as tuberculosis[45], Hansen's disease, and AIDS-associated mycobacterial infections[21,46]. Rifampicin is an antibiotic belonging to the rifamycin class[21]. It targets specifically the DNA-dependent RNA synthesis of the bacterium[21]. It interferes with the initiation phase of the RNA synthesis by modifying the *rpoB* gene, which prevents the interaction between the DNA-dependent RNA polymerase and its respective promoter site from occurring because the *rpoB* gene codes for a sigma factor of the DNA-dependent RNA polymerase[21]. From previous literature, it had been shown that rifampicin is able to penetrate the biofilm of *S. epidermidis* RP62A, but it cannot effectively inhibit or obliterate the growth of the RP62A strain[18,69]. Normal growth is observed after approximately 12 hours of exposure to rifampicin[69]. Thus, even though rifampicin is a well-established antibiotic used to treat *Staphylococcus* infections in clinical settings, it is ineffective in the treatment of *Staphylococcus* infections caused by *S. epidermidis* RP62A[69].

In an effort to combat antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), the prescription of antibiotics has become more regulated. Aside from synthetic antibiotics, biofilm-producing bacteria can also be resistant to natural antimicrobial agents such as essential oils[14,19-20,38,57-58]. D-limonene is a terpenoid primarily found as a major component derived from extracted citrus oils such as orange and lemon[49,58]. The use of D-limonene products itself accounts for approximately 92% of the total annual volume reported in the United States[1]. The daily per capita intake of D-limonene in the U.S. was reported in 2011 as 27,906 µg/person[1]. Previous studies on *Escherichia coli*[8], *Staphylococcus aureus*[19,49], and *Bacillus cereus*[49], have shown D-limonene to be effective as a natural antimicrobial bacteriostatic and bactericidal agent[18]. Its antibiotic property is exhibited through its catabolic mechanism on the bacterium's plasma membrane[8]. It is shown that D-limonene can cause membrane toxicity in bacteria[14] by changing the fatty acid composition of the phospholipid bilayer membrane[15]. Its citrus-like scent and low toxicity level has led to its incorporation in disinfectants as well as a proposed food preservative[19,58-59].

Although D-limonene has been shown to possess an ability to inhibit bacterial growth in many bacterial strains, there has been no research today on testing D-limonene's effect on biofilm-producing *S. epidermidis*. Thus, the aim of this study is to test the effect of D-limonene as an antimicrobial agent against biofilm-forming *S. epidermidis* RP62A in comparison to the parental strain ATCC 12228. Since it has been shown in previous studies that D-limonene can effectively inhibit the growth of various bacterial species, it is hypothesized that D-limonene will also be able to effectively inhibit the growth of biofilm-forming *S. epidermidis* RP62A. The significance of this research is the potential clinical use of D-limonene alone or in combination with antibiotic against hospital-acquired *S. epidermidis* infections.

2. Materials and Methods

2.1 Strains and Growth Conditions

S. epidermidis strains ATCC 12228 and RP62A were purchased from ATCC (Manassas, VA). The strains were grown in an aerobic atmosphere at 37°C in Trypticase Soy Agar (TSA) and/or Broth (TSB). They were grown in TSB with or without D-limonene and rifampicin at various concentrations as described below, both purchased from Sigma-Aldrich (St. Louis, MO). The bacterial concentrations of both strains were determined using the DU®720 General Purpose UV/Vis Spectrophotometer (Beckman Coulter, Pasadena, CA) at OD_{600}.

2.2 Growth Analysis of *S. epidermidis* strains ATCC 12228 and RP62A

Overnight cultures of ATCC 12228 and RP62A were diluted to 10^6 CFU/mL, which correlates with about OD_{600} = 0.02, by adding to 50 mL of fresh TSB containing varying concentrations of D-limonene (76.4 mg/L, 152.8 mg/L, 229.2 mg/L, 305.6 mg/L, 382 mg/L, and 458.4 mg/L). Additionally, each strain was diluted in 50 mL of TSB containing a combination of 382 mg/L of D-limonene and 50 µg/mL of rifampicin. As controls, each strain was simultaneously grown in TSB only, and also in 50 mL of TSB containing 50 µg/mL of rifampicin for each trial performed. All other controls were also grown with the treatment groups simultaneously. The flasks were then incubated overnight at 37°C without agitation. The growth of both strains was measured at OD_{600} in two-hour intervals from 0 to 24 hours, for each condition.

2.3 Statistical Analysis

One-way ANOVA with Tukey's Honest Significant Difference Post-Hoc tests were conducted using VassarStats (http://www.vassarstats.net/anova1u.html). The samples were run as independent variables and weighted. Statistical difference was defined as significant when $P < 0.01$.

3. Results

3.1 D-Limonene Susceptibility

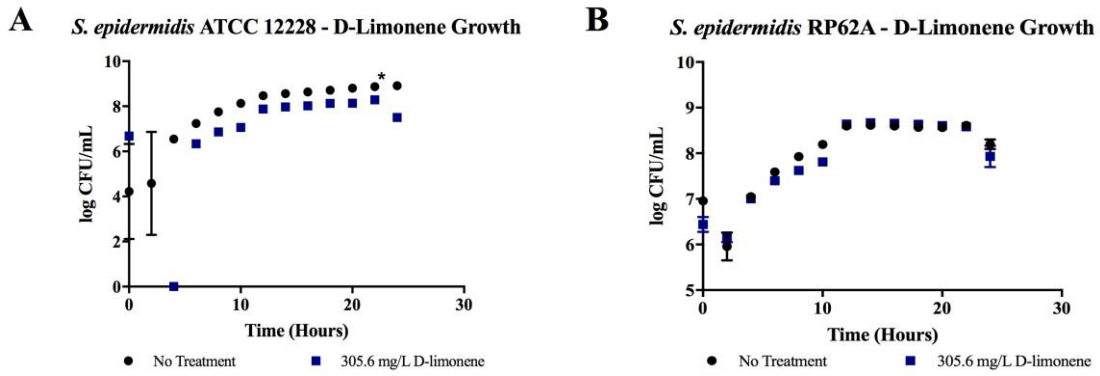

Figure 1. Growth analysis when exposed to D-limonene

Figure 1A. Growth curve demonstrating the growth of *S. epidermidis* ATCC 12228 under exposure to 0 mg/L (no treatment) and 305.6 mg/L of D-limonene. Figure 1B. Growth curve demonstrating the growth of *S. epidermidis* RP62A under exposure to 0 mg/L (no treatment) and 305.6 mg/L of D-limonene.

Both curves were constructed with OD_{600} values from two data trials (trial 3 = 0, 2, 4, 6, 8, 10, 24 hours, trial 4 = 0, 12, 14, 16, 18, 20, 22, 24 hours). Difference in the stationary phase of the ATCC 12228 strain and the RP62A strain under exposure to 0 mg/L of D-limonene (no treatment) is not significant. Difference in the stationary phase of ATCC 12228 between 0 mg/L of D-limonene (no treatment) condition and 305.6 mg/L of D-limonene condition is significant ($P < 0.01$, one-way ANOVA). Difference in the stationary phase of RP62A between 0 mg/L of D-limonene (no treatment) condition and 305.6 mg/L of D-limonene condition is not significant. Difference in the stationary phase of the ATCC 12228 strain and the RP62A strain under exposure to 305.6 mg/L of D-limonene is significant ($P < 0.01$, one-way ANOVA).

Growth analysis indicates that the growth rate of ATCC 12228 was reduced when exposed to D-limonene concentrations at 305.6 mg/L (Figure 1A). Exposure to D-limonene concentrations above 305.6 mg/L were

similar in growth to 305.6 mg/L of D-limonene (data not shown). However, exposure to D-limonene concentrations below 305.6 mg/L did not affect the growth rate of ATCC 12228, and thus no difference in growth was observed from the no treatment condition (data not shown). The stationary phase of ATCC 12228 between 0 mg/L of D-limonene (no treatment) condition and 305.6 mg/L of D-limonene condition is significantly different from each other ($P < 0.01$). Furthermore, its doubling time under exposure to 0 mg/L of D-limonene (no treatment) was 1.99 hours, but its doubling time under exposure to 305.6 mg/L of D-limonene was 11.24 hours. The growth rate of the ATCC 12228 strain under exposure to 305.6 mg/L of D-limonene was reduced almost 6 times when compared to its growth rate under exposure to 0 mg/L of D-limonene (no treatment). This shows that the doubling time of the ATCC 12228 strain increased in the presence of D-limonene at and above 305.6 mg/L. Thus, both are suggestive that D-limonene acts as a moderate bacteriostatic agent against the ATCC 12228 strain by slowing down its growth.

In contrast, it is evident from the growth analysis that RP62A strain growth rate was not impacted when exposed to 305.6 mg/L of D-limonene (Figure 1B). Concentrations of D-limonene above and below 305.6 mg/L were observed to have no effect on the growth rate of RP62A when exposed (data not shown). The stationary phase of RP62A between the 0 mg/L of D-limonene (no treatment) condition and the 305.6 mg/L of D-limonene condition is not significantly different from each other ($P > 0.01$). Moreover, the doubling time of RP62A under exposure to the no treatment control (0 mg/L of D-limonene) was 2.60 hours. Similarly, the doubling time of RP62A under exposure to 305.6 mg/L of D-limonene was 2.48 hours. This demonstrates that the doubling time of the RP62A strain under exposure to 305.6 mg/L of D-limonene remains closely similar to its doubling time under exposure to no D-limonene. Thus, results from both strains suggest that D-limonene has minimal static effect on the growth of the RP62A strain.

3.2 Rifampicin Susceptibility

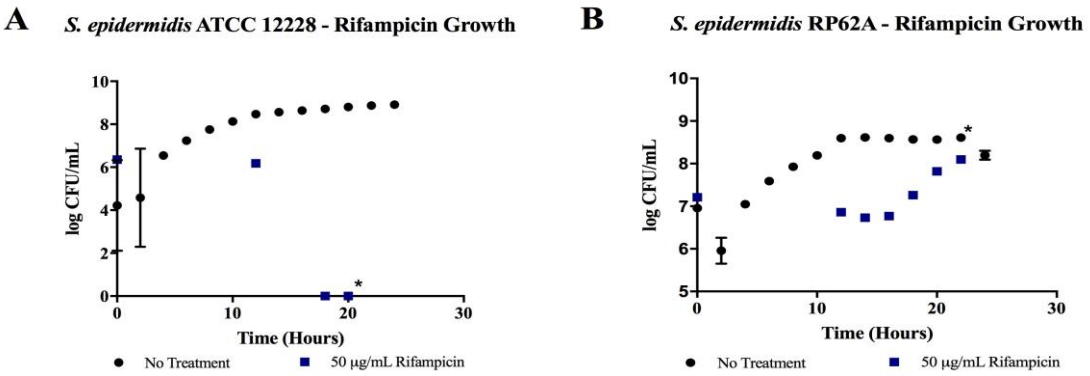

Figure 2. Growth analysis when exposed to rifampicin

Figure 2A. Growth curve demonstrating the growth of *S. epidermidis* ATCC 12228 under exposure to 50 µg/mL of rifampicin, and also its growth under exposure to 0 mg/L of D-limonene (no treatment) as a comparison. Figure 2B. Growth curve demonstrating the growth of *S. epidermidis* RP62A under exposure to 50 µg/mL of rifampicin, and also its growth under exposure to 0 mg/L of D-limonene (no treatment) as a comparison.

Both curves were constructed with OD_{600} values from two data trials (trial 3 = 0, 2, 4, 6, 8, 10, 24 hours, trial 4 = 0, 12, 14, 16, 18, 20, 22, 24 hours). Difference in the stationary phase of ATCC 12228 between 0 mg/L of D-limonene (no treatment) condition and 50 µg/mL of rifampicin condition is significant ($P < 0.01$, one-way ANOVA). Difference in the overall growth of RP62A between 0 mg/L of D-limonene (no treatment) condition and 50 µg/mL of rifampicin condition is significant ($P < 0.01$, one-way ANOVA).

The growth of the ATCC 12228 strain was inhibited when exposed to 50 µg/mL of rifampicin (Figure 2A). The stationary phase of ATCC 12228 between 0 mg/L of D-limonene (no treatment) condition and 50 µg/mL of rifampicin condition is significantly different from one another ($P < 0.01$). In addition to no signs of

growth, the cell density was also observed to have decreased over time. Thus, it suggests that rifampicin acts as a bactericidal agent against the ATCC 12228 strain.

Conversely, the growth of the RP62A strain was initially inhibited when first exposed to 50 µg/mL of rifampicin (Figure 2B). However, after 16 hours, it started to grow normally in this condition (Figure 2B). The results correlate with previous research on the RP62A strain, where it was similarly observed to have started normal growth after 12 hours of exposure to rifampicin[69]. Although the difference in overall growth of the RP62A strain between 0 mg/L of D-limonene (no treatment) condition and 50 µg/mL of rifampicin condition is significant ($P < 0.01$), there was a lag in growth of RP62A in the first 16 hours. However, after 16 hours of exposure, normal growth was observed and the concentration of RP62A continued to increase. Despite the increasing RP62A concentration, the difference in growth of the RP62A strain between the two conditions at 22 hours of exposure remains significant ($P < 0.01$), since it appears that the sample exposed to rifampicin was still within exponential growth.

3.3 D-limonene-Rifampicin Drug Cocktail Susceptibility

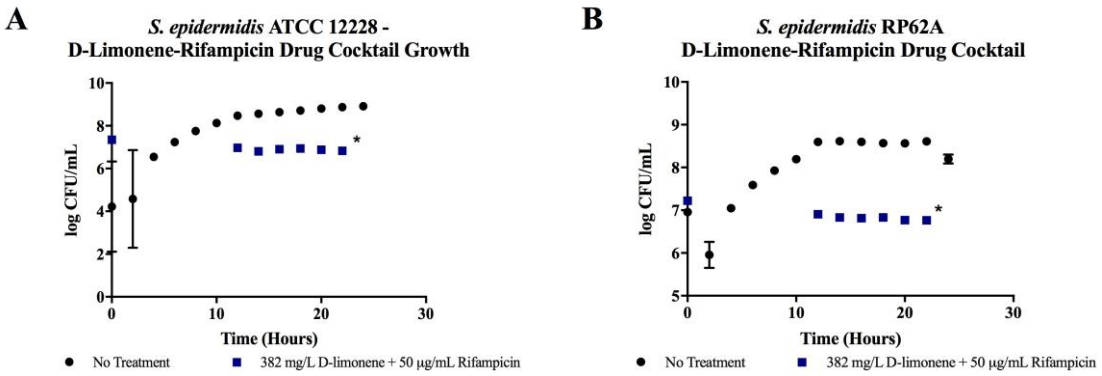

Figure 3. Growth analysis when exposed to D-limonene-rifampicin drug cocktail

Figure 3A. Growth curve demonstrating the growth of *S. epidermidis* ATCC 12228 under exposure to the D-limonene-rifampicin drug cocktail (382 mg/L of D-limonene + 50 µg/mL of rifampicin), and also its growth under exposure to 0 mg/L of D-limonene (no treatment) as a comparison. Figure 3B. Growth curve demonstrating the growth of *S. epidermidis* RP62A under exposure to the D-limonene-rifampicin drug cocktail (382 mg/L of D-limonene + 50 µg/mL of rifampicin), and also its growth under exposure to 0 mg/L of D-limonene (no treatment) as a comparison.

Both curves were constructed with OD_{600} values from two data trials (trial 3 = 0, 2, 4, 6, 8, 10, 24 hours, trial 4 = 0, 12, 14, 16, 18, 20, 22, 24 hours). Difference in the stationary phase of ATCC 12228 between 0 mg/L of D-limonene (no treatment) condition and D-limonene-rifampicin drug cocktail condition is significant ($P < 0.01$, one-way ANOVA). Difference in the stationary phase of RP62A between 0 mg/L of D-limonene (no treatment) condition and D-limonene-rifampicin drug cocktail condition is significant ($P < 0.01$, one-way ANOVA). Difference in the stationary phase of ATCC 12228 and RP62A under exposure to the D-limonene-rifampicin drug cocktail is not significant.

The growth of the ATCC 12228 strain was significantly inhibited when exposed to the D-limonene-rifampicin drug cocktail (Figure 3A). The stationary phase of the ATCC 12228 strain between 0 mg/L of D-limonene (no treatment) condition and D-limonene-rifampicin drug cocktail condition is significantly different from each other ($P < 0.01$). This shows that the drug cocktail is able to significantly inhibit the growth of ATCC 12228. Thus, it suggests that the D-limonene-rifampicin drug cocktail works effectively against the ATCC 12228 strain by significantly inhibiting its growth.

Furthermore, the growth of the RP62A strain was also significantly inhibited when exposed to the D-limonene-rifampicin drug cocktail condition (Figure 3B). The stationary phase of the RP62A strain between 0 mg/L of D-limonene (no treatment) condition and D-limonene-rifampicin drug cocktail condition is

significantly different from each other ($P < 0.01$). This shows that the drug cocktail is also able to significantly inhibit the growth of RP62A. Thus, it suggests that the D-limonene-rifampicin drug cocktail works effectively against the RP62A strain as well by inhibiting its growth.

4. Discussion

Staphylococcus epidermidis is found to exist as various strains, such as the ATCC 12228 and RP62A strains. One of the major differences between the strains is that the ATCC 12228 strain is unable to produce a biofilm, while the RP62A strain does produce a biofilm. This suggests that difference in the initial growths observed between both strains may be related to strain variation. *S. epidermidis* RP62A that is found in the clinical setting is a greater challenge to treat and eradicate because of its capability to produce a biofilm[27,36,39]. The RP62A strain is suggested to be more tolerant to D-limonene than the ATCC 12228 strain because of this particular constituent[5,37]. The biofilm that protects the bacteria may act as a barrier against antimicrobial agents, and should be further studied.

Prior to the experiment, a relationship curve of bacterial concentrations versus optical density at 600 nm was performed to determine the cell density for each strain and was monitored over 24 hours simultaneously. Cell density for each strain was diluted each time to 10^6 CFU/mL (correlates with about $OD_{600} = 0.02$), utilizing the respective growth curve for calculation. The rationale behind choosing 10^6 CFU/mL as initial concentration is that *S. epidermidis* concentrations below 10^6 CFU/mL, such as 10^4 CFU/mL, increased the time *S. epidermidis* spent in a lag phase, and thus further slowed down their growth. On the other hand, *S. epidermidis* concentrations above 10^6 CFU/mL, such as 10^8 CFU/mL, saturated the bacterial culture. Therefore, it is suggested that 10^6 CFU/mL is an optimal *S. epidermidis* concentration for growth.

According to the results, in the D-limonene condition, D-limonene acts as a bacteriostatic agent against the ATCC 12228 strain by slowing its growth at a minimum inhibitory concentration of 305.6 mg/L, but it has no observable effect on the RP62A strain. Speculation as to the reason D-limonene has no effect on the growth of the RP62A strain is thought to be due to the capability of RP62A to form a biofilm. The biofilm surrounds the *S. epidermidis* RP62A cells, thus blocking D-limonene from accessing its target sites, which are the cell membrane and cell wall[41,42]. This may be the reason RP62A is able to retain resistance to D-limonene and maintain normal growth.

In the rifampicin control condition, rifampicin was found to act as a bactericidal agent against the ATCC 12228 strain. However, for the RP62A strain, normal growth was observed after 16 hours of exposure similar to the observations of Zheng and Stewart[69]. It is anticipated that the reason rifampicin has no effect on the growth of the RP62A strain after 16 hours of exposure is due to a mutation that the RP62A strain may undergo towards rifampicin sensitivity by modifying its *rpoB* gene during the first 16 hours of exposure leading to rifampicin resistance[21]. This speculation is based on previous studies that have shown resistance to rifamycin-class antibiotics in various bacterial species, such as *Escherichia coli* and *Mycobacterium tuberculosis*, due to the modification of the *rpoB* gene[21]. The *rpoB* gene codes for an alternative sigma factor that aids in the binding of RNA polymerase to the promoter site during the initiation phase of RNA synthesis in *S. epidermidis*[21]. Rifamycin binding to *rpoB* gene may therefore block transcription initiation. Therefore, it is predicted that mutation of *rpoB* gene abrogates its binding to rifamycin, in turn RNA polymerase is able to initiate transcription, and thus confers rifampicin resistance to the RP62A strain, allowing normal growth.

In the D-limonene-rifampicin drug cocktail condition, the drug cocktail was found to work effectively against both the ATCC 12228 and RP62A strains. Since results showed that the RP62A strain is resistant to both D-limonene and rifampicin, the individual agents were combined, forming a drug cocktail, in order to observe if there would be a synergic effect in the inhibition of growth of the RP62A strain. As demonstrated, the combination of D-limonene and rifampicin did indeed result in an effective inhibition of the RP62A growth. A study on the effect of the rifampicin-vancomycin drug cocktail on *S. epidermidis* RP62A showed the combination of the two drugs was more effective in inhibiting the growth of the RP62A strain than individually[18]. The rifampicin-vancomycin drug cocktail was not only able to prevent the development of rifampicin resistance in the RP62A strain, but as well as improve the ability of vancomycin to penetrate through the biofilm of the RP62A strain[18]. As a result, both factors contributed to the effectiveness of the rifampicin-vancomycin drug cocktail to inhibit the growth of the RP62A strain[18]. It is rare for bacteria to undergo two or more different mutations simultaneously. In order for the RP62A strain to survive and grow in the presence of the D-limonene-rifampicin drug cocktail, two different mutations would have to occur at

the same time, due to the fact that D-limonene and rifampicin have different target sites as discussed above. Thus, the D-limonene-rifampicin drug cocktail is able to effectively work against the RP62A strain in inhibiting its growth because it is unlikely that the RP62A strain had undergone two different mutations simultaneously. With this result, it is hoped that the D-limonene-rifampicin drug cocktail will be further evaluated and used in clinical settings to prevent initial nosocomial *Staphylococcus* infections from developing.

Future research may include determining the role of the *icaADBC* gene locus in *S. epidermidis* RP62A's D-limonene resistance by performing qRT-PCR to evaluate the expression level of the *icaADBC* gene locus, specifically the *icaA* gene. If the expression of the *icaA* gene is not found to be responsible, the expression levels of other genes, that are also responsible for biofilm formation, may also be done, such as the *rpoB* gene. The *rpoB* gene is responsible for controlling the expression of the *icaADBC* gene locus[27]. Furthermore, future work might focus on studying the synergic effect of other drug cocktail combinations, such as combining D-limonene with other rifamycin-class antibiotics, with regards to the inhibition of growth of the RP62A strain. Additionally, D-limonene may be combined with other essential oils that have been attributed with having antimicrobial properties in order to determine their values as alternatives to synthetic antibiotics in the clinical setting[19,37].

5. Conclusion

In conclusion, the hypothesis is not supported because the study showed that D-limonene alone does not work effectively in inhibiting the growth of *S. epidermidis* RP62A. However, the research demonstrated a synergic effect in that D-limonene is effective in inhibiting the growth of *S. epidermidis* RP62A when combined with the antibiotic rifampicin. Thus, this study provides promising results, which suggests that D-limonene may be utilized as a therapy in preventing initial hospital-acquired *Staphylococcus* infections in combination with other antibiotics.

6. Acknowledgements

The authors wish to express their appreciation to the Manhattanville College Castle Scholars Honors Program for providing the Elizabeth McCormack grant that funded this research. They also express their appreciation to faculty research advisors Dr. Anna Yeung-Cheung and Dr. Christopher J. Pappas for their guidance and time throughout this study. In addition to that, they would like to sincerely thank Dr. Ira Schwartz's Lab (New York Medical College, Valhalla, NY), and the Biology Department at Manhattanville College for providing research reagents and supplies, equipment, and the laboratory space to complete this study.

7. References Cited

1. Adams, T. B., Gavin, C. L., McGowen, M. M., Waddell, W. J., Cohen, S. M., Feron, V. J., . . . Smith, R. L. (2011). The FEMA GRAS assessment of aliphatic and aromatic terpene hydrocarbons used as flavor ingredients. *Food Chem Toxicol*, 49, 2471-2494. doi:10.1016/j.fct.2011.06.011
2. Arciola, C. R., Baldassarri, L., & Montanaro, L. (2001). Presence of *icaA* and *icaD* genes and slime production in a collection of staphylococcal strains from catheter-associated infections. *J Clin Microbiol, 39*(6), 2151-2156. doi:10.1128/JCM.39.6.2151-2156.2001
3. Bergamini, T. M., McCurry, T. M., Bernard, J. D., Hoeg, K. L., Corpus, R. A., James, B. E., . . . Cheadle, W. G. (1996). Antibiotic efficacy against *Staphylococcus epidermidis* adherent to vascular grafts. *J Surg Res, 60*(1), 3-6.
4. Brennan, T. C., Kromer, J. O., & Nielsen, L. K. (2013). Physiological and transcriptional responses of *Saccharomyces cerevisiae* to D-limonene show changes to the cell wall but not to the plasma membrane. *Appl Environ Microbiol, 79*(12), 3590-3600. doi:10.1128/AEM.00463-13

5. Brooks, J. L., & Jefferson, K. K. (2014). Phase variation of poly-N-acetylglucosamine expression in *Staphylococcus aureus*. *PLoS Pathog, 10*(7), e1004292. doi:10.1371/journal.ppat.1004292
6. Cafiso, V., Bertuccio, T., Santagati, M., Campanile, F., Amicosante, G., Perilli, M. G., . . . Stefani, S. (2004). Presence of the *ica* operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. *Clin Microbiol Infect, 10*(12), 1081-1088. doi:10.1111/j.1469-0691.2004.01024.x
7. Chen, M., Yu, Q., & Sun, H. (2013). Novel strategies for the prevention and treatment of biofilm related infections. *Int J Mol Sci, 14*(9), 18488-18501. doi:10.3390/ijms140918488
8. Cho, S. H., Naber, K., Hacker, J., & Ziebuhr, W. (2002). Detection of the *icaADBC* gene cluster and biofilm formation in *Staphylococcus epidermidis* isolates from catheter-related urinary tract infections. *Int J Antimicrob Agents, 19*(6), 570-575.
9. Chueca, B., Pagan, R., & Garcia-Gonzalo, D. (2014). Differential mechanism of *Escherichia coli* Inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. *PLoS One, 9*(4), e94072. doi:10.1371/journal.pone.0094072
10. Clumeck, N., Marcelis, L., Amiri-Lamraski, M. H., & Gordts, B. (1984). Treatment of severe staphylococcal infections with a rifampicin-minocycline association. *J Antimicrob Chemother*, 13(Suppl C), 17-22. doi: 10.1093/jac/13.suppl_C.17
11. Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., & Gotz, F. (1999). The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun, 67*(10), 5427-5433.
12. Cramton, S. E., Ulrich, M., Gotz, F., & Doring, G. (2001). Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun, 69*(6), 4079-4085. doi:10.1128/IAI.69.6.4079-4085.2001
13. Das, J. R., Bhakoo, M., Jones, M. V., & Gilbert, P. (1998). Changes in the biocide susceptibility of *Staphylococcus epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. *J Appl Microbiol, 84*(5), 852-858.
14. Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., & Mauriello, G. (2007). Membrane toxicity of antimicrobial compounds from essential oils. *J Agric Food Chem, 55*(12), 4863-4870. doi:10.1021/jf0636465
15. Di Pasqua, R., Hoskins, N., Betts, G., & Mauriello, G. (2006). Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J Agric Food Chem, 54*(7), 2745-2749. doi:10.1021/jf052722l
16. Dixson, S., Brumfitt, W., & Hamilton-Miller, J. M. T. (1984). Activity of rifampicin against *Staphylococci*, with special reference to multiresistant strains. *J Antimicrob Chemother*, 13(Supp. C), 7-16. doi: 10.1093/jac/13.suppl_C.7
17. Drancourt, M., & Raoult, D. (2002). *rpoB* gene sequence-based identification of *Staphylococcus* species. *J Clin Microbiol, 40*(4), 1333-1338.
18. Dunne, W. M., Jr., Mason, E. O., Jr., & Kaplan, S. L. (1993). Diffusion of rifampin and vancomycin through a *Staphylococcus epidermidis* biofilm. *Antimicrob Agents Chemother, 37*(12), 2522-2526.
19. Espina, L., Gelaw, T. K., de Lamo-Castellvi, S., Pagan, R., & Garcia-Gonzalo, D. (2013). Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes. *PLoS One, 8*(2), e56769. doi:10.1371/journal.pone.0056769
20. Espina, L., Pagan, R., Lopez, D., & Garcia-Gonzalo, D. (2015). Individual Constituents from Essential Oils Inhibit Biofilm Mass Production by Multi-Drug Resistant *Staphylococcus aureus*. *Molecules, 20*(6), 11357-11372. doi:10.3390/molecules200611357
21. Floss, H. G., & Yu, T. W. (2005). Rifamycin-mode of action, resistance, and biosynthesis. *Chem Rev, 105*(2), 621-632. doi:10.1021/cr030112j
22. Gad, G. F., El-Feky, M. A., El-Rehewy, M. S., Hassan, M. A., Abolella, H., & El-Baky, R. M. (2009). Detection of *icaA, icaD* genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. *J Infect Dev Ctries, 3*(5), 342-351.
23. Gagnon, R. F., Richards, G. K., & Wiesenfeld, L. (1991). *Staphylococcus epidermidis* biofilms: Unexpected outcome of double and triple antibiotic combinations with rifampin. *ASAIO Trans, 37*(3), M158-60.

24. Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O., & Gotz, F. (1998). Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J Biol Chem, 273*(29), 18586-18593.

25. Giacometti, A., Cirioni, O., Ghiselli, R., Goffi, L., Mocchegiani, F., Riva, A., . . . Saba, V. (2000). Polycationic peptides as prophylactic agents against methicillin-susceptible or methicillin-resistant *Staphylococcus epidermidis* vascular graft infection. *Antimicrob Agents Chemother, 44*(12), 3306-3309.

26. Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., . . . Fraser, C. M. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol, 187*(7), 2426-2438. doi:10.1128/JB.187.7.2426-2438.2005

27. Handke, L. D., Slater, S. R., Conlon, K. M., O'Donnell, S. T., Olson, M. E., Bryant, K. A., . . . Fey, P. D. (2007). *SigmaB* and *SarA* independently regulate polysaccharide intercellular adhesin production in *Staphylococcus epidermidis*. *Can J Microbiol, 53*(1), 82-91. doi:10.1139/w06-108

28. Kavanaugh, N. L., & Ribbeck, K. (2012). Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl Environ Microbiol, 78*(11), 4057-4061. doi:10.1128/AEM.07499-11

29. Kloos, W. E., & Bannerman, T. L. (1994). Update on clinical significance of coagulase-negative *Staphylococci*. *Clin Microbiol Rev, 7*(1), 117-140.

30. Kunin, C., Brandt, D., & Wood, H. (1969). Bacteriologic Studies of Rifampin, a New Semisynthetic Antibiotic. *The Journal of Infectious Diseases, 119*(2), 132-137. Retrieved from http://www.jstor.org/stable/30102288

31. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., . . . Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet, 357*(9264), 1225-1240.

32. Li, H., Xu, L., Wang, J., Wen, Y., Vuong, C., Otto, M., & Gao, Q. (2005). Conversion of *Staphylococcus epidermidis* strains from commensal to invasive by expression of the *ica* locus encoding production of biofilm exopolysaccharide. *Infect Immun, 73*(5), 3188-3191. doi:10.1128/IAI.73.5.3188-3191.2005

33. McKenney, D., Hubner, J., Muller, E., Wang, Y., Goldmann, D. A., & Pier, G. B. (1998). The *ica* locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. *Infect Immun, 66*(10), 4711-4720.

34. Monzon, M., Oteiza, C., Leiva, J., & Amorena, B. . (2001). Synergy of different antibiotic combinations in biofilms of *Staphylococcus epidermidis*. *Journal of Antimicrobial Chemotherapy, 48*(6), 793-801. doi:10.1093/jac/48.6.793

35. Muthaiyan, A., Biswas, D., Crandall, P. G., Wilkinson, B. J., & Ricke, S. C. . (2012). Application of orange essential oil as an antistaphylococcal agent in a dressing model. *BMC Complementary & Alternative Medicine, 12*(125). doi:10.1186/1472-6882-12-125

36. Neely, A. N., & Maley, M. P. (2000). Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol, 38*(2), 724-726.

37. Nuryastuti, T. (2010). Environmental signals affecting *icaA*-expression in *Staphylococcus epidermidis* biofilms. (Doctoral Dissertation), University of Groningen. Retrieved from http://dissertations.ub.rug.nl

38. Nuryastuti, T., van der Mei, H. C., Busscher, H. J., Iravati, S., Aman, A. T., & Krom, B. P. (2009). Effect of cinnamon oil on *icaA* expression and biofilm formation by *Staphylococcus epidermidis*. *Appl Environ Microbiol, 75*(21), 6850-6855. doi:10.1128/AEM.00875-09

39. Otter, J. A., Vickery, K., Walker, J. T., deLancey Pulcini, E., Stoodley, P., Goldenberg, S. D., . . . Edgeworth, J. D. (2015). Surface-attached cells, biofilms and biocide susceptibility: implications for hospital cleaning and disinfection. *J Hosp Infect, 89*(1), 16-27. doi:10.1016/j.jhin.2014.09.008

40. Otto, M. (2009). *Staphylococcus epidermidis*--the 'accidental' pathogen. *Nat Rev Microbiol, 7*(8), 555-567. doi:10.1038/nrmicro2182

41. Pasqua, R. D., Hoskins, N., Betts, G., & Mauriello, G. (2006). Changes in Membrane Fatty Acids Composition of Microbial Cells Induced by Addiction of Thymol, Carvacrol, Limonene, Cinnamaldehyde, and Eugenol in the Growing Media. *Journal of Agricultural and Food Chemistry*, 54, 2745-2749. doi:10.1021/jf052722I

42. Pasqua, R. D., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., & Mauriello, G. (2007). Membrane Toxicity of Antimicrobial Compounds from Essential Oils. *Journal of Agricultural and Food Chemistry*, 55, 4863-4870. doi:10.1021/jf0636465

43. Peters, G., & Pulverer, G. (1984). Pathogenesis and management of *Staphylococcus epidermidis* 'plastic' foreign body infections. *J Antimicrob Chemother, 14 Suppl D*, 67-71.

44. Pettit, R. K., Weber, C. A., Kean, M. J., Hoffmann, H., Pettit, G. R., Tan, R., . . . Horton, M. L. (2005). Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob Agents Chemother, 49*(7), 2612-2617. doi:10.1128/AAC.49.7.2612-2617.2005

45. Pharmacists, A. S. o. H.-S. (2012). Rifampin. Retrieved from https://www.nlm.nih.gov/medlineplus/druginfo/meds/a682403.html

46. Rifampin. (May 28, 2016). Retrieved from https://aidsinfo.nih.gov/drugs/109/rifampin/0/professional

47. Rodriguez-Martinez, J. M., & Pascual, A. (2006). Antimicrobial resistance in bacterial biofilms. *Reviews in Medical Microbiology, 17*(3), 65-75. doi:0.1097/01.revmedmi.0000259645.20603.63

48. Rumi, M. V., Huguet, M. J., Bentancor, A. B., & Gentilini, E. R. (2013). The *icaA* gene in staphylococci from bovine mastitis. *J Infect Dev Ctries, 7*(7), 556-560. doi:10.3855/jidc.2670

49. Rupp, M. E., & Fey, P. D. (2001). In vivo models to evaluate adhesion and biofilm formation by *Staphylococcus epidermidis*. *Methods Enzymol, 336*, 206-215.

50. Rupp, M. E., Fey, P. D., Heilmann, C., & Gotz, F. (2001). Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J Infect Dis, 183*(7), 1038-1042. doi:10.1086/319279

51. Rupp, M. E., Ulphani, J. S., Fey, P. D., Bartscht, K., & Mack, D. (1999). Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect Immun, 67*(5), 2627-2632.

52. Rupp, M. E., Ulphani, J. S., Fey, P. D., & Mack, D. (1999). Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. *Infect Immun, 67*(5), 2656-2659.

53. Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G., & Jabbouri, S. (2005). Extracellular carbohydrate-containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. *Infect Immun, 73*(5), 3007-3017. doi:10.1128/IAI.73.5.3007-3017.2005

54. Schelz, Z., Molnar, J., & Hohmann, J. . (2006). Antimicrobial and Antiplasmid Activites of Essentail Oils. *Fitoterapia, 77*(4), 279-285. doi:10.1016/j.fitote.2006.03.013

55. Sieniawska, E., Los, R., Baj, T., Malm, A., & Glowniak, K. (2013). Antimicrobial efficacy of *Mutellina purpurea* essential oil and α-pinene against *Staphylococcus epidermidis* grown in planktonic and biofilm cultures. *Industrial Crops and Products, 51*, 152. doi:10.1016/j.indcrop.2013.09.001

56. Smith, K., Gould, K. A., Ramage, G., Gemmell, C. G., Hinds, J., & Lang, S. (2010). Influence of tigecycline on expression of virulence factors in biofilm-associated cells of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother, 54*(1), 380-387. doi:10.1128/AAC.00155-09

57. Sokovic, M., & and van Griensven, L. J.L.D. (2006). Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*. *European Journal of Plant Pathology, 116*, 211-224. doi:10.1007/s10658-006-9053-0

58. Solorzano-Santos, F., & Miranda-Novales, M. G. (2012). Essential oils from aromatic herbs as antimicrobial agents. *Curr Opin Biotechnol, 23*(2), 136-141. doi:10.1016/j.copbio.2011.08.005

59. Sun, J. (2007). D-Limonene: safety and clinical applications. *Altern Med Rev, 12*(3), 259-264.

60. Svensson, E., Hanberger, H., Nilsson, M., & Nilsson, L. E. (1997). Factors affecting development of rifampicin resistance in biofilm-producing *Staphylococcus epidermidis*. *J Antimicrob Chemother, 39*(6), 817-820.

61. Vandeventer, P. E., Weigel, K. M., Salazar, J., Erwin, B., Irvine, B., Doebler, R., . . . Niemz, A. (2011). Mechanical disruption of lysis-resistant bacterial cells by use of a miniature, low-power, disposable device. *J Clin Microbiol, 49*(7), 2533-2539. doi:10.1128/JCM.02171-10

62. Vuong, C., Kocianova, S., Voyich, J. M., Yao, Y., Fischer, E. R., DeLeo, F. R., & Otto, M. (2004). A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem, 279*(52), 54881-54886. doi:10.1074/jbc.M411374200

63. Wang, X., Niu, C., Sun, G., Dong, D., Villaruz, A. E., Li, M., . . . Gao, Q. (2011). *ygs* is a novel gene that influences biofilm formation and the general stress response of *Staphylococcus epidermidis*. *Infect Immun, 79*(3), 1007-1015. doi:10.1128/IAI.00916-10

64. Wehrli, W. (1983). Rifampin: Mechanisms of Action and Resistance. *Reviews of Infectious Diseases, 5*, S407-S411. Retrieved from http://www.jstor.org/stable/4453139

65. Wei, W., Cao, Z., Zhu, Y. L., Wang, X., Ding, G., Xu, H., . . . Li, Y. (2006). Conserved genes in a path from commensalism to pathogenicity: comparative phylogenetic profiles of *Staphylococcus epidermidis* RP62A and ATCC 12228. *BMC Genomics, 7*, 112. doi:10.1186/1471-2164-7-112

66. Wladyka, B., Piejko, M., Bzowska, M., Pieta, P., Krzysik, M., Mazurek, L., . . . Mak, P. (2015). A peptide factor secreted by *Staphylococcus pseudintermedius* exhibits properties of both bacteriocins and virulence factors. *Sci Rep, 5*, 14569. doi:10.1038/srep14569

67. Wojtyczka, R. D., Orlewska, K., Kepa, M., Idzik, D., Dziedzic, A., Mularz, T., . . . Wasik, T. J. (2014). Biofilm formation and antimicrobial susceptibility of *Staphylococcus epidermidis* strains from a hospital environment. *Int J Environ Res Public Health, 11*(5), 4619-4633. doi:10.3390/ijerph110504619

68. Zhang, Y. Q., Ren, S. X., Li, H. L., Wang, Y. X., Fu, G., Yang, J., . . . Wen, Y. M. (2003). Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol, 49*(6), 1577-1593.

69. Zheng, Z., & Stewart, P. S. (2002). Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother, 46*(3), 900-903.