The Inhibitory Effect of the Combination Treatment of Rifampicin and D-limonene on the Growth and Biofilm formation of *Staphylococcus epidermidis* RP62A

ABSTRACT

Aims: To discover an alternative chemical that could potentially be used in combination with antibiotics to inhibit the growth of *Staphylococcus epidermidis* RP62A

Study design: The studies of the growth inhibition in both the biofilm forming strain of *S. epidermidis* RP62A and the wild-type strain ATCC 12228, were conducted with different concentrations of rifampicin and D-limonene using the measurement of the optical density.

Place and Duration of Study: All experiments were conducted in the Department of Biology at Manhattanville College between September 2015 and December 2019.

Methodology: Different concentrations of rifampicin (0.0025 μg/mL, 0.005 μg/mL, 0.01 μg/mL and 0.02 μg/mL) and D-limonene (152.8 μg/mL, 305.6 μg/mL, 611.2 μg/mL and 1222.4 μg/mL) were used in the study. The minimal inhibitory concentrations (MIC) of rifampicin and D-limonene on RP62A and ATCC 12228 were obtained. The combination of rifampicin and D-limonene on the inhibition of the growth of RP62A and ATCC 12228 was conducted using a microtiter plate by measuring optical density. The alamarBlue® assay was further used to evaluate the viability of RP62A and ATCC 12228 with the combination treatment. The biofilm assay was then conducted on RP62A with the combination treatment of rifampicin and D-limonene.

Results: The MIC of the combination treatment of rifampicin and D-limonene on RP62A was found to be 0.01 μg/mL and 1222.4 μg/mL respectively. A combination treatment of 0.005 μg/mL of rifampicin with 611.2 μg/mL of D-limonene significantly inhibited the growth and biofilm formation of RP62A than the same concentration of rifampicin.

Conclusion: The addition of the D-limonene to the rifampicin was found to be effective in inhibiting the growth and biofilm formation of RP62A. This indicates that the combination of alternative mineral oil may have the potential to lower the antibiotic concentration in the inhibition of the growth of the bacteria *S. epidermidis.*

Keywords: rifampicin, D-limonene, Staphylococcus epidermidis, biofilm, combination treatment, growth inhibition

1. INTRODUCTION

Staphylococcus epidermidis is a Gram-positive, coagulase-negative staphylococci (CoNS) bacterium that colonizes the human skin and poses a threat to the health of individuals [1, 2]. It is considered an opportunistic pathogen that facilitates nosocomial infections through adherence on medical devices, such as intravascular catheters, contributing to 60-70% of nosocomial infections [2, 3, 4]. However, *S. epidermidis* flora can be beneficial by providing minimal skin irritation, increasing the lysate size against other bacteria, and the production of T-cells in the spleen and lymph nodes [5, 6, 7].

21 Infections caused by CoNS bacteria, like *Staphylococcus*, have become difficult to treat due to the ability of the bacteria to
22 form a biofilm [8]. The infection transmission risk is thus increased by the presence of *S. epidermidis* in hospitals, causing
23 CoNS sepsis in neonates and immunocompromised patients [3,9].

24 Biofilms are clusters of bacterial cells that adhere to each other and often to a surface, contributing to the cause of
25 persistent infections [10]. The biofilm extracellular matrix of *S. epidermidis* can adhere onto other surfaces through its
26 polysaccharide intercellular adhesin (PIA) [11]. The biofilm of the mutant strain *S. epidermidis* RP62A provides antibiotic
27 resistance, causing a difficulty when treating infections [12]. The *icaABCD* gene operon has encoded proteins responsible
28 for PIA biosynthesis associated in the biofilm formation of *S. epidermidis* [13]. The biofilm formation of *S. epidermidis*
29 RP62A depends on a specific accumulation-associated protein (Aap) [14]. The aggregation of the Aap protein in the zinc
30 dependent G5 domains of the LPXTG motif in *S. epidermidis* forms fibrils. The zinc ions and proteolytic cleavage of Aap
31 are responsible for the accumulation of biofilm cells and the biofilm effect [2, 14]. A study conducted by Wojtyczka *et al.*
32 showed that antibiotic resistance against the CoNS bacteria biofilm increased the number of nosocomial infections,
33 consequently posing a threat to human health [8].

34 *Staphylococcus* infections are routinely treated by antibiotics. About 75-90% of *S. epidermidis* isolates are resistant
35 against methicillin, which is one of the most used antibiotics for staphylococcal infections in hospitals [15]. *S. epidermidis*
36 can also be resistant to rifamycin, tetracycline, clindamycin, and gentamycin [16]. Although different antibiotics are
37 constantly being developed, microbial resistance keeps emerging among different types of bacteria [17]. The antibiotic
38 resistance of microbes originated through their adaptation to different antibiotics, their overuse, and the degree to which
39 resistance occurs [17]. Rifampicin is one of the most widely used antibiotics to treat nosocomial infections [18]. Rifampicin
40 targets the *rpoB* gene of the sigma factor of DNA-dependent RNA polymerase in the mRNA synthesis of the bacterium,
41 causing a change in its promoter site [18]. However, after hours of exposure, rifampicin is unable to fully inhibit the growth
42 of the biofilm of RP62A, despite its ability to penetrate it [19].

43 The increase of antibiotic resistance has facilitated the search for other alternatives to treat microbial infections [17].
44 Essential oils have been widely studied as antimicrobial agents in the last decades. A study conducted by Di Pasqua *et al.*
45 showed that the addition of essential oils disrupted the structure of the cell envelope of a variety of bacteria, they also
46 decreased their amount of unsaturated fatty acids, and proved the antimicrobial properties through their membrane
47 toxicity [20]. One of the essential oils used in that study was D-limonene, which is a monoterpene composed of citrus oils,
48 has low toxicity, and lacks any mutagenic or carcinogenic properties [20, 21]. A D-limonene dose less than 1,650 mg/kg a
49 day is considered safe to ingest [21]. Another study showed that a high D-limonene intake increased the survival in
50 lymphoma-bearing mice [22]. The preliminary study of the effectiveness of D-limonene in combination with rifampicin on
51 the biofilm-forming *S. epidermidis* RP62A strain was done in our laboratory, however, no minimum inhibitory
52 concentrations of D-limonene and rifampicin were achieved [23]. Individual constituents of essential oils, like D-limonene,
53 have some nonspecific antimicrobial influence that can postpone the “assembly-disassembly” cycle of biofilm formation
54 during the incubation period of a similar Staphylococcal strain, *S. aureus* [24]. This shows the significance of measuring
55 the dependence and inhibitory effects of limonene on biofilm formation at different growth phases [24].

56 In the current study, the minimal inhibitory concentration (MIC) of the combination of D-limonene and rifampicin was
57 evaluated on *S. epidermidis* RP62A. Additionally, an alamarBlue® assay was used to test the cell viability with the
58 combination treatment. Further application of this study is to reduce the unaccompanied, high concentration use of the
59 antibiotic rifampicin alone, and to provide an alternate treatment to the biofilm forming *S. epidermidis* strain.

60 2. MATERIAL AND METHODS

61 2.1 Strains and growth conditions

62 *S. epidermidis* strains wild type ATCC 12228 and biofilm-forming RP62A were purchased (American Type Culture
63 Collection, VA) and stored at -80°C. Both strains were grown in aerobic conditions in Trypticase Soy Agar (TSA) and
64 Trypticase Soy Broth (TSB) (Sigma-Aldrich, MO) in the Excella E24 Incubator Shaker (New Brunswick Scientific, CT) at
65 100 RPM at 37°C for 20 hours. The bacterial concentration of these two strains was measured using a DU Series 700
66 UV/Vis Spectrophotometer (Beckman Coulter, CA). Based on the growth curve computed in our previous study, the
67 bacterial concentrations of the strains at Optical Density OD_{600} =1 were 7.5 x 10^8 CFU/mL for ATCC 12228 and 4.5 x 10^8
68 CFU/mL for RP62A [23].

69 2.2 Minimal inhibitory concentration of rifampicin and D-limonene on *S. epidermidis* ATCC 12228 and 70 RP62A

71 Both strains were grown on Mueller-Hinton agar (MHA) (BD Difco, MD) with 2% sodium chloride (Sigma-Aldrich, MO) for
72 20 hours at 37°C. Bacterial colonies from each strain were transferred onto sterile 1X phosphate buffer saline (PBS)

(Wards Science, NY), and the growth was analyzed at OD_{600}. Both strains were then diluted to a final concentration of 10^6 CFU/mL. Two flat-bottom 96-well plates (Corning, NY) were used to inoculate each strain with treatments in a total volume of 200 µL. The highest concentration of each treatment was added into the first well of each row. Two-fold dilutions were then performed with the addition of Mueller-Hinton broth (MHB) (Sigma-Aldrich, MO) containing 2% sodium chloride and bacterial suspension at a final concentration of 5×10^4 CFU/mL along with the addition of either rifampicin (Sigma-Aldrich, MO) (0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL) or D-limonene (Sigma-Aldrich, MO) dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO) (152.8 µg/mL, 305.6 µg/mL, 611.2 µg/mL and 1222.4 µg/mL). Wells containing MHB alone, and MHB with bacteria served as control. Each microtiter plate was incubated in the incubator shaker at 100 RPM at 37°C for 20 hours. The same volume of DMSO was also added to MHB to grow the same concentration of bacteria as an additional control. The growth was measured at OD_{570} after 20 hours using a Thermo Fisher Scientific accuSkan GO UV/Vis Microplate Spectrophotometer (Hampton, NH). Each treatment condition was run in triplicate with a minimum of three trials per condition. The pH of all treatments was measured using an Orion Star A111 pH meter (Thermo Fisher Scientific, MA).

2.3 Combination treatment of rifampicin and D-limonene on the growth of *S. epidermidis* ATCC 12228 and RP62A in microtiter plate

After the initial results, two different concentrations of D-limonene (611.2 µg/mL and 1222.4 µg/mL) were selected for further analysis in the inhibition of the growth of both strains in combination with four concentrations of rifampicin (0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL). The same amount of final bacteria concentration of 5×10^4 CFU/mL were added to each well, forming a total volume of 200 µL. Each treatment condition was run in triplicate with a minimum of three trials completed. Wells containing MHB alone, MHB with bacteria, MHB and different concentrations of D-limonene or rifampicin served as controls. Each microtiter plate was incubated in the incubator shaker at 100 RPM at 37°C and the growth was measured at OD_{570} after 20 hours.

2.4 The inhibition of the growth analysis on the combination treatment on ATCC 12228 and RP62A after 6 hours and 20 hours in conical tubes

As a result of the microtiter plate experiment, the concentration of 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and their combination were further evaluated on the growth inhibition on both ATCC 12228 and RP62A in 15 mL conical tubes. Both strains were grown in 2 mL of MHB with 2% sodium chloride in plastic conical tubes (Thermo Fisher Scientific, MA). All combination treatments along with the control of either rifampicin or D-limonene were added to the tubes, mixed, incubated overnight in the incubator shaker at 100 RPM for 37°C. Bacterial concentration at OD_{570} was measured after 20 hours. The treatments were run in triplicates and at least five trials were performed. Further, nine additional tubes that contained MHB and bacteria only were incubated for 6 hours under the same condition. The OD_{570} was measured after 6 hours of incubation and the same concentration of rifampicin, D-limonene, and combination of rifampicin and D-limonene was added to these nine tubes. These tubes were then incubated for an additional 14 hours and the OD_{570} was measured again.

2.5 The alamarBlue® assay

A 96-well plate set up for the alamarBlue® assay using 100 µL of both bacteria with 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and their combination along with the control were performed. Half the plate contained RP62A, and the other half contained ATCC 12228. Following the assay protocol, 10 µL of alamarBlue®(AB) reagent (Thermo Fisher Scientific, MA) were added to the well for both the 6-hour and 20-hour trials as described before. The plate was placed in the incubator at 37°C and the optical density was read twice after 1 and 2 hours. A Multiskan FC Microplate Spectrophotometer (Thermo Fisher Scientific, MA) was used to measure the absorbance, OD_{570} and OD_{600}. All samples were run in triplicates, and a minimum of three trials were done.

The alamarBlue® assay formula (Formula 1) [25] was used to determine the percentage of cell reduction in each well.

$$\frac{(E_{oxi}600 \times A_{570}) - (E_{oxi}570 \times A_{600})}{(E_{red}570 \times C_{600}) - (E_{red}600 \times C_{570})} \times 100$$

Formula 1: alamarBlue® assay cell reduction percentage equation [25]

121 **2.6 Biofilm assay of the combination treatment on RP62A**

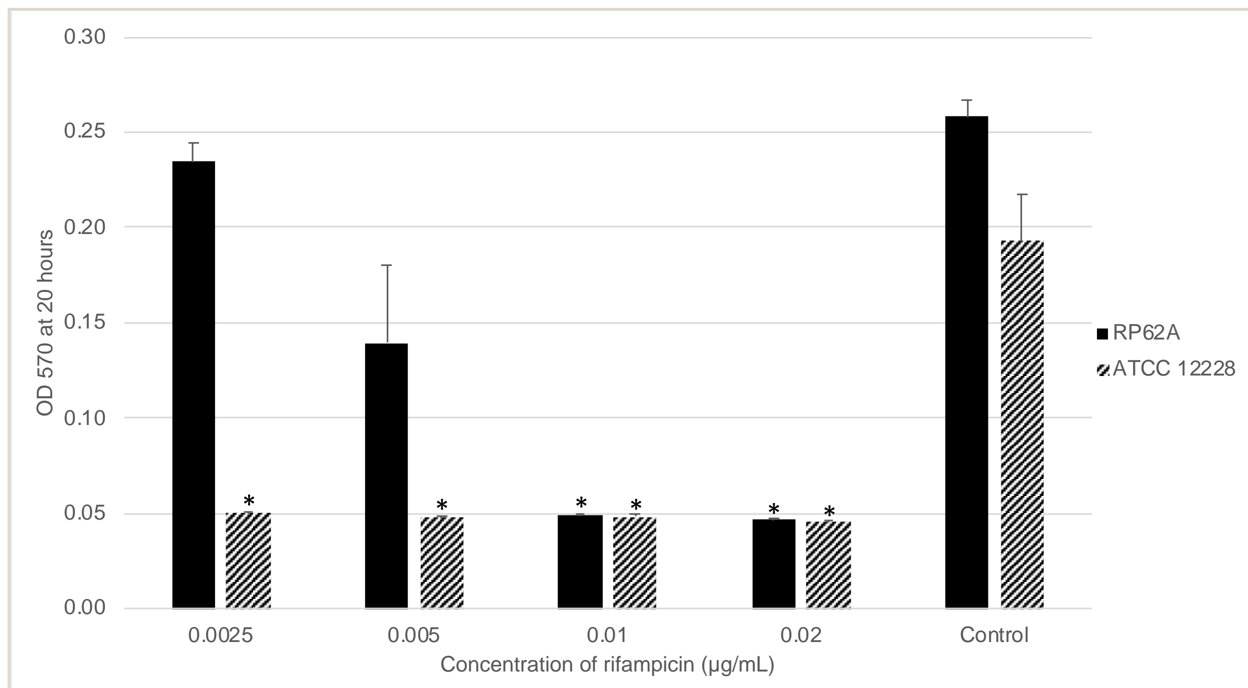
122 The biofilm assay was performed on the 96 well plate on RP62A only with the 0.005 µg/mL of rifampicin, 611.2 µg/mL of
123 D-limonene and the combination treatment along with the control using the protocol from Stephanovic' *et al* [26]. Bacteria
124 were grown as previously described on a microtiter plate for 20 hours at 37°C. The biofilm assay was then performed and
125 OD_{570} was measured. All samples were run in triplicates with a minimum of three trials were done.

126 **2.7 Statistical analysis**

127 A One-way ANOVA and Tukey's Honest Significant Difference (HSD) tests were used when comparing the growth of both
128 strains on different concentrations of rifampicin, D-limonene, and the combination with the control. The biofilm formation of
129 RP62A after the treatment was added from the beginning or after 6 hours of growth were compared with the control in
130 both the microtiter plate assay and the alamarBlue® assay. The significance value was set at $P < .05$. Statistical analyses
131 and other measurements presented (average of the bacterial growth, standard error of mean (SEM) and figures) were
132 performed using SPSS version 26 or Excel.

133 **3. RESULTS**
134 **3.1 Growth analysis of rifampicin and D-limonene treatment on ATCC 12228 and RP62A**

135 The growth of ATCC 12228 was significantly inhibited with all concentrations tested of rifampicin. After 20 hours, there
136 was a significant difference in the growth inhibition using 0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL of
137 rifampicin when compared to the control of the bacteria alone without any treatment, $P < .05$ (Figure 1). However, there
138 was a significant difference in the growth inhibition on RP62A with 0.01 µg/mL and 0.02 µg/mL of rifampicin when
139 compared to the control, $P < .05$ (Figure 1). The lower concentrations of rifampicin of 0.005 µg/mL and 0.0025 µg/mL
140 could only inhibit the growth of RP62A but not the ATCC 12228. In addition, there was a significant difference in growth
141 inhibition after 20 hours with the concentration of 1222.4 µg/mL of D-limonene when compared to the control in both
142 ATCC 12228 and RP62A ($P < .05$) (Figure 2). All lower concentrations of limonene (152.8 µg/mL, 305.6 µg/mL and 611.2
143 µg/mL) did not inhibit the growth of both bacteria. Thus, the MIC of the rifampicin and D-limonene on RP62A was found to
144 be 0.01 µg/mL and 1222.4 µg/mL respectively. For either rifampicin or D-limonene treatment, growth inhibition was more
145 prevalent against ATCC 12228 than RP62A. DMSO did not inhibit the growth of both bacteria (data not shown). The pH of
146 D-limonene alone was 4.2 and compared to a 7.1 when added to MHB. The rifampicin alone or when added to MHB and
147 the combination of rifampicin and D-limonene were found to have a neutral pH of about 7.2.



148

149 Figure 1. The effect of different concentrations of rifampicin on the growth (OD_{570} + SEM) of *S. epidermidis* RP62A and
150 ATCC 12228, (*The significance of $P <.05$ for the treatment when compared to the control), SEM: standard error of mean.

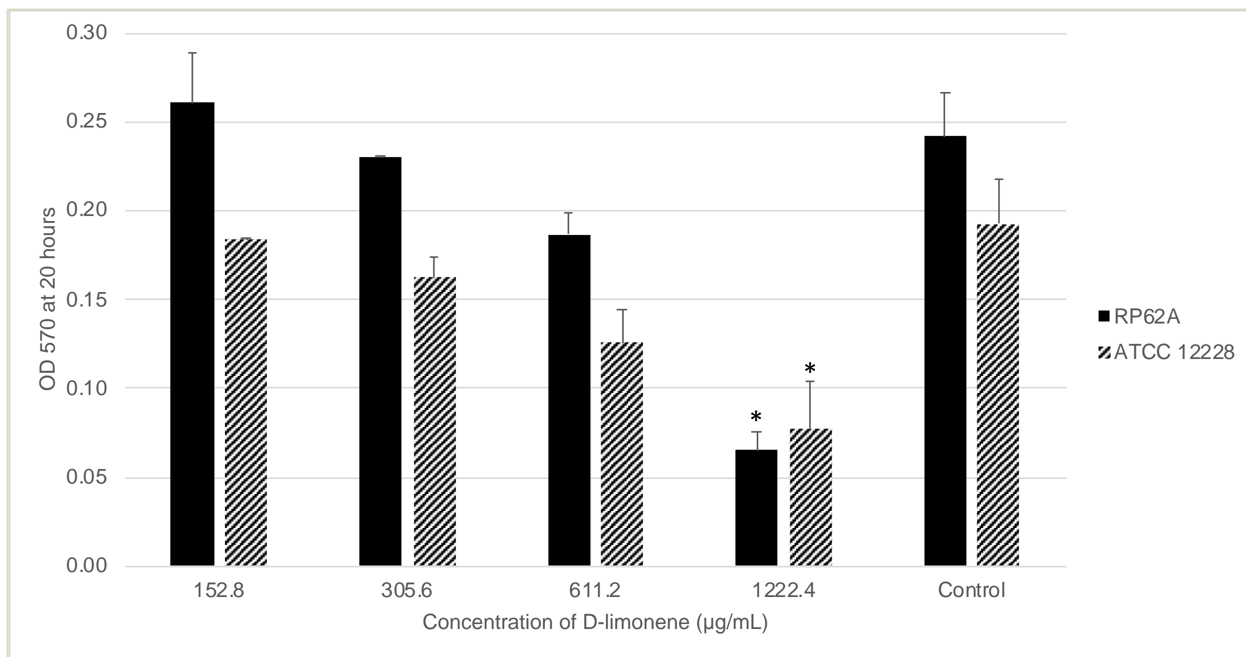
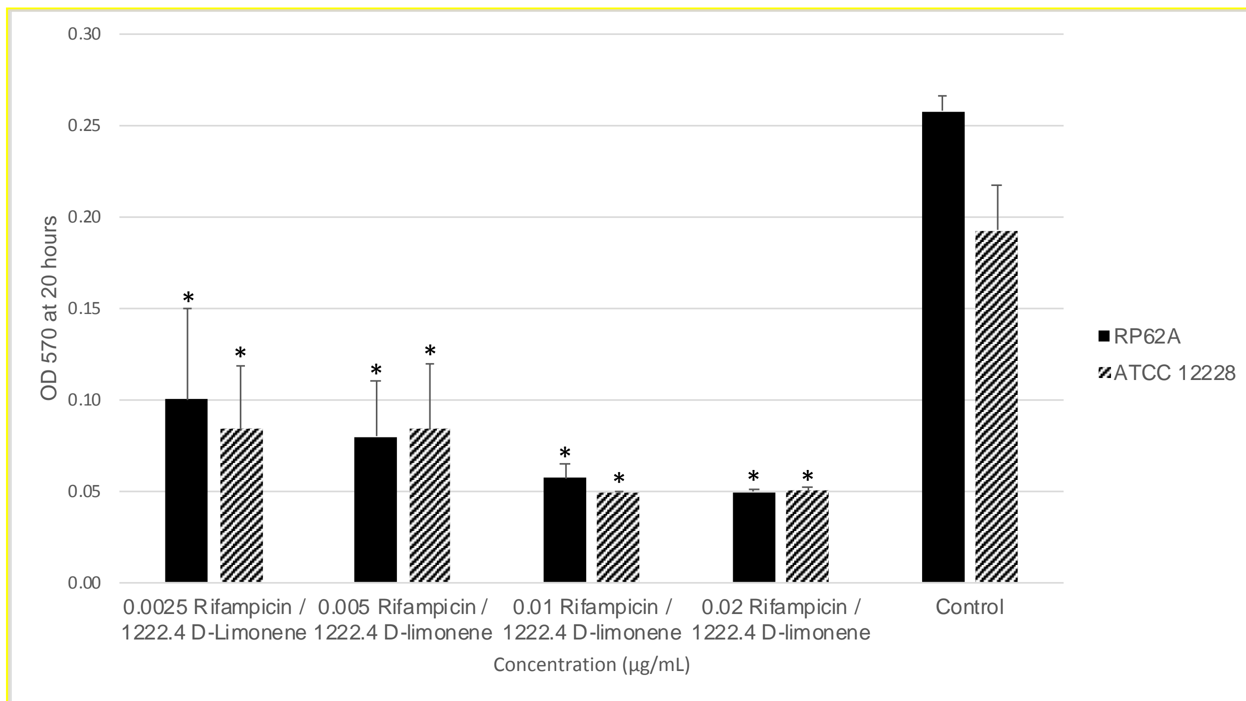


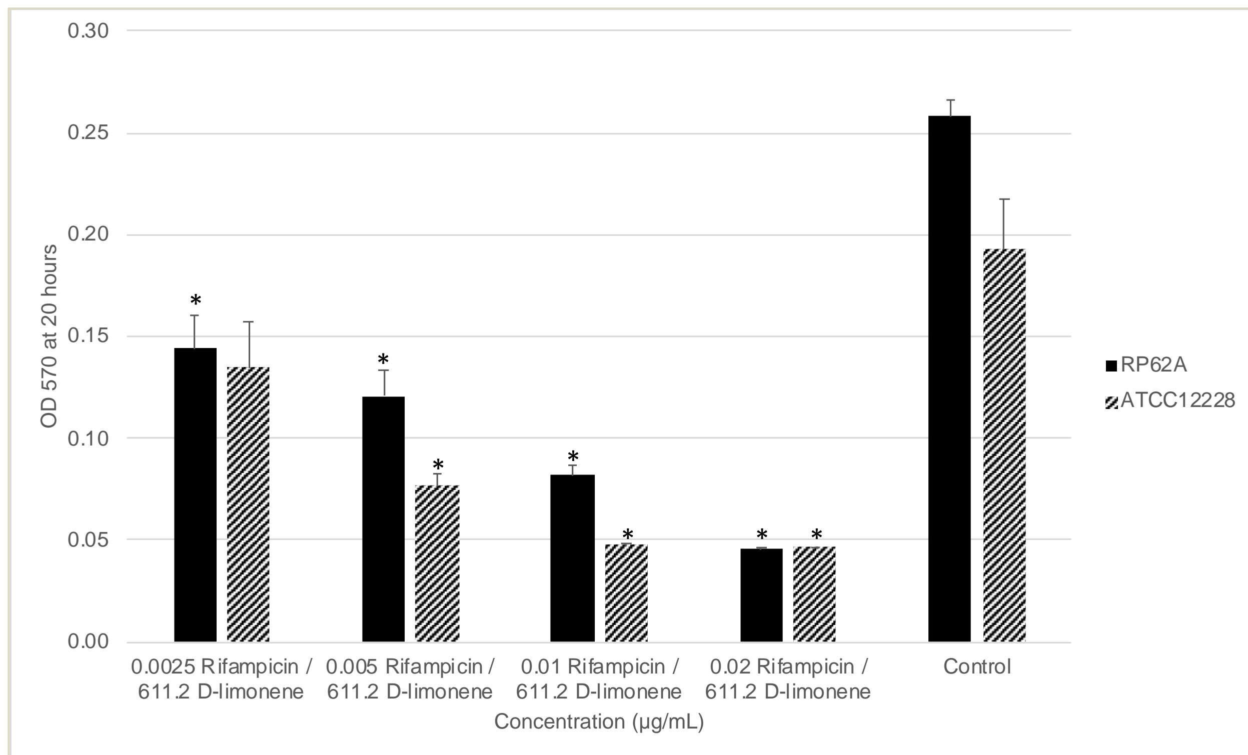
Figure 2. The effect of different concentrations of D-limonene on the growth (OD_{570} + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of *P* <.05 for the treatment when compared to the control), SEM: standard error of mean.

3.2 Growth analysis on the combination treatment on ATCC 12228 and RP62A in microtiter plate

Two different concentrations of D-limonene (611.2 µg/mL and 1222.4 µg/mL) were used in combination with four different concentrations of rifampicin (0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL) to study the growth inhibition on ATCC 12228 and RP62A. When 1222.4 µg/mL of D-limonene concentration were combined to all different concentrations of rifampicin, the inhibition of the growth of both strains was significant when compared to the control ($P < .05$) (Figure 3). However, when 611.2 µg/mL of D-limonene was used in combination with the four different concentrations of rifampicin, all treatments showed significant growth inhibition on RP62A except the combination with 0.025 µg/mL of rifampicin had shown no significant effect on the growth on ATCC 12228 (Figure 4).



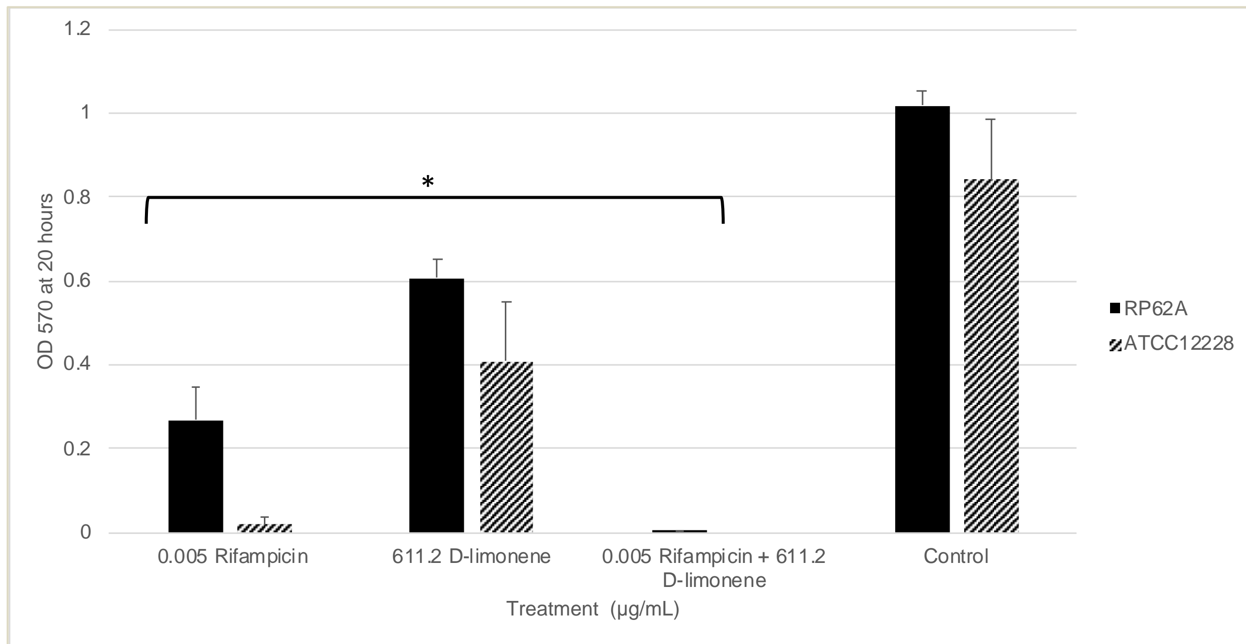
163 Figure 3. The effect of different concentrations of the rifampicin with 1222.4 µg/mL of D-limonene on the growth (OD_{570} + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of *P* <.05 for the treatment when compared to the control), SEM: standard error of mean.



169 Figure 4. The effect of different concentrations of the rifampicin with 611.2 µg/mL of D-limonene on the growth (OD_{570} + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of *P* <.05 for the treatment when compared to the control), SEM: standard error of mean.

3.3 Inhibition of growth on the combination treatment on ATCC 12228 and RP62A in the conical vials

In the following experiments, a concentration of 0.005 µg/mL of rifampicin was chosen due to its inability to inhibit the bacterial growth completely. A concentration of 611.2 µg/mL of D-limonene was also chosen as the next lower concentration than the MIC in the combination treatment. After 20 hours of incubation, the growth of ATCC 12228 and RP62A in conical tubes was significantly inhibited when treated with 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination of both rifampicin and D-limonene when compared to the control of bacteria alone without treatment (*P*<.05) (Figure 5). However, the effect of the combination treatment resulted in almost no growth with zero absorbance in both strains (Figure 5). In another experiment, after both bacteria were grown for 6 hours, all treatment conditions were tested and incubated for 14 hours. Significant results were also shown with the inhibition of the growth of ATCC 12228 and RP62A with the treatments when compared to the control for all conditions (*P*<.05) (data not shown).

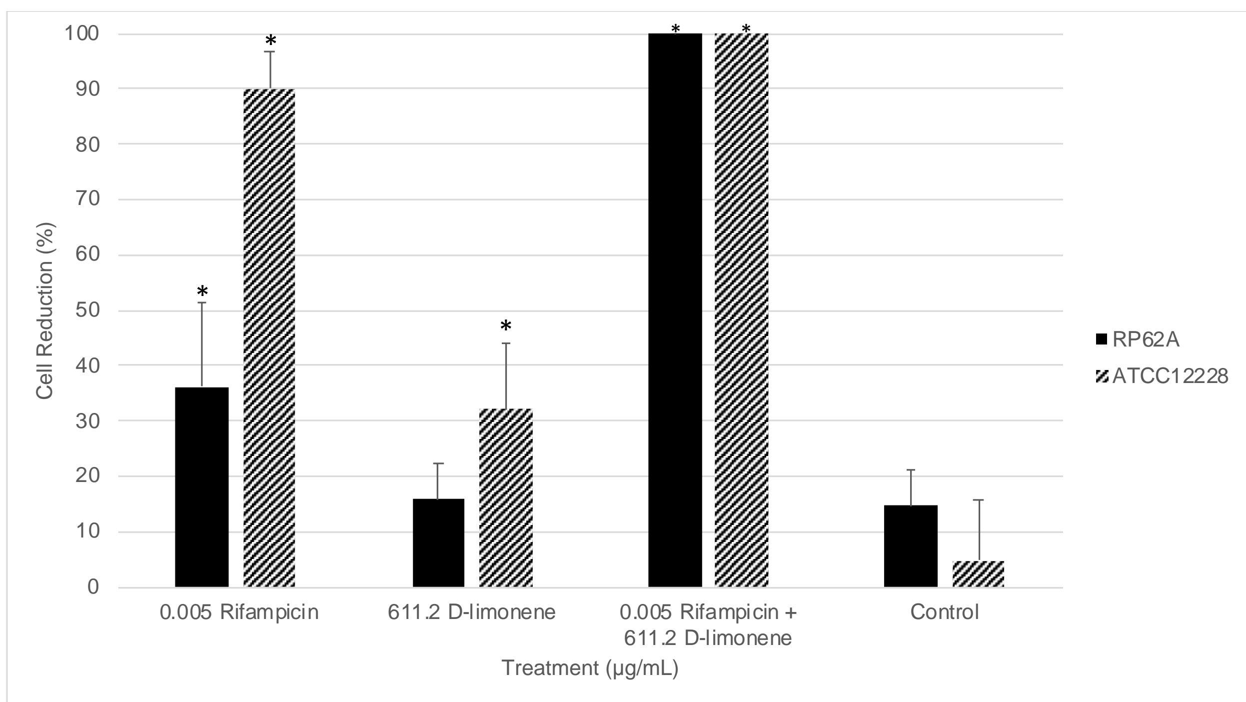


183

184 Figure 5. The effect of the growth (OD_{570} + SEM) of *S. epidermidis* RP62A and ATCC 12228 in the plastic conical vials
185 with 0.005 μg/mL of rifampicin, 611.2 μg/mL of D-limonene and the combination after 20 hours of incubation, (*The
186 significance of $P < .05$ for the treatment when compared to the control), SEM: standard error of mean.

187 **3.4 The alamarBlue® assay of the combination treatment on cell reduction of ATCC 12228 and RP62A**

188 The results of the alamarBlue® assay after the 20 hours incubation of 0.005 μg/mL of rifampicin treatment on ATCC 12228
189 had an average of 89.9% of cell reduction when compared to 36.1% of cell reduction on RP62A (Figure 6). When the
190 611.2 μg/mL of D-limonene alone was added, there was a 15.9 % cell reduction on RP62A and a 32.1% cell reduction on
191 ATCC 12228 (Figure 6). Meanwhile, the combination treatment of 0.005 μg/mL of rifampicin and 611.2 μg/mL of D-
192 limonene had an average of 96.2% cell reduction on RP62A and a 100% cell reduction on ATCC 12228 (Figure 6).
193 However, after 6 hours of the growth of the bacteria, treatments were added to the bacteria to continue to grow for the
194 next 14 hours, the result of the alamarBlue® assay was inconclusive (data not shown).

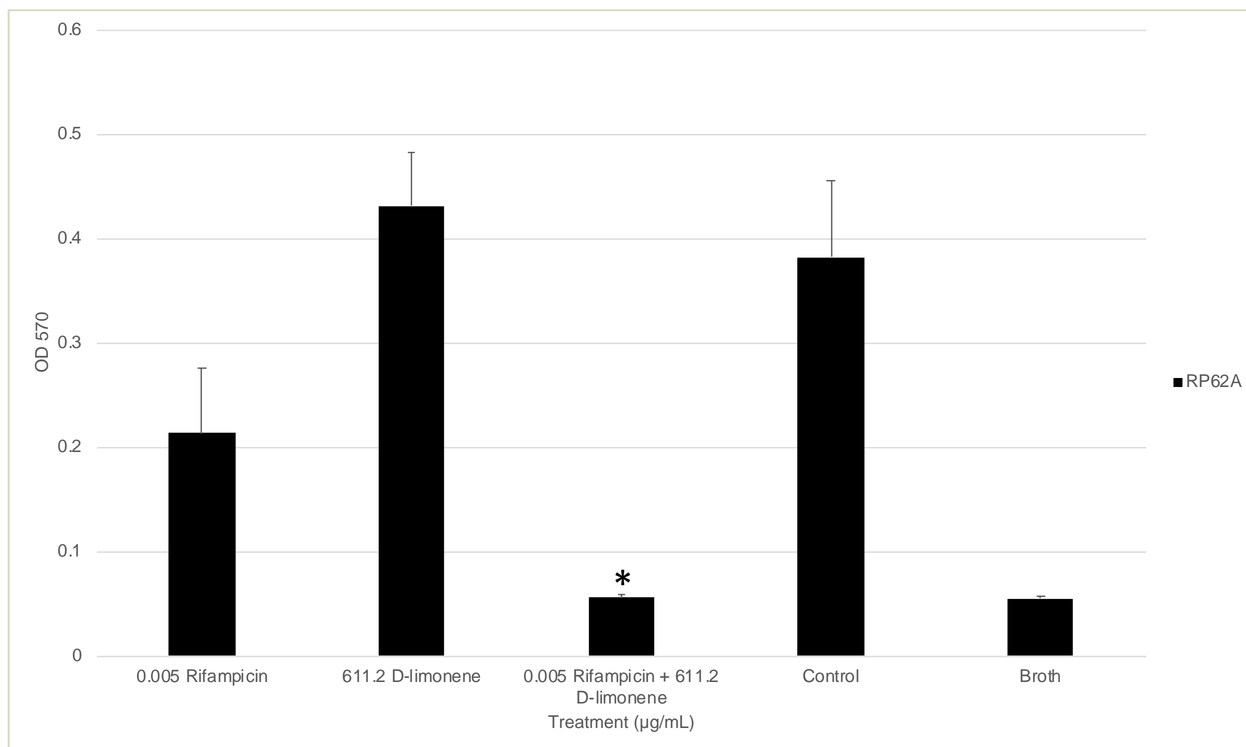


195

196 Figure 6. The percentage (+SEM) of cell reduction after the treatment of 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-
197 limonene and the combination using the alamarBlue® assay. The reduction potential at OD_{570} and OD_{600} of *S. epidermidis*
198 RP62A and ATCC 12228 was measured and calculated, (*The significance of $P<.05$ for the treatment when compared to
199 the control), SEM: standard error of mean.

200 **3.5 The Biofilm assay analysis on the combination treatment of RP62A**

201 The biofilm assay of RP62A was performed in a 96-well plate, result showed the combination treatment of 0.005 µg/mL of
202 rifampicin and 611.2 µg/mL of D-limonene had significantly inhibited the biofilm formation of RP62A when compared to the
203 control of bacteria alone (*P*<.05) (Figure 7). The broth was used as an additional control and had a slight background
204 OD_{570} absorbance of 0.05 in the biofilm assay.



205

206 Figure 7. The biofilm assay (OD_{570} + SEM) of *S. epidermidis* RP62A with the treatment of 0.005 µg/mL of rifampicin, 611.2
207 µg/mL of D-limonene and the combination, (*The significance of $P<.05$ for the treatment when compared to the control),
208 SEM: standard error of mean.

209 **4. DISCUSSION**
210 The goal of this study was to determine the effective combination of the concentrations of rifampicin and D-limonene on
211 the inhibition of the growth and biofilm formation of *S. epidermidis* RP62A compared to the same concentration of
212 rifampicin alone. A combination of 0.005 µg/mL of rifampicin and 611.2 µg/mL of D-limonene was found to inhibit the
213 growth of both RP62A and ATCC 12228 and limit the biofilm formation of RP62A, rather than when using the same
214 concentration of 0.005 µg/mL rifampicin alone. The results of this study showed that there was only a reduction of 9.15 %
215 in growth of RP62A with the treatment of 0.0025 µg/mL of rifampicin, however, there was a 74.08%. reduction in growth of
216 ATCC 12228 with the same concentration of rifampicin. In contrast, the growth of RP62A was greatly reduced by 81.12%
217 and 81.82% respectively when compared to the control when treated with 0.01 and 0.02 µg/mL of rifampicin. The 0.005
218 µg/mL of rifampicin with a 46% reduction in the growth of RP62A was then chosen in this study due to its inability to inhibit
219 the bacterial growth completely.

220 Previous research showed that the resistance of rifampicin on RP62A can be due to several mutations on the *rpo*B gene,
221 the beta subunit of the RNA polymerase in the bacteria [27]. The capabilities of D-limonene of manipulating the
222 phospholipid bilayer membrane were shown in *E. coli* [28]. In a recent study on the effects of the limonene on the Gram-
223 positive *Listeria monocytogenes,* results showed that limonene can increase the cell membrane permeability with the
224 leakage of nucleic acids and proteins and can hinder the ATP synthesis [29]. This means that D-limonene may provide an
225 additive effect in manipulating the bilayer membrane and providing an easy access for rifampicin to cross it and alter the
226 growth of the bacteria despite the presence of the biofilm.

In another study using 0.1 µg/mL of rifampicin in assessing the inhibition of the growth of RP62A, the results showed that the slow growth of the bacteria and biofilm formation may prevent the bactericidal effect of the antibiotic [19]. An *in vitro* study by Fazly Bazzaz *et al.* showed that the MIC of rifampicin for *S. epidermidis* DSMZ3270 biofilm-forming strain was 0.03 mg/mL in its free form and solid lipid nanoparticles loaded with rifampicin can reduce the production of the biofilm as opposed to the free form [30]. The use of 0.005 µg/mL of rifampicin in this study seems lower, however, with the combination use with D-limonene, the additional inhibitory effect was found. We also found that rifampicin had a profound reduction in bacterial growth in reference to wild-type ATCC 12228 when compared to the biofilm formation in RP62A.

The D-limonene is a generally recognized as safe (GRAS) product and was found to be able to be used in food preservation alone or in combination with lethal heat treatments [21, 28]. This essential oil is known for its antimicrobial properties and was able to inhibit the growth of *E. coli* O157:H7 and the biofilm production of multi-drug resistant *S. aureus* [21, 24, 31]. The MIC of D-limonene for RP62A and ATCC 12228 strains was found to be 1222.4 µg/mL in our current study. We decided to use the next lower concentration of 611.2 µg/mL of D-limonene with different combinations of rifampicin in the latter growth inhibition studies. Our results indicate that the combination of 611.2 µg/mL of D-limonene with 0.005 µg/mL of rifampicin could achieve the cell reduction of RP62A to 96.2%, when compared to only using the 611.2 µg/mL of D-limonene alone producing a 15.9% cell reduction.

From the first set of combination treatment trials using microtiter plates, the bacterial growth reflected by the measurement of optical density readings was found to be lower than using the conical vials. We believe the volume of the conical tubes of 2 mL versus the 0.2 mL tubes may create a difference to achieve a better growth of the bacteria and resulted in higher optical density measurement. A study by Nicolau Korres *et al.* showed *Klebsiella pneumoniae* Subsp. *pneumoniae* and the positive control of RP62A formed biofilm on glass and polystyrene plastic [32]. It is important to note that the 96-well plate used is made up of polystyrene while the conical vial tubes are made up of polypropylene which may explain the absence of biofilm ring observed in the conical vial tubes. In another study with the use of essential oil on different biofilm-forming coagulase-negative staphylococci, the inhibition of the growth of *S. warneri* by Polytoxinol^{TM} was found to be at the initial phase of the bacterial cell adherence to the polystyrene surface [33]. Thus, the optical density readings were found to be lower in the combination treatment trials using the 96-well plates than the conical tubes. The OD_{570} was used throughout the growth studies and the results were comparable and better than the OD_{600}, this may be due to the biofilm formation of RP62A and the nature of the 96-well plates.

When we studied the combination treatment used on a different growth phase of the bacteria, our results indicated that the combination treatment was effective in the inhibition of the bacterial growth from the beginning and the start of the log phase in both RP62A and ATCC 12228. In our previous study, both bacteria started to grow in the log phase after 6 hours of incubation [23]. Further, the alamarBlue® assay is widely used to study cell viability and metabolic function [34]. In our study of assessing the alamarBlue® assay, the results showed that there was almost a total of 100% of cell viability reduction using the combination treatment on RP62A and ATCC 12228. However, a more significant result was found when treatment was added in earlier on, as biofilm may start to form after 6 hours of incubation. A previous study found the biofilm production of *S. epidermidis* strain occurs in 2-stages; first with the adhesion to the surface and later with the formation of the multilayer cell clusters [35]. Thus, we believe that once the biofilm is formed in RP62A, the combination treatment may not be effective.

Lastly, the microtiter biofilm assay confirmed the ability of the combination treatment of rifampicin and D-limonene to inhibit the biofilm formation of RP62A. However, the control well of broth alone produced a residual color and thus, an optical density reading. Other modified biofilm assay methods may be used in the future to obtain better results [36]. The pH of D-limonene alone had an acidic pH of 4.0, while D-limonene and rifampicin and the combination in the MHB were found to be neutral. The acidic property of D-limonene can cause the inhibition of the growth of bacteria. Further, the essential oil extract of *Citrus limon* of the pericarp contains about 69.9% of limonene and is different from the pure chemical form we used in the study [37]. The 97% pure form of the D-limonene was used throughout our studies and for the future experiment, the pure oil extract of limonene can be used for the evaluation of the inhibition of the biofilm-forming *S. epidermidis*.

5. CONCLUSION

A combination of 0.005 µg/mL of rifampicin with 611.2 µg/mL of D-limonene inhibited the growth of the biofilm-forming *S. epidermidis* RP62A. The alamarBlue® assay confirmed the effect of the combination treatment on the inhibition of the growth and the biofilm formation of RP62A. The results of this study hold a promising future in using alternative combination treatments to treat Staphylococcus infections in hospital settings and to further decrease the dosage and usage of prescribed antibiotics.

ACKNOWLEDGEMENTS

We would like to thank the Tribeta Biological Honor Society for providing us grants to complete this project. We would also thank Manhattanvlle College for providing internal funding to support the research project. We would also like to thank

Natcha Rummaneethorn, Charlene Caoili and Thimmy Garbenius for their contribution to the research. Finally, we would like to thank Dr. Christopher J. Pappas for his guidance and the Department of Biology at Manhattanville College for supporting this research.

COMPETING INTERESTS DISCLAIMER

Authors have declared that no competing interests exist. Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

REFERENCES

1. Jones, D., Deibel, RH., Niven, CF. Identity of *Staphylococcus epidermidis*. J Bacteriol. 1963;85:62-67. https://doi.org/10.1128/jb.85.1.62-67.1963
2. Otto, M. *Staphylococcus epidermidis* – the "accidental" pathogen. Nat Rev Microbiol. 2009;*7*(8):555–567. https://doi.org/10.1038/nrmicro2182
3. Nguyen, T H., Park, MD., Otto, M. Host response to *Staphylococcus epidermidis* colonization and infections. Front Cell Infect Microbiol. 2017;7:90. https://doi.org/10.3389/fcimb.2017.00090
4. Bryers, JD. Medical biofilms. Biotechnol Bioeng. 2008; 100(1):1–18. https://doi.org/10.1002/bit.21838
5. Lai, Y., Di Nardo, A., Nakatsuji, T., Leichtle, A., Yang, Y., Cogen, A. L. et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nat Med. 2009;15(12):1377–82. https://doi.org/10.1038/nm.2062
6. Lai, Y., Cogen, AL., Radek, KA., Park, HJ., MacLeod, DT., Leichtle, A. et al. Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. J Invest Dermatol. 2010;130(9):2211–21. https://doi.org/10.1038/jid.2010.123
7. Scharschmidt, TC., Vasquez, KS., Truong, H-A., Gearty, SV, Pauli, ML., Nosbaum, A. et al. A wave of regulatory T cells into neonatal skin mediates tolerance to commensal microbes. Immunity. 2015;43(5):1011–21. https://doi.org/10.1016/j.immuni.2015.10.016
8. Wojtyczka, R., Orlewska, K., Kępa, M., Idzik, D., Dziedzic, A., Mularz, T. et al. Biofilm formation and antimicrobial susceptibility of *Staphylococcus epidermidis* strains from a hospital environment. Int J Environ Res Public Health. 2014;11(5):4619–4633. https://doi.org/10.3390/ijerph110504619
9. Cheung, GYC., Otto, M. Understanding the significance of *Staphylococcus epidermidis* bacteremia in babies and children. Curr Opin Infect Dis. 2010; 23(3):208–16. https://doi.org/10.1097/QCO.0b013e328337fecb
10. Vestby LK, Grønseth T, Simm R, Nesse LL. Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics (Basel)*. 2020;9(2):59. doi:10.3390/antibiotics9020059
11. Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., et al. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacterio. 1996;178(1):175–83. 10.1128/jb.178.1.175-183.1996
12. Stewart, PS., Costerton, JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358(9276):135-138. https://doi.org/10.1016/S0140-6736(01)05321-1
13. Gerke, C., Kraft, A., Süßmuth, R., Schweitzer, O., Götz, F. Characterization of the N-Acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. J Biol Chem. 1998; 273:18586–18593. 10.1074/jbc.273.29.18586
14. Conrady, DG., Brescia, CC., Horii, K., Weiss, AA., Hassett, DJ. Herr, AB. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proc Natl Acad Sci. U.S.A. 2008;105(49):19456–61. https://doi.org/10.1073/pnas.0807717105

335 15. Diekema, DJ., Pfaller, MA., Schmitz, FJ., Smayevsky, J., Bell, J., Jones, RN. et al. Survey of infections due to
336 *Staphylococcus* species: Frequency of occurrence and antimicrobial susceptibility of isolates collected in the
337 United States, Canada, Latin America, Europe, and the Western Pacific Region for the SENTRY Antimicrobial
338 Surveillance Program, 1997–1999. Clin Infect Dis. 2001;32(s2):S114–S132. https://doi.org/10.1086/320184
339 16. Rogers, KL., Fey, PD., Rupp, ME. Coagulase-negative staphylococcal infections. Infect Dis Clin North Am. 2009;
340 23:73–98. https://doi.org/10.1016/j.idc.2008.10.001
341 17. Spellberg, B., Bartlett, JG., Gilbert, DN. The future of antibiotics and resistance. N Eng J Med. 2013;368(4):299–
342 302. https://doi.org/10.1056/NEJMp1215093
343 18. Floss, HG., Yu, T-W. Rifamycin - mode of action, resistance, and biosynthesis. Chem Rev. 2005;105:621–632.
344 https://doi.org/10.1021/cr030112j
345 19. Zheng, Z., Stewart, PS. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. Antimicrob Agents
346 Chemother. 2002;46(3):900–3. https://doi.org/10.1128/AAC.46.3.900-903.2002
347 20. Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., Mauriello, G. Membrane toxicity of antimicrobial
348 compounds from essential oils. J Agric Food Chem. 2007;55(12):4863–4870. https://doi.org/10.1021/jf0636465
349 21. Sun, J. D-Limonene: Safety and Clinical Applications. Altern Med Rev. 2007;12(3):259–264. Retrieved from
350 http://www.anaturalhealingcenter.com/documents/Thorne/articles/Limonene12-3.pdf
351 22. Del Toro-Arreola, S., Flores-Torales, E., Torres-Lozano, C., Del Toro-Arreola, A., Tostado-Pelayo, K., Guadalupe
352 Ramirez-Dueñas, M. et al. Effect of D-limonene on immune response in BALB/c mice with lymphoma. Int
353 Immunopharmacol. 2005;5(5):829–838. https://doi.org/10.1016/J.INTIMP.2004.12.012
354 23. Rummaneethorn, N., Caoili, CM. D-limonene Increases efficacy of rifampicin as an inhibitor of *in vitro* growth of
355 opportunistic *Staphylococcus epidermidis* RP62A. In The National Conference On Undergraduate Research
356 (NCUR) 2016 (pp. 487–498). Asheville, North Carolina. Retrieved from
357 https://apps.cur.org/ncur2021/archive/Display_NCUR.aspx?id=95371
358 24. Espina, L., Pagán, R., López, D., García-Gonzalo, D. (2015). Individual constituents from essential oils inhibit
359 biofilm mass production by multi-drug resistant *Staphylococcus aureus*. Molecules. 2015;20(6):11357–11372.
360 https://doi.org/10.3390/molecules200611357
361 25. alamarBlue® Assay U.S. Patent No. 5,501,959 by Thermo Fisher Scientific
362 https://tools.thermofisher.com/content/sfs/manuals/PI-DAL1025-1100_TI%20alamarBlue%20Rev%201.1.pdf
363 26. Stepanović, S., Vuković, D., Dakić, I., Savić, B., Švabić-Vlahović , M. A modified microtiter-plate test for
364 quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40:175–179. DOI: 10.1016/s0167-
365 7012(00)00122-6
366 27. Yu, ML., Greenwood-Quaintance KE., Brinkman, CL., Lee, JYH., Howden, BP., Patel, R. Rifampicin resistance in
367 *Staphylococcus epidermidis*: molecular characterisation and fitness cost of rpoB mutations. Int J Antimicrob.
368 Agents. 2018;51(5):670-677. doi: 10.1016/j.ijantimicag.2017.12.019
369 28. Espina, L., Gelaw, TK., de Lamo-Castellvi, S., Pagan, R., Garcia-Gonzalo, D. Mechanism of bacterial inactivation
370 by (+)-limonene and its potential use in food preservation combined processes. PLoS One. 2013;8(2):e56769.
371 https://doi.org/10.1371/journal.pone.0056769
372 29. Han,Y., Sun, Z., Chen, W. Antimicrobial susceptibility and antibacterial mechanism of limonene against *Listeria*
373 *monocytogenes*. Molecules (Basel). 2020;25(1):33. https://doi.org/10.3390/molecules25010033
374 30. Fazly Bazzaz, BS., Khameneh, B., Zarei, H., Golmohammadzadeh, S. Antibacterial efficacy of rifampicin loaded
375 solid lipid nanoparticles against *Staphylococcus epidermidis* biofilm. Microb Pathog. 2016;93:137-144.
376 https://doi.org/10.1016/j.micpath.2015.11.031
377 31. Van Vuuren, SF., Viljoen, AM. Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in
378 combination. Flavour Fragr J. 2007;22(6):540-544. Doi: https://doi.org/10.1002/ffj.1843
379 32. Nicolau Korres, AM., Aquije, GM., Buss, DS., Ventura, JA., Fernandes, PM., Fernandes, AA. Comparison of
380 biofilm and attachment mechanisms of a phytopathological and clinical isolate of *Klebsiella pneumoniae* Subsp.
381 *pneumoniae*. Sci World J. 2013;925375. doi:10.1155/2013/925375
382 33. Al-Shuneigat J., Cox SD., Markham JL. Effects of a topical essential oil-containing formulation on biofilm-forming
383 coagulase-negative staphylococci. Lett Appl Microbiol. 2005;41(1):52-5. DOI: 10.1111/j.1472-765X.2005.01699.x
384 34. Rampersad SN. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in
385 cell viability bioassays. Sensors (Basel). 2012;12(9):12347-12360. doi:10.3390/s120912347

386 35. Mack D., Riedewald J., Rohde H, Magnus T., Feucht HH., Elsner HA., Laufs R., Rupp ME. Essential functional
387 role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. Infect Immun.
388 1999;67(2):1004-8. doi: 10.1128/IAI.67.2.1004-1008.1999.
389 36. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp. 2011;(47):2437. doi:10.3791/2437
390 37. Klimek-Szczykutowicz, M., Szopa, A., & Ekiert, H. *Citrus limon* (Lemon) Phenomenon-A Review of the Chemistry,
391 Pharmacological Properties, Applications in the Modern Pharmaceutical, Food, and Cosmetics Industries, and
392 Biotechnological Studies. Plants (Basel, Switzerland). 2020; 9(1):119. https://doi.org/10.3390/plants9010119