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Article

Cocktail therapy to inhibit multispecies biofilm in cystic fibrosis patients

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SUMMARY

Bacterial biofilms cause 80% of life-threatening chronic infections such as urinary tract infections, wound infections, indwelling catheter infections, and severe pneumonia in immunocompromised patients such as those with cystic fibrosis (CF). ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter*) pathogens are known to form dense biofilms highly resistant to antibacterial treatments. This can lead to antibiotic use at higher concentrations, resulting in antibiotic resistance and toxicity. Furthermore, biofilms exist in a dense multispecies form where they communicate with each other by a mechanism called Quorum Sensing (QS), making them hostile to several antibiotics. The focus of our project was to use a combination treatment consisting of FDA-approved concentrations of three different Quorum Quenching (QQ) agents that specifically target the QS mechanisms between the three major interspecies biofilm-pathways within the CF lung: chlorogenic acid to inhibit *Pseudomonas fluorescens-Staphylococcus epidermidis* signaling, carvacrol to inhibit *P. fluorescens-Burkholderia pyrrocinia* signaling, and 6-gingerol to inhibit *P. fluorescens-Candida albicans* signaling. Computational analysis of the CF lung microbiome using QIIME2 and PICRUSt2 confirmed the necessity of QS in multispecies biofilm pathways. Docking analysis further supported the binding affinities of each treatment and targeted enzymes. The combination treatment demonstrated a near 80% efficacy in inhibiting *P. fluorescens-S. epidermidis-B. pyrrocinia-C. albicans* biofilm. Treatment safety was supported by a cytotoxicity assay on human alveolar epithelial cells. These findings can be translated into developing novel adjuvants to deliver the cocktail treatment *in vivo*, thus reducing morbidity and mortality from chronic biofilm-related infections and saving millions of dollars in product decontamination.

INTRODUCTION

Biofilms are a complex aggregation of microbial cells that are encased within an extracellular matrix that can form and attach to a variety of surfaces, such as rocks, cooking vessels, and medical implants. A biofilm matrix is a self-produced polymer consisting of polysaccharide, protein, and DNA. Bacterial biofilms cause 80% of all chronic microbial infections in the body, including urinary tract infections, wound infections, in indwelling catheters, nosocomial infections, and lung infections in cystic fibrosis (CF) patients (1). Although biofilm is rarely studied in a multispecies context, the microbial community of a biofilm commonly contains more than one species of bacteria, with each species influencing the other's gene expression and growth. Current treatments focus primarily on mono-species rather than multispecies biofilm despite multispecies biofilm being more virulent (2). Most ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter*) pathogens are opportunistic and therefore with immunosuppression in CF, these microbes often become virulent, causing deadly infections that take thousands of lives each year (1). The infected CF lung microbiome in particular is known to contain multiple microorganisms cooperating and communicating with one another in the same biofilm. Among the major pathogens in the CF lung are *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Candida albicans* (3, 4). The primary method of communication between these microbes is quorum sensing (QS), or the production of signaling molecules called autoinducers (AIs). AIs allow both intra and interspecies communication, which results in the alteration of gene expression.

AIs accumulate as the bacterial population in the environment becomes increasingly dense and bind to bacterial transcription factors to express genes that encode for more virulence factors within biofilm communities (4). This type of communication allows for the persistence and virulence of biofilm. One solution to disrupt this communication is quorum quenching (QQ), or the mechanism of QS inhibition by preventing AI production, detection and binding, or enzymatically degrading AIs (7). Furthermore, considering the increasing prominence of multispecies biofilm in CF patients, novel QQ agents to mitigate biofilm growth would drastically reduce morbidity and mortality from these infections.

The compound 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) is one of the AIs secreted by *P. aeruginosa*. HQNO interferes with the flow of electrons to the cytochrome bc 1 complex during the electron transport chain, causing electrons to be prematurely donated to O2 (9). The resulting production of reaction oxygen species (ROS) causes bacterial cell autolysis and the release of DNA, facilitating biofilm

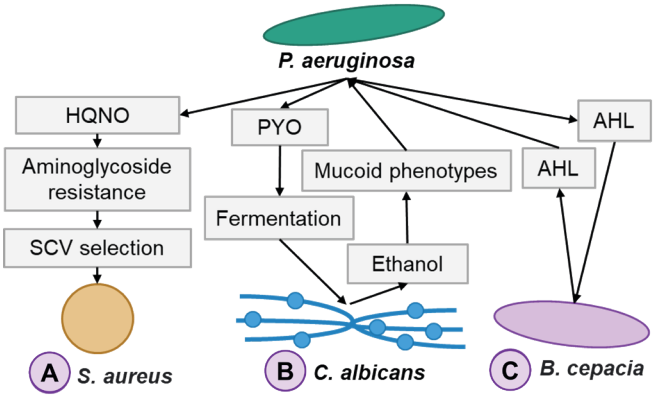

Figure 1: Primary interspecies biofilm pathways in the CF lung. A) *P. aeruginosa* induces 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) production and thereby aminoglycoside resistance in *S. aureus* strains, selecting for highly resistant small colony forming variants (SCV). **B)** *P. aeruginosa* secretes pyocyanin (PYO), inducing *C. albicans* fermentation and ethanol production that selects for mucoid phenotypes of *P. aeruginosa*. **C)** *P. aeruginosa* secretes 3-oxo-dodecanoyl HSL (3OC12-HSL), which is detected by *B cepacia*.

formation (9). HQNO also enhances antibiotic resistance and biofilm formation in S. aureus strains that selectively grow as small colony variants (SCVs) such as methicillin resistant *Staphylococcus aureus* (MRSA) (9, 10) **(Figure 1)**. CA is a natural phenolic compound and antioxidant, touted to significantly inhibit the formation of *P. aeruginosa* biofilm and swarming motility by downregulating QS related genes thus inhibiting the PqsR transcription factor, which facilitates the transcription of pqsA encoding for HQNO (12) **(Figure 2A)**.

Phenazine production, particularly pyocyanin, in *P. aeruginosa* plays a key role in co-colonization of *C. albicans* and *P. aeruginosa* (a fungus and bacteria, respectively). Pyocyanin [5-methyl-1(5H)-phenazinone], a secondary metabolite produced by nearly all *P. aeruginosa* strains triggers tissue damage by its redox cycling and production of ROS, thereby inducing oxidative stress and promoting the release of extracellular DNA (eDNA) in the outermost matrix of the biofilm (15). eDNA acts as a scaffold for the biofilm, protecting the biofilm from external environmental hazards including antibiotics. In mixed biofilms with *C. albicans, P. aeruginosa* upregulates its production of pyocyanin and other phenazines relative to single-species biofilms (3,4), exerting a switch from respiration to fermentation, which in turn upregulates *C. albicans* ethanol production (4). This production of ethanol in turn stimulates *P. aeruginosa* biofilm formation, resulting in mucoid phenotypes with enhanced growth rates (3, 4) **(Figure 1)**. *In silico* studies have supported 6-gingerol utilizing hydrogen bonding to bond to the QS receptor LasR, which regulates the transcription of the *phz* operon that encodes for phenazines such as pyocyanin (17).

Synergistic interactions between *P. aeruginosa* and *B. cepacia* (both gram negative bacteria) through QS systems that rely on N-Acyl homoserine lactone (AHL) signal molecules to coordinate expression of virulence factors associated with the formation of biofilms (4). In mixed biofilms, *B. cepacia* can recognize several AHL signals produced by *P. aeruginosa*, and AHL-quorum signaling systems are pivotal in the role of *P. aeruginosa-B. cepacia* multispecies biofilm formation (3-5, 13). Carvacrol is a natural phenolic compound that inhibits

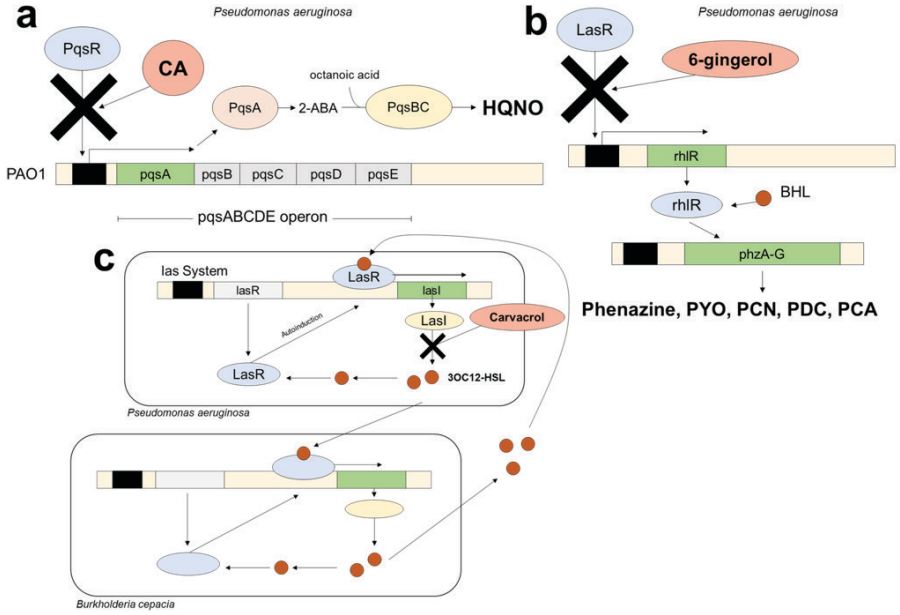

Figure 2: Schematic representation of QS inhibiting treatment mechanisms. A) CA inhibits binding of PqsR therefore inhibiting transcription of PqsA, abscisic acid (2-ABA), and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) production. **B)** 6-gingerol inhibits transcription of rhlR therefore inhibiting N-butanoyl-l-homoserine lactone (BHL) induced production of phenazines, pyocyanin (PYO), Phenazine-1-carboxamide (PCN), phenazine-1,6-dicarboxylic acid (PDC), and Phenazine-1-carboxylic acid (PCA). **C)** Carvacrol inhibits production of 3OC12-HSL synthesis by LasI, thereby inhibiting AHL reception by *B. cepacia*.

LasI enzyme and down-regulates the cascade of signals, pathways, and expression of virulence of P. aeruginosa. Docking analysis on the interactions between carvacrol and LasI proteins found that carvacrol inhibited P. aeruginosa AHL production by nearly 60% (14) **(Figure 2C)**.

In an effort to mitigate the growth of deadly bacterial biofilm commonly found in CF patients, the goal of this study was to identify novel inhibitory agents to bacterial quorum sensing between *P. aeruginosa*, *S. aureus*, *B. cepacia*, and *C. albicans in vitro*. We used QQ agents as they are safe, widely studied, and effective in inhibiting both single and multispecies biofilm formation (7, 8). Chlorogenic acid (CA), carvacrol, and 6-gingerol were tested as potential QQ agents to disrupt the interactions between *P. aeruginosa* and *S. aureus*, *P. aeruginosa* and *C. albicans*, and *P. aeruginosa* and *B. cepacia*, respectively.

RESULTS

Treatments Reduced Total Biofilm Viability

We treated dual and multispecies biofilm groups consisting of *Pseudomonas fluorescens*, *Staphylococcus epidermidis*, *Burkholderia pyrrocinia*, and *C. albicans* with appropriate concentrations of CA, carvacrol, and 6-gingerol (individually for dual-species groups and in combination for multispecies group). Biofilm groups were then measured for their viability. A lower RFU (Relative Fluorescence Unit) value indicates a lower number of viable bacteria in the biofilm, according to resazurin assay. Our results showed that the dual and multispecies biofilm control groups with no treatment (*P. fluorescens* + *S. epidermidis*, *P. fluorescens* + *B. pyrrocinia*, *P. fluorescens* + *C. albicans*, and *P. fluorescens* + *S. epidermidis* + *B. pyrrocinia* + *C. albicans*) had RFUs of 4.102, 4.050, 3.992, and 4.002, respectively ($p < 0.01$). The biofilm rings encased in the control tubes were visibly darker and more prominent compared to those in the treated groups. The multi-species biofilm exhibited a slightly lower RFU compared to the *P.fluorescens* + *S.epidermidis* biofilm due to competition of antibiotic resistant bacterial and fungal strands. Of treatments applied to *P. fluorescens* + *S. epidermidis*, CA exhibited the greatest inhibitory effect on biofilm formation, with an RFU of 1.199 ($p < 0.01$) and inhibition efficacy of nearly 70% **(Figure 3)**. Of treatments applied to *P. fluorescens* + *B.pyrrocinia*, carvacrol exhibited the greatest inhibitory effect on biofilm formation, with an RFU of 1.701 ($p < 0.01$) and inhibition efficacy of nearly 60% **(Figure 3)**. Of treatments applied to *P. fluorescens* + *C. albicans*, 6-gingerol exhibited the greatest inhibitory effect on biofilm formation, with an RFU of 2.222 ($p < 0.01$) and inhibition efficacy of nearly 45% **(Figure 3)**. Finally, of treatments applied to *P. fluorescens* + *S. epidermidis* + *B. pyrrocinia* + *C. albicans*, the combination treatment of CA, carvacrol, and 6-gingerol exhibited the greatest inhibitory effect on biofilm formation, with an RFU of 0.829 ($p < 0.01$) and inhibition efficacy of nearly 80% **(Figure 3)**. Additionally, a cytotoxicity assay on mammalian cells revealed that the alveolar epithelial cells treated with triple cocktail treatment grew 52 viable cells in all 5 blocks of the hemocytometer compared to 55 viable cells in the control group ($p < 0.01$), demonstrating that the cocktail therapy did not inhibit the growth of mammalian cells. This suggests the treatment's potential in vivo safety.

Qiime2 Analysis Supported Biofilm Pathogens

The computing environment Qiime2 was utilized to examine the relative abundance of bacterial species in sputum (mucus produced by lungs) samples from patients with and without CF. Evaluation of the output plot revealed *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Burkholderia* to be predominant bacterial genera among CF sputum samples. Among all CF patient sputum samples, *Pseudomonas* exhibited the most consistently high relative abundance within several CF patients, from 23-78%. While *Burkholderia* was not consistently seen in many CF patients, it

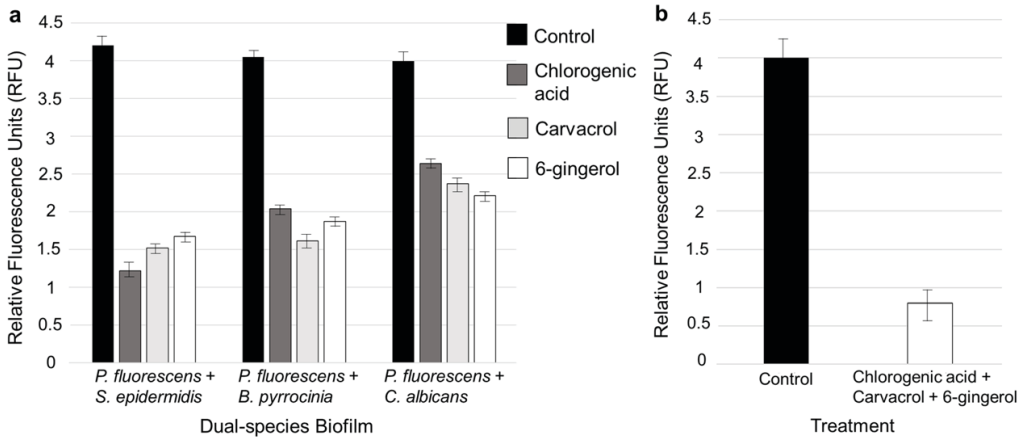

Figure 3: Combinations of co-cultured *P. fluorescens*, *S. epidermidis*, *B. pyrrocinia*, and *C. albicans* treated with CA, carvacrol, and/or 6-gingerol. A) Relative fluorescence units (RFU) and inhibition efficacies (%) of *P. fluorescens* co-cultures with *S. epidermidis*, *B. pyrrocinia*, or *C. albicans* treated with CA, carvacrol, or 6-gingerol. **B)** Relative fluorescence units (RFU) of *P. fluorescens-S. epidermidis-B. pyrrocinia-C. albicans* multispecies cultures treated with CA + carvacrol + 6-gingerol. Error bars represent standard error.

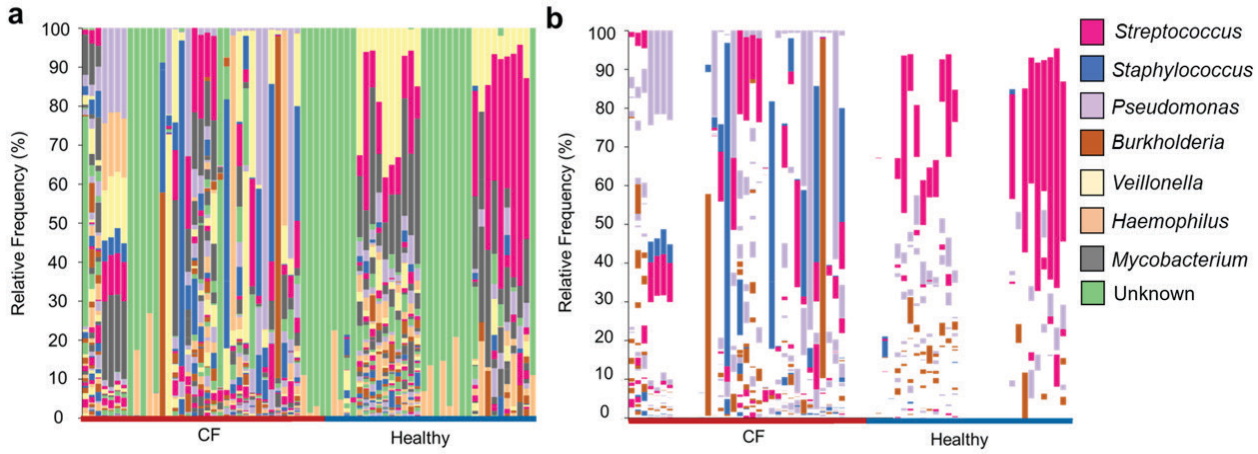

Figure 4: Relative frequency of bacterial strains present in CF vs. healthy patients. Qiime2 analysis was performed on 16S rRNA data from lung microbiome sputum samples. **A)** Relative frequency of all bacterial strains in CF and healthy patients. **B)**. Relative frequency of *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Burkholderia* bacterial strains in CF and healthy patients.

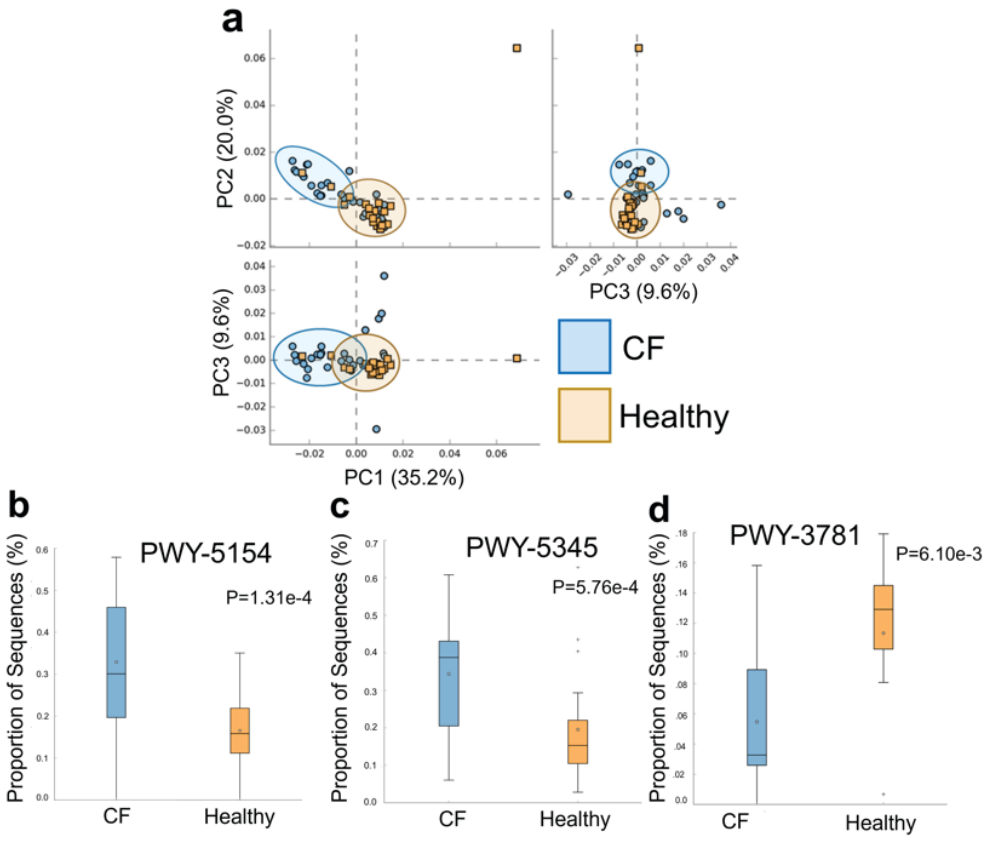

Figure 5: Metabolic pathway and enzyme expression of CF vs. healthy lung microbes. 16S rRNA from lung microbiome sputum samples was analyzed using PICRUSt2. **A)** Principal component analysis plot of bacterial strains in CF vs. healthy patients. **B)** L-arginine biosynthesis III via N-acetyl-L-citrulline expression in CF vs. healthy patients. **C)** L-methionine biosynthesis by sulfhydrylation expression in CF vs. healthy patients. **D)** Aerobic respiration I via cytochrome c expression in CF vs. healthy patients. All CF vs. healthy data was statistically significant, with $p < 0.001$.

exhibited a near 88% relative abundance within one CF patient and nearly 46% relative abundance in another, making it the single most abundant bacterial strain in one patient sample examined in this study **(Figure 4)**. Finally, *Staphylococcus* and *Streptococcus* also demonstrated high relative abundance ranging from 12-80% **(Figure 4)**. *Streptococcus* was more abundantly seen in healthy patient samples than CF patients; nearly every healthy sputum sample that was classified had anywhere from 6-61% relative abundance of this genus. *Staphylococcus*, *Pseudomonas*, and *Burkholderia* were seen to have higher relative abundance in CF sputum samples compared to healthy samples **(Figure 4B)**. This may indicate that *Staphylococcus*, *Pseudomonas*, *Burkholderia*, and more virulent bacterial biofilm forming species are able to outcompete less fit *Streptococcus* strains in CF patients. Finally, of the samples investigated, combinations of *Staphylococcus*, *Pseudomonas*, and *Burkholderia* were seen in nearly every CF sample, particularly *Pseudomonas* and *Staphylococcus*, indicating high likelihood of interspecies interactions between these strains in the CF lung **(Figure 4B)**.

PICRUSt2 Analysis Supported Biofilm Pathways

We then investigated upregulated metabolic pathways in CF patients using PICRUSt2 to compare sputum samples from healthy and CF patients. Predicted functional profiling using PICRUSt2 revealed that pathways for L-arginine biosynthesis III via N-acetyl-L-citrulline (pathway 5154 on MetaCyc) and L-methionine biosynthesis by sulfhydrylation (pathway 5345 on MetaCyc) were significantly enriched in CF samples compared to healthy samples **(Figure 5B-C)** (18). Interestingly, aerobic respiration I via cytochrome c (pathway 3781 on MetaCyc) was more enriched in healthy samples compared to CF samples **(Figure 5D)**. Finally, principal component analysis displayed a strong correlation within CF sputum strain and healthy sputum strain groups, supporting a clear distinction between the groups **(Figure 5A)**.

Molecular Docking Supported Treatment Efficacy

Molecular docking studies were conducted to assess the binding affinities of each treatment (CA, carvacrol, and 6-gingerol) when bound to docking sites on their respective targeted enzymes. AutoDock Vina (20) was used to dock each of the three receptor structures (PqsR, LasI, and LasR) with each receptor molecule (CA, carvacrol, and 6-gingerol, respectively). CA, which docks to PqsR, had the highest estimated affinity score of -7.1 kcal/mol, whereas the LasI and LasR structures had slightly lower affinities of -6.3 kcal/mol and -5.7 kcal/mol with carvacrol and 6-gingerol, respectively. Affinity scores lie within the typical range of scores associated with federally approved medications, suggesting the treatment's potential inhibition efficacy in-vitro. 3D structures of each complex were generated using PyMOL to identify potential hydrogen bonding between each treatment and their associated enzymes **(Figure 6A-C)**. CA formed ten hydrogen bonds with LEU-153, ALA-134, ASN-206, GLN-203, SER-201, ARG-209, and ALA-103 **(Figure 6A)**. 6-gingerol formed two hydrogen bonds with ARG 82 **(Figure 6B)**, and carvacrol formed no hydrogen bonds with LasI **(Figure 6C)**.

DISCUSSION

We investigated the efficacy of different treatments on multiple biofilms. BSL-1 organisms were used for experimentation due to safety concerns and resource limitations, however, they are nearly genetically identical

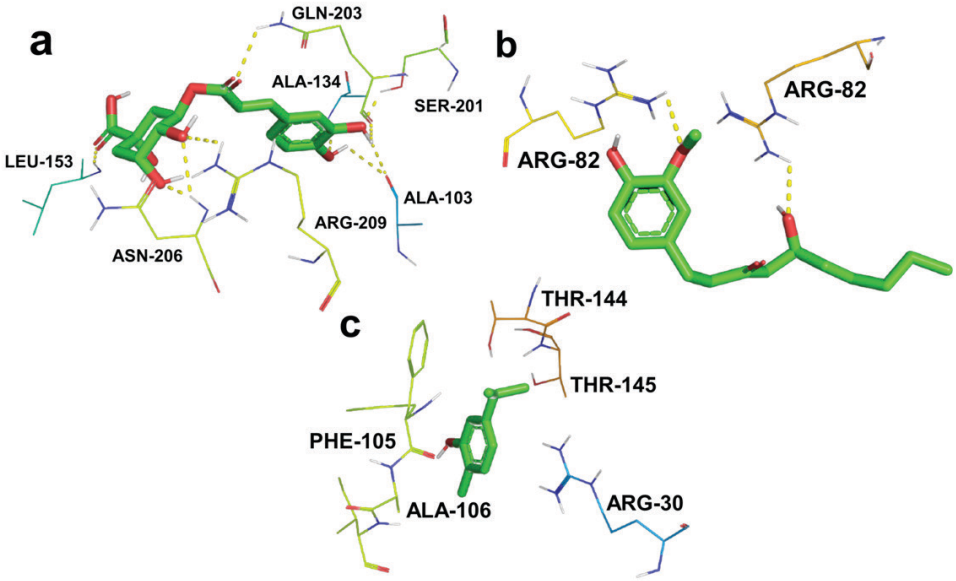

Figure 6: Autodock Vina analysis of protein-ligand interactions. A) Interaction analysis of PqsR binding with CA. **B)** Interaction analysis of LasR binding with 6-gingerol. **C)** Interaction analysis of LasI binding with carvacrol.

to the BSL-2 organisms the study originally planned to test on. *P. fluorescens* and *S. epidermidis* dual-species biofilm was best inhibited by CA as indicated by its low RFU value, suggesting that the pathway of HQNO production not only plays a pivotal role in the co-existence of these biofilm species, but also that CA effectively targeted the Pqs QS system. The *P. fluorescens* and *B. pyrrocinia* dual species biofilm was best inhibited by carvacrol, suggesting that the pathway of N-acetylcysteine QS molecule production not only plays a pivotal role in the co-existence of these biofilm species, but also that carvacrol effectively targeted the las QS system. Interactions between carvacrol's hydroxyl group with the amine group on the active site of the LasI enzyme could show promising inhibitory effects on LasI activity, demonstrating a potential reduction of the expression of lasR, biofilm, and swarming motility. The *P. fluorescens* and *C. albicans* dual species biofilm was best inhibited by 6-gingerol, suggesting that the pathway of phenazine production not only plays a pivotal role in the co-existence of these biofilm species, but also that 6-gingerol effectively targeted the rhl QS system. Finally, the multispecies consortium (*P. fluorescens*, *S. epidermidis*, *B. pyrrocinia*, and *C. albicans*) treated with all treatments (CA, carvacrol, and 6-gingerol) showed a significant reduction of biofilm growth, presenting its efficacy as a potential combination treatment. While further *in vivo* testing is needed, CF patients could be treated with this cocktail therapy in conjunction with smaller doses of antibiotics to cause a reduction in chronic bacterial biofilm-related infections, and thus also minimize the side effects associated with high concentrations of antibiotics (1-5).

Computational analysis of the infected and baseline CF lung microbiome supported the prevalence of these microbial species in the CF lung. Pathway and enzyme analysis supported the necessity of QS, and particularly HQNO, as a key player in the formation of multispecies biofilm. The upregulation of aerobic respiration I via cytochrome c in healthy patients compared to CF patients can be explained by HQNO being a potent inhibitor of mitochondrial NDH-2 in many species. HQNO is a known inhibitor of the mitochondrial cytochrome bc1 complex by binding to the quinol binding site (Qi). This leads to rupture of the organelle and eventually cellular apoptosis, which initiates eDNA release in the extracellular polymeric biofilm matrix, further supporting the role of HQNO in biofilm formation, infection, and virulence. Pathways such as this one should be investigated further.

Docking analysis also suggested the presence binding affinity between each treatment and targeted enzyme (LasR, LasI, and PqsR), further supporting the efficacy of these treatments on their targeted pathways of multispecies biofilm formation. In the future, fungal data within the CF lung microbiome will be investigated using Qiime2, and MICOM (Python package for metabolic modeling of microbial communities) will be used to study the role of fungi in multispecies biofilm.

The triple cocktail therapy investigated in this study has significant potential both as a treatment and prophylaxis. These findings may be applicable to the development of novel methods in treating patients with other chronic biofilm-related microbial infections besides pulmonary infections in cystic fibrosis, such as urinary tract infections, endocarditis, chronic wounds, and infections in indwelling catheters. This treatment can be delivered in conjunction with antibiotics, thus reducing morbidity and mortality from chronic microbial infections that account for millions of deaths across the world along with billions of dollars lost in equipment damage and product contamination (1-5).

MATERIALS AND METHODS

Preparing Bacterial and Fungal Inoculum

Pseudomonas fluorescens, *Staphylococcus epidermidis*, *Candida albicans*, and *Burkholderia pyrrocinia* were streaked on tryptic soy agar plates and grown at room temperature for 48-72 hours. Then, 10 mL of tryptic soy broth was inoculated with a small sample of each bacterial/fungal culture from the agar plates and gently vortexed until a homogeneous solution was achieved.

Preparing Treatment Stock Solutions

Samples of 100 mL of tryptic soy broth were each vortexed with 750 µL of carvacrol, 3 mg of CA, and 3.2 mg of 6-gingerol to prepare the carvacrol 0.75%, CA 30 µg/mL, and 6-gingerol 32 µg/mL stock solutions, respectively.

Preparing Control Dual-Species and Multispecies Biofilm

To prepare the control biofilm samples, 3 mL of tryptic soy broth was added to 0.3 mL of the *P. fluorescens* inoculum. Each of the 0.3 mL of *S. epidermidis*, *B. pyrrocinia* or *C albicans* inoculum were individually added to each of three sets of *P. fluorescens* inoculum tubes to begin culture of dual-species biofilm. The remaining three tubes were all cultured with 0.3 mL of all bacterial and fungal inoculum together to begin formation of multispecies biofilm, meant to model the diverse microbiome of the CF lung. 100 µL of the multispecies inoculum was plated onto each of the selective agar media (Mannitol-salt for *S. epidermidis*, Cetrimide for *P. fluorescens*, Sabouraud dextrose for *C. albicans*, *B. pyrrocinia* - selective agar for B. pyrrocinia) to isolate individual species and ensure no competitive inhibition of the microbes occurred.

Preparing the Experimental Dual-Species and Multispecies Biofilm

To prepare the experimental biofilm samples 3 mL of each treatment stock solution (carvacrol 0.75%, CA 30 µg/mL, and 6-gingerol 32 µg/mL) was pipetted into separate borosilicate tubes. 0.3 mL of *P. fluorescens* inoculum and 0.3 mL of *B. pyrrocinia* were added to each carvacrol 0.75% tube, 0.3 mL of *P. fluorescens* inoculum and 0.3 mL of *S. epidermidis* inoculum were added to each CA 30 µg/mL tube, and 0.3 mL of *P. fluorescens* inoculum and 0.3 mL of *C. albicans* inoculum

were added to each 6-gingerol 32 µg/mL tube. The remaining three tubes were all cultured with 1 mL of each treatment stock solution and 0.3 mL of each microbial stock solution. All twenty-four tubes (12 control, 12 experimental for a total of three trials) were then incubated at room temperature for three days to develop bacterial biofilm.

Quantifying Biofilm Viability using a Resazurin Assay

Liquid and free-floating planktonic cells were disposed of from each borosilicate tube and 3 mL of clean tryptic soy broth was added then gently vortexed to harvest the biofilm bacteria as a homogeneous mixture. 200 µL of each control and experimental trial was pipetted into each cell of a 96-well plate, each of which were treated with alamarBlue® reagent (Resazurin) and incubated at room temperature for 2 hours. Fluorescence of each dilution was recorded using a fluorescence reader with excitation set to 550 nm and emission to 600 nm.

Analyzing Cytotoxicity using Mammalian Cell Lines

A549 human alveolar epithelial cells were grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1 µG/mL Puromycin, with and without 3 mL of the treatment solution. After 72 hours, viable cells were counted in the control and experimental group using a hemocytometer after staining with trypan blue.

Qiime2

Computing environment Qiime2 was used to process and analyze the amplicon library data generated by targeting the 16S rRNA gene in bacterial communities to gain insight on the relative abundance of bacterial taxa. Sequencing data from 16s rRNA was collected from a total of 83 sputum samples from various patient studies (both infected and baseline CF patients as well as healthy patients) through National Center for Biotechnology Information's (NCBI) sequence read archive. Bacterial 16s rRNA genes were sequenced from different hypervariable regions (generally V4 or V3-V5). Raw sequences were filtered and denoised using DADA2, and no sequence base pairs were cut prior to denoising. Finally, single and paired sequence data was merged and imported in Qiime2 to investigate the relative abundance of bacterial species present in the samples.

PICRUSt2

PCIRUSt2 was used for the identification of potential biofilm-associated microbial functional signatures based on 16S rRNA gene sequencing of NCBI data. Outputs from Qiime2 were analyzed in PICRUSt2 to investigate the enzymatic pathways involved in the CF lung microbiome.

Molecular Docking of Proteins LasI, LasR, and PqsR of *P. fluorescens*

Crystallographic structures of LasI (PDB 1RO5), LasR (PDB 2UV0), and PqsR (PDB 4JVC) proteins were obtained from the Protein Data Bank and used as model receptors (19). Structures of Carvacrol, 6-gingerol, and CA ligands were obtained from PubChem and tested for the possibility of binding to each protein (20). AutoDock Vina was used for docking studies to study protein-ligand interactions and obtain affinity energy. PyMOL viewer (version v1.3r1) was used to convert protein-ligand docked structures from .pdbqt to .pdb format, and Mgl Tools was used to prepare protein and ligand files for docking (21).

Statistical Analysis

A one-way ANOVA test was conducted on each dual-species group (for a total of three tests) to determine whether differences between the mean RFUs of each treatment group were statistically significant. Additionally, a T-test was conducted on the multispecies group to determine whether differences between the mean RFUs of the control and experimental groups were statistically significant. A one-prop Z-test was conducted for on the percent of viable and non-viable cells in the viability assay. Finally, STAMP was used to create figures and conduct statistical significance testing for PICRUSt data. An alpha value of < 0.05 was taken as significant for all statistical tests.

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