INVESTIGATION OF NUTRIENTS AS TREATMENTS OF BACTERIAL BIOFILMS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Using nutrients bacteria utilize to grow as treatments of bacterial biofilms have been investigated. This dissertation examines the problem of the prevention and treatment of biofilms by: (i) testing the effect of the nutrient β-phenylethylamine (PEA) on growth, ATP content of biofilm, and biofilm biomass, (ii) testing the effect of ethyl acetoacetate (EAA) and other small molecules on growth, ATP content of biofilm, and biofilm biomass, and (iii) investigating three applications of PEA and EAA.

PEA could be used as both, prevention and treatment for bacterial biofilms. We observed a reduction of growth, ATP content of biofilm, and biofilm biomass of *Escherichia coli* K-12 AJW678 with increasing concentrations regardless of when PEA was added. PEA and EAA were able to reduce growth, ATP content of biofilm, and biofilm biomass for multiple bacterial strains, but the efficacy of the nutrients were strain dependent. PEA was found to effectively reduce growth, ATP content of biofilm, and biofilm biomass when used in multiple applications. When PEA was physically integrated in polyurethane, we observed at least a 20 % reduction of ATP content of biofilm for all the bacterial strain tested. To mimic a clinically relevant environment, biofilms were formed in silicone tubing and treatment of PEA and EAA were administered similar to antibiotic lock treatments (ALT). The PEA and EAA treatments reduced ATP content and biofilm biomass of multiple pathogens. PEA was also effective on ATP content, biofilm biomass, and cell counts when used in a microfluidic system.

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LIST OF ABBREVIATIONS

PEA ..... β-phenylethylamine

EAA ..... ethyl acetoacetate

ALT ..... antibiotic lock treatments

NDSU ..... North Dakota State University

EPS ..... extracellular polymeric substance

WHO ..... World Health Organization

UPEC ..... Uropathogenic *E. coli*

TJR ..... total joint replacement

MRSA ..... methicillin resistant *S. aureus*

VRSA ..... vancomycin resistant *S. aureus*

SHS ..... Superheated steam

AI ..... autoinducers

c-di-GMP ..... cyclic di-GMP

MSDS ..... Material Safety Data Sheets

GP ..... DL-α-glycerol phosphate

AAA ..... acetoacetic acetate

EHEC ..... enterohemolytic *E. coli*

DMSO ..... dimethyl sulfoxide

LB ..... Luria Bertani

TSA ..... tryptic soy agar

TSB ..... tryptone soy broth

PBS ..... phosphate buffered saline

BB ..... beef broth

CV ..... crystal violet

ATP ..... adenosine triphosphate

NE ..... norepinephrine

DHMA ..... 3,4-dihydroxymandelic acid

PEA-PU..... PEA-polyurethane

CFU..... colony forming units

CLABSI ..... central line-associated bloodstream infection

CHAPTER 1. LITERATURE REVIEW[1, 2]

Biofilms

Biofilms are group of bacteria that can attach to a variety of surfaces and can occur in many natural, environmental, clinical, and industrial settings [1]. Formation of biofilms occurs in several steps and in earlier models it has been postulated that each step is characterized by a specific cell surface organelle [2-5]. Reversible attachment is the first step of biofilm formation, where the bacterium may loosely attach to a surface and detach again. Flagella, fimbriae, and pili aid in the initial contact with the surface. Irreversible attachment occurs when the bacteria start to produce adhesions and some extracellular polymers. During the maturation phase the attached cells produce an extracellular polymeric substance (EPS) matrix which helps define the three-dimensional structure of the biofilm [2, 6-8]. This makes bacterial biofilms 100-1000 times harder to treat than planktonic bacteria with physical, chemical, and antibiotic treatment. The EPS and biofilm formation is one way bacteria protect themselves from antibiotics, biocides, and other chemical treatments [9-11]. The biofilm also provides a perfect environment for the exchange of genetic material, particularly antibiotic resistance genes, by putting bacteria in close contact with each other [12, 13]. The accumulation of mutations and other genes can confer resistance over time [14-16] (for a review see [9, 17]). Evidence supports that the development of biofilm facilitates the development of antibiotic resistance.

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[1] **Schroeder, M.**, Spadafore, M., Prüß, B.M. *β-phenylethylamine, a small molecule with a large impact*. WebMedCentral. 2013. 4(9). WMC003577. The material in this chapter was co-authored by Meredith Schroeder and Birgit M. Prüß. Meredith Schroeder was instrumental in the development of the conclusions that are advanced here. Meredith Schroeder also drafted and revised all versions of this chapter. Birgit M. Prüß served as proofreader and checked the math in the statistical analysis conducted by Meredith Schroeder.

[2] **Schroeder, M.**, Brooks, B.D., and Brooks, A.E. *The complex relationship between virulence and antibiotic resistance*. Genes. 2017. 8(1): 39. The material in this chapter was co-authored by Meredith Schroeder and Amanda E. Brooks. Meredith Schroeder was instrumental in the development of the conclusions that are advanced here. Meredith Schroeder also drafted and revised all versions of this chapter. Amanda E. Brooks served as proofreader and helped develop the concepts discussed in this chapter.

Additionally, antibiotic resistance is thought to be conferred through sub-populations of bacteria within the biofilm that are resistant to antibiotics, known as persister cells. Some persister cells produce enzymes that degrade antibiotics, whereas others use efflux pumps to eliminate the antibiotics [17]. The environment within the biofilm applies oxidative stresses to the bacteria, which has been shown to drive the bacteria into a highly mutative state [18-20]. Persister cells can also become metabolically inactive, which has critical implications for developing new treatments to prevent bacterial biofilms. In general, most antibiotic's mode of action is dependent on the metabolic activity of bacteria, for example β-lactams target cell wall synthesis during bacterial division, while quinolones work by interrupting DNA replication [17, 21-24]. In vitro work has shown that persister cells can stop actively growing and this makes the bacteria within the biofilm less susceptible to antibiotic effects [25]. This increased survival of metabolically inactive cells in comparison to actively growing bacteria in the presence of antibiotics is known as drug-indifference [22, 23, 26-29]. Biofilms facilitate antibiotic resistance, with more than 2 million Americans annually suffering from an antibiotic-resistant infection [30]. This has a significant economic burden (~$20 billion in direct healthcare costs [31]) on the world's healthcare systems. Recently CNBC reported that the increasing occurrence of antibiotic resistant infections is a significant health risk, while the World Health Organization (WHO) listed antibiotic resistant infections as one of the top three threats to global public health [32].

Bacterial biofilms role in the health care industry

Biofilms play a particularly important role in a clinical settings, 65 to 80 % of bacterial infections in humans have been attributed to biofilms, examples of biofilm associated infectious diseases include catheter-associated urinary tract infections [33], periodontitis [34], and otitis [35], as well as *Pseudomonas aeruginosa* infections of cystic fibrosis patients [36]. Table 1 summarizes

bacterial biofilm that forms on medical devices and implants. The bacterial pathogens that are attributed to the infection, and the medical device that these pathogens form biofilms on are indicated. Medical devices, such as catheters and implants, provide the perfect surface for biofilm formation and have been shown to contribute to chronic bacterial infections.

Table 1. Common examples of biofilm formation on medical devices.

Bacterial Pathogens	Medical Device	Biomaterial	Ref.
Uropathogenic *E. coli, P. aeruginosa, Acinetobacter baumanii, Klebsiella ornithinolytica, K. pneumonia, Enterococcus spp., Serratia marcescens,*	Urinary catheter	Polyurethane, silicone	[37-39]
	Contact lenses	Silicone hydrogel	[40, 41]
Cronobacter sakazakii, Enterobacter cancerogenus, K. pneumoniae, S. marcescens, Yersinia enterocolitica	Enteral feeding tubes	Polyurethane, polyvinylchloride	[42, 43]
C. sakazakii, E. cloaceae, P. aeruginosa, Acinetobacter spp., Y. enterocolitica	Orthodontic	Acrylic	[44]
Coagulase-negative *Staphylococci, Staphylococcus aureus,* enteric Gram-negative bacilli, *Candida spp.*	Intravenous catheter	Polyurethane, silicone	[45, 46]
S. aureus, K. pneumoniae, P. aeruginosa, and A. calcoaceticus-baumannii	Joint implants	Titanium, stainless steel, cobalt-chromium, ceramics, hydroxyapatite, polyethylene, polymethylmethacrylate (PMMA) cement	[47, 48]

One example of biofilm associated infections involving medical devices is infection by *S. aureus* and others on indwelling catheters. It is estimated that currently in the United State about 150 million intravascular catheters are used every year for critically ill patients, such as patients with chronic renal failure, cancer patients, ICU patients, and patients with long-term illness [46]. Annually in the U.S., millions of people die and billions of dollars in additional healthcare costs are the result of biofilm associated infections in the health care industry. Long-term use of a

catheter can result in overuse, low blood flow from clotting or narrowing of the vein, and infection all leading to the failure of the vascular access over time. When this occurs patients are left with one last option to provide treatments, a central line (or central venous catheter). A central line is a long, flexible tube usually made of polytetrafluoroethylene (Teflon), polyurethane, or silicone. This requires surgical implantation, where the catheter is threaded into the jugular vein and guided into the superior vena cava. Vascular access catheters are already the most expensive treatment option, but an infection can cost a patient on average an additional $15,000-$56,000. Hemodialysis patient's incidence of catheter infections have been cited to be as high as 29.6 % and of those patients that contract an infection about 25 % will die [49-51].

Another example of the negative impacts biofilms can have in health care is during total joint replacement (TJR) surgeries. Each year world-wide millions of people receive TJR surgery providing pain relief, restoring function, and improving patient quality of life [52]. In spite of advances in orthopedic procedures and biomaterials, infection remains a major complication in TJR surgery, with rates as low as 1-2 % to as high as 4-12 % [53]. Infections can result in implant removal and hardware replacement, which drives the risk of infection to a staggering 20-30 % and each revision surgery is estimated to cost $42,000-$56,000 (U.S) [54]. These infections result in a significant loss of life (over 1,000 deaths per year), many a direct result of the growing frequency of antibiotic resistant infections. Last-resort antibiotics, such as methicillin and vancomycin, which were once very effective at treating biofilm related infections are becoming insufficient due to overuse. Bacterial biofilm infections caused by antibiotic resistant strains, such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA) are becoming more prevalent in the health care industry (for a review see [55]).

Bacterial biofilms role in the food processing industry

In the food processing industry, bacterial biofilms can facilitate outbreaks of foodborne illness. Livestock, such as cattle, poultry, pigs, and seafood make up large portion of the food we consume and are a huge part of the agriculture industry. Microorganisms including pathogens can be naturally found in the intestinal tract of livestock, such as *E. coli* O157:H7 which is considered part of the normal flora in the stomach of ruminants [56]. Pathogens can be spread to other livestock when they are shed through feces and contaminate the soil and water in their environment. Infected animals that are not properly disinfected prior to slaughter can transmit bacterial pathogens to equipment, surfaces, and containers used in different stages of the process. [57]. Bacteria can spread to previously uninfected surfaces via biofilm formation, leading to outbreaks of infectious disease. Many bacterial pathogens have been associated with foodborne illnesses, such as *Pseudomonas spp.*, *S. marcescens*, *E. coli spp.*, and *S. simulans*. These bacteria strains have been identified as bacteria that can attach to the polyurethane and polyvinylchloride surfaces that line the conveyor belts used during different stages in food processing [58].

Annually it is estimated that 48 million Americans ingest bacteria contaminated food that results in illness and costs $77.7 billion in health care costs, workers compensation, and loss of product. Foodborne outbreaks have resulted in 300,000 hospitalizations and up to 5,000 deaths per year due to illness [59]. This could be attributed to the increased consumption of livestock and seafood over the years, which has greatly increased the likelihood for consumers to ingest food contaminated with bacterial pathogens. In addition, the demand for these foods, globalization, advancements in technology, and a competitive market have immensely changed the way we produce and process food [60]. The problem of foodborne outbreaks is compounded by the increasing emergence of antibiotic resistant bacteria due to the overuse of antibiotics, which can

be added to the animal feed and have even been used in fisheries for prevention and treatment of diseases [61, 62]. More importantly sub therapeutic doses of antibiotics have been shown to enhance growth of the animals, making it an attractive option for farmers [63]. Use of sub therapeutic doses of antibiotics can reduce the normal flora of the animal, which usually outcompete more pathogenic microorganisms.

Current treatments of bacterial biofilms

Despite many advances in treatment options, none have been able to completely eliminate biofilm related infections. One strategy to reduce biofilm associated infection in catheters has been to use antibiotic-lock therapy (ALT). ALT is when high concentrations of antibiotic solutions are left to dwell in the catheter while the catheter is not in use. Traditional antibiotics like vancomycin, cefazolin, ceftazidime, ciprofloxacin, gentamicin, and ampicillin are typically used for ALT treatment. ALT treatments have been shown to be effective at reducing catheter related blood stream infections of cancer patients, however no significant conclusions could be drawn due to differences in administration and dwell time [64]. Systemic antibiotic prophylaxis has traditionally been used to treat TJR and other infections associated with implants. However, while this treatment option is generally considered to be effective, problems with systemic antibiotic delivery include systemic side effects and low antibiotic concentration reaching the site of infection. This unintentionally contributes to the problem of antibiotic resistance [65, 66]. In food processing, strong chemicals have been used to reduced biofilm formation on the surface of food processing equipment., such as halogens, peroxygens, acids, and quaternary ammonium compounds. The chemical chlorine has been used in food processing due to its oxidizing and disinfecting power, but recently has been shown to cause resistance in biofilms of *Listeria monocytogenes* with repeated exposure (DeBeer and others 1994) [67].

Treatments of lactic acid and peracetic acid have also been shown to effectively reduce *Salmonella spp.* on pieces of chicken [68].

Another strategy has been to incorporate traditional antibiotics and antimicrobials directly into the materials of biomedical devices or the surface used to coat food processing equipment to prevent biofilm formation. Intravascular catheters integrated with chlorhexidine-silver sulfadiazine or minocycline-rifampicin, reduced bacterial colonization and blood-stream associated infections. However, recent studies have shown that the antimicrobial effect of chlorhexidine and silver sulfadiazine coated catheters only lasted 48 hours and may not be sufficient for reducing catheter related infections [69, 70]. Catheter materials or implants coated or embedded with metal nanoparticles have also been found to be effective at reducing biofilm formation of bacterial pathogens. Both silver and copper nanoparticles have been used due to their anti-microbial properties (for a review, please, see [71, 72]). In vitro, Ag/Cu-coated catheters have been shown to prevent the attachment of MRSA. The Ag/Cu coated catheter was also shown, using a rat animal model, to reduce the occurrence of catheter-related infections and bacteremia in comparison to non-coated catheters [73]. However, these materials are accompanied by high production costs, limited lifetimes, the emergence of resistant bacterial strains, and have even been shown to increase antibiotic resistance in some bacterial strains [74-76]. In addition, high doses of silver can have toxic effects on the human body and have been shown to accelerate thrombin formation and platelet activation when catheters were coated with silver nanoparticles [77, 78].

In TJR surgeries, vancomycin has been used to coat the surface of titanium metal implants and when tested against *S. epidermidis* it was shown to significantly decrease biofilm formation [79]. However, the use of antibiotics could expedite the emergence and frequency of

antibiotic resistant bacterial infections. While in food processing, quaternary ammonium salts have been used to modify surface materials used in food packaging, which has been shown to effectively kill fungi, gram positive bacteria, and gram negative bacteria [80]. Another group of scientists have developed a rechargeable antimicrobial material integrated with halamines, which has been shown to be effective against *L. monocytogenes* and *E. coli* O157:H7 and could provide continuous sanitation of food processing surfaces [81].

Finally, physical cleaning or physical alteration of the surface properties of biomaterials or medical devices is another approach to reducing biofilm formation on particular materials. Physical cleaning using wet heat, dry heat, pasteurization, and super-heated steam have all been used as sterilization methods to reduce biofilm formation in the food processing industry, as well as to sterilize instruments in the medical industry [82, 83]. Superheated steam (SHS) has been shown to be effective at reducing *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *S. enteritidis* phage type 30, and *L. monocytogenes* on almonds and pistachios [82]. Surface properties that have been altered and have been shown to have some antimicrobial effects, include chemical composition, hydrophilicity and hydrophobicity [84], surface roughness [85], and surface charge. Increased surface roughness has been shown to actually increase biofilm formation and promote bacterial settlement of *Geobacter sulferreducens* grown on steel cathodes [86]. Anti-adhesion materials could be used to help prevent early biofilm formation, however thus far the efficacy of these materials hasn't been promising with *in vivo* testing. None of these strategies have completely solved the problem of bacterial biofilm related infections in the medical and food processing industry, but novel approaches are desperately needed.

Current research on the treatments of bacterial biofilms

Current research is focused on reducing biofilm formation and the emergence of antibiotic resistance through signal transduction pathways, in order to disrupt cellular communication by introducing or modulating external signals to regulate bacterial phenotypes [87]. Communication amongst bacteria is essential for many cellular functions, such as biofilm formation, sporulation, competence, antibiotic resistance, and regulation of a variety of other virulence factors [88-90]. Bacteria communicate through two major signal transduction pathways, quorum sensing and two-component systems. Quorum sensing is a form of cell to cell communication that is responsive to changes in cellular density. In order to communicate, bacteria produce chemical signal molecules, called autoinducers (AI), which are released into the external environment to be taken up by surrounding bacteria. As bacterial density increases, the concentration of AI in the immediate environment also increases, which interacts with cell signal receptors on surrounding bacteria [91-93]. Acylated homoserine lactone AI mediate communication between gram-negative bacteria. Two additional autoinducers; AI-1 and AI-2, mediate species-specific communication molecules and interspecies communication molecules, respectively [94-97]. Other secreted peptides, also known as autoinducing peptides, facilitate gram-positive bacteria quorum sensing [98]. In addition to using quorum sensing as a means to communicate for biofilm formation, bacteria can also use quorum sensing to increase virulence. One study found that quorum sensing molecules enhanced attachment and biofilm formation of the strain *Acidovorax citrulli* [99]. Another example of this is that in *E. coli,* production of virulence factors and toxins are facilitated through quorum sensing [100]. Increased biofilm formation increases persistence of bacterial strains, which increases the virulence of the bacteria.

Enhancing virulence genes that increase biofilm formation can facilitate the exchange of antibiotic resistance genes.

Two-component signaling is a mechanism by which bacteria sense and respond to the environment around them. Two-component systems work by the binding of an external signaling molecule to a histidine kinase receptor protein that causes a phosphorylation cascade event which results in a response [101, 102]. Gene expression can be regulated through two-component systems in bacteria, which has been shown to impact virulence of bacterial strains [103]. In *E. coli*, there are 37 two-component systems that are triggered by a variety of different environmental changes [104]. One example of a two-component system *E. coli* has is the EnvZ/OmpR whose response regulator is OmpR and responds to changes in external osmolarity. Through a regulatory cascade event, OmpR stimulates curli production by activating the regulatory gene *csgD* , which then up regulates *csgBAC* operon that encodes for the structural components of curli [105]. This causes increased expression of *csgBAC,* resulting in increased biofilm formation [106]. WalK/WalR, a two-component system in *S. aureus* that controls multiple cellular functions, is essential to virulence and is being investigated as a potential target to combat staph infections [107]. Regardless of the specific bacterial communication or sensing mechanism, it is clear that such regulation enhances virulence and may provide targets for drug development that provide decreased chances for bacterial resistance due to 1) the lack of a selective life or death pressure and 2) fewer fitness advantages.

Another bacterial signal that has been shown to regulate many bacterial behaviors is the intracellular signaling molecule cyclic di-GMP (c-di-GMP). Elevated levels of c-di-GMP in the cell have been shown to play a key role in allowing bacteria to switch from the planktonic phenotype and the biofilm phenotype, while low levels of c-di-GMP allow bacteria to become

motile and disperse from the biofilm phenotype [108, 109]. This switching is accomplished by controlling the levels of c-di-GMP through the synthesis and degradation of c-di-GMP. c-di-GMP is synthesized by the enzyme diguanylate cyclases from two molecules of GTP and it is degraded by the enzyme phosphodiesterases into 5′-phosphoguanylyl-(3′-5′)-guanosine and/or GMP [110, 111]. Currently, it is thought that high intracellular concentrations of c-di-GMP are found to be associated with the biofilm phenotype, while low intracellular concentrations have been associated with the motile or planktonic phenotype. This has been seen in several bacteria species, *e.g. E. coli*, *P. aeruginosa*, and *S. enterica* serovar Typhimurium (for a review see [112]) [113]. However, c-di-GMP has been shown to modulate a variety of factors impacting biofilm formation, including flagella rotation to type IV pili retraction, EPS production, expression of surface adhesion molecules, antimicrobial resistance, secondary metabolite production, and biofilm dispersion [108].

As an example, LED209 is a treatment in development that focuses on reducing biofilm formation and the emergence of antibiotic resistance by disrupting QseC/QseB, which acts like both a quorum sensing molecule and two component system. As an example, in *E. coli*, AI-2 has been suggested to regulate motility and biofilm formation through QseB/QseC [114]. QseC is the membrane-bound quorum-sensing histidine protein kinase that phosphorylates QseB, which is the transcription factor that regulates virulence gene expression [115]. Expression of virulence and motility genes, whose products are involved in adherence, motility, and pathogenesis, can be induced by the direct binding of norepinephrine (NE) to QseC [115, 116]. In EHEC it has been shown that that QseB/QseC transcriptionally regulates the master flagella regulator, FlhD/FlhC, which is responsible for swimming motility [117]. Our own research has shown a correlation between flagella and chemotaxis gene expression, which impacts biofilm formation [118].

Additionally, NE utilizes the QseC/QseB signaling pathway to induce transcription of TynA (the periplasmic tyramine oxidase) and FeaB (aromatic aldehyde dehydrogenase), which converts NE to 3,4-dihydroxymandelic acid (DHMA) [119-121]. Chemotaxis can then occus when DHMA binds to the serine chemoreceptor Tsr [122].. Thus, NE can indirectly induce a chemotaxis response [122]. The treatment LED209 works by inhibiting the binding of NE to QseC, which is not toxic to the bacteria and does not inhibit pathogen growth. As a result, LED209 blocks any QseC-dependent virulence gene expression and in vivo this has shown to reduce the virulence of pathogens, including EHEC and *S. enterica* [114, 123]. The advantage to treatments, like LED209, that target signal transduction pathways avoid applying selective pressure on the bacteria and thus avoid inducing drug resistance. The relationship between NE, QseC/QseB, TynA/FeaB, DHMA, and Tsr is show in Figure 1.

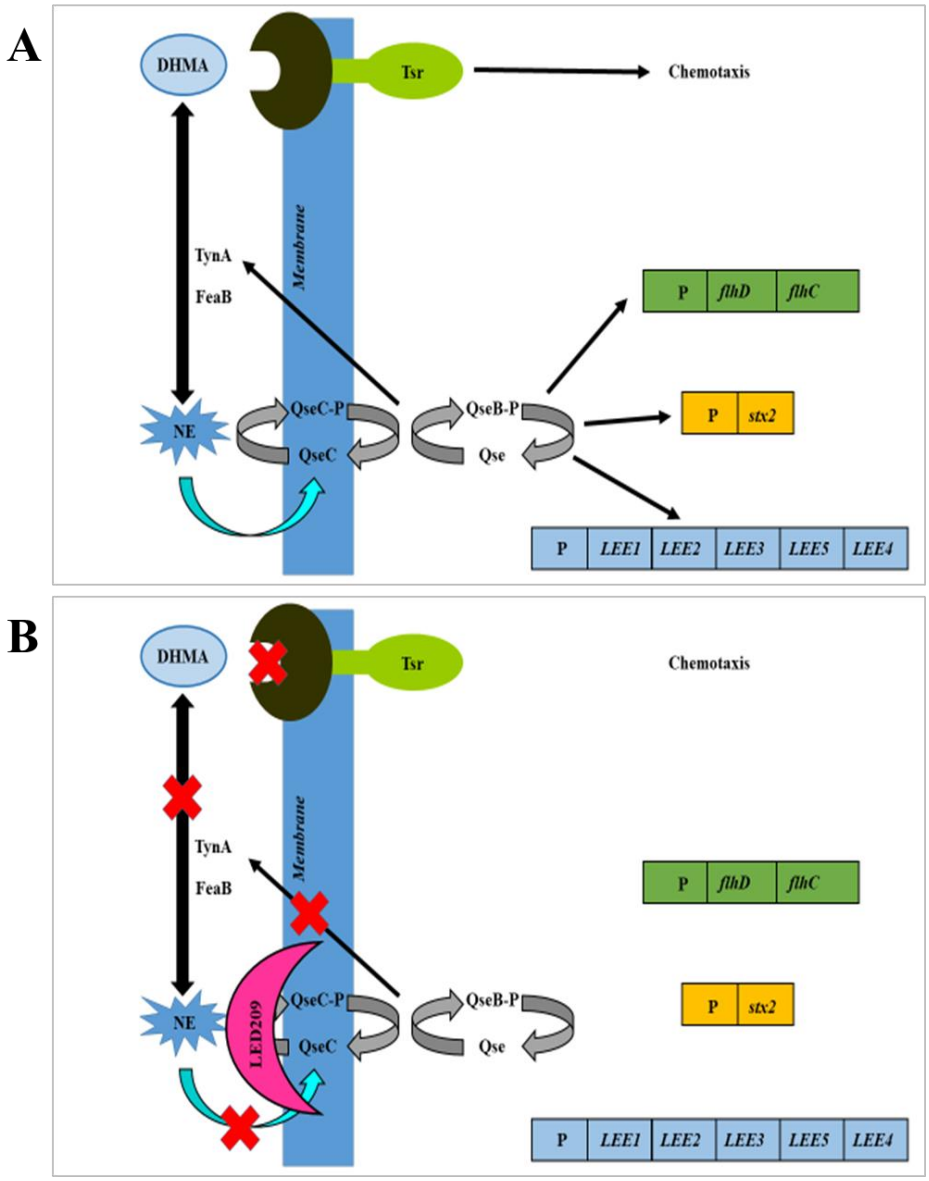

Figure 1. LED209 effect on norepinephrine regulation of virulence genes through QseC/QseB two-component system. Panel A shows how norepinephrine regulates virulence genes through the QseC/QseB two-component system. Panel B shows how LED209 impacts norepinephrine regulation of virulence genes through the QseC/QseB two-component system.

B-phenylethylamine and other small molecules/nutrients with antimicrobial effects

PEA is known under a variety of names including β-phenylethylamine, β-phenethylamine, and phenylethylamine. According to the International Union of Pure and Applied Chemistry, the proper name of PEA is 2-phenylethylamine. Its molecular formula is

denoted by $C_8H_{11}N$. The general information on and the chemical properties of PEA are summarized in Table 2.

Table 2. General information on and chemical properties of PEA. Information was taken from the Compound database from the NIH (http://pubchem.ncbi.nlm. nih.gov) and the Material Safety Data Sheets (MSDS) from TCI America.

	Solvent independent properties	**Solvent dependent properties**			**Reference**
		In ddH_2O	In lipid	In Plasma	
Alternative names	phenylethylamine, β-phenylethylamine, 2-phenylethylamine, benzeneethanamine, phenethylamine, β-phenethylamine, 2-phenethylamine	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Molecular Formula	$C_8H_{11}N$	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Molecular weight	121.17964 g/mol	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Companies that sell PEA	Forest Health, Vitacost, Amazon, Walmart	NA	NA	NA	NA
Toxicity	Mouse LD_{50} (oral) 400 mg/kg	NA	NA	NA	MSDS, TCI America
Solubility	NA	High solubility	Low solubility	High solubility	MSDS, TCI [124, 125]
Half life	NA	NA	NA	~5-10min	[125]

The occurrence of PEA and its derivatives has previously been reviewed [126]. PEA can be found in many algae [127], fungi and bacteria [128] as well as a variety of different plant species [129]. PEA is the decarboxylation product of phenylalanine and can naturally be synthesized by several bacterial species and fungi [128, 130, 131]. In several bacterial species, the decarboxylation is catalyzed by the enzyme tyrosine decarboxylase, which also converts tyrosine to another trace amine, tyramine [132, 133]. PEA is naturally found in food, such as chocolate, trees, shrubs, vines, herbs (such as clover), and vegetables (such as beans and peas) [134]. PEA is also naturally found in the brains of humans and other mammals [135, 136]. This is attributed to the high solubility of PEA in plasma and its ability to cross the blood-brain barrier [137]. Like its α-methylated

derivative, amphetamine, PEA has stimulant effects which lead to the release of so called biogenic amines, including dopamine and serotonin [138, 139]. Unlike amphetamine, PEA is rarely found in high concentrations in the human body, due to its oxidative deamination to phenylacetic acid by the enzyme B monoamine oxidase [140]. Phenylacetic acid has an effect that is similar to the activity of the natural endorphins, an effect that is known as a "runner's high". Walmart and Amazon have capitalized on the positive effects of PEA, selling it as a nutritional supplement, mood elevator, and weight loss supplement at dosages around 500 mg per day or more [141]. Altogether, PEA appears to have a number of positive effects on human health without the risks of its structural relatives.

PEA can also be chemically manufactured in the laboratory setting. Two different pathways that lead to the chemical synthesis of PEA have been established in the 1940s and 1950s. First, PEA is produced by reduction of a nitrile into an amine [142]. A second, simpler way of producing PEA is to reduce ω-nitrostyrene with lithium aluminum hydride in ether [143]. The experimental procedure that employs the use of lithium aluminum in reduction reactions follows the mechanism used in a Grignard synthesis. Recent literature focuses on the biological synthesis of PEA, rather than the chemical one. 2-phenylethylamine can be synthesized by *E. coli* overexpressing ω-transaminase [144]. Likewise, the PEA biosynthetic enzyme from *E. faecium* can be expressed in *E. coli*, which leads to large amounts of L-phenylalanine and tyrosine decarboxylase activity [145]. Intriguingly, PEA can serve as a substrate for the synthesis of other drugs, such as sulfonamides that are being used as anti-microbials [146].

Ethyl acetoacetate (EAA) is known under a variety of names including ethyl 3-oxobutanoate, ethyl acetylacetate, ethyl 3-oxobutyrate, and diacetic ether. Its molecular formula is denoted by $C_6H_{10}O_3$ or $CH_3COCH_2COOC_2H_5$ and has a molecular weight of 130.143 g/mol.

EAA is considered part of the chemical family beta keto-acids and derivatives, which are organic compounds containing an aldehyde substituted with a keto group on the C_3 carbon atom. EAA can naturally be found in coffee and coffee products, strawberry, and yellow passion fruit. EAA is considered general regarded as safe by the FDA and is approved as a food additive where it is used as flavoring agent (Flavis No 9.402; FDA 21 CFR 172.515). According to the MSDS by Science Lab, the LD_{50} for the toxicity in rats after oral application is 3.98 g kg^{-1} of body weight (www.sciencelab.com). The intermediate of EAA has been commonly used in the production of a wide variety of compounds, including amino acids, analgesics, antibiotics, antimalarial agents, antipyrine and aminopyrine, and vitamin B1. It also aids in the manufacturing of dyes, inks, lacquers, perfumes, plastics, and yellow paint pigments [147].

DL-α-glycerol phosphate (GP) is known under a variety of names including Sn-Glycerol 3-phosphate, D-Glycerol 1-phosphate, Sn-glycerol-3-phosphate, (R)-glycerol 1-phosphate, and multiple variations of these names. Its molecular formula is denoted by $C_3H_9O_6P$ and has a molecular weight of 172.073 g/mol [148]. GP is a chemical intermediate in the glycolysis metabolic pathway and produced from glycerol by the enzyme glycerol kinase. The production of GP is used in glycolysis by rapidly generating NAD+ to be used as energy in bacteria and mammals [149, 150]. L-lyxose (referred to in this manuscript as lyxose) is known under a variety of names including Aldehydo-L-lyxose, L-Lyx, and (2R,3R,4S)-2,3,4,5-tetrahydroxypentanal. Its molecular formula is denoted by $C_5H_{10}O_5$ and has a molecular weight of 150.13 g/mol [151]. Lyxose is an aldopentose sugar, which is a monosaccharide containing five carbon atoms, and includes a functional aldehyde group. Lyxose rarely occurs in nature, but has been shown to be an essential component of glycolipids that make up the cell wall of *Mycobacterium phlei* [152].

PEA, acetoacetic acetate (AAA), GP and lyxose were identified in a screen of 95 carbon and 95 nitrogen sources to have antimicrobial effects on *E. coli* O157:H7. PEA was found to have the greatest inhibitory effect on *E. coli* O157:H7 growth, bacterial cell counts, and biofilm amounts. In liquid beef broth medium, PEA reduced biofilm amounts, bacterial cell counts, and planktonic growth of *E. coli* O157:H7. In another experiment, bacterial cell counts of *E. coli* O157:H7 were determined from beef meat pieces that were treated with different dilutions of PEA and subsequently inoculated with the bacteria; this resulted in a 90 % reduction of bacterial cell counts when the beef was treated with a concentration of PEA at 150 mg/ml [153]. Additionally, other studies in our lab have investigated the antimicrobial efficacy of AAA on the growth and biofilm amounts of a variety of pathogens relevant to the food processing industry [153, 154]. AAA is a derivative of acetic acid, which has been used in the food industry for years as a cleaning technique. Acetic acid has been shown to be effective at reducing *Campylobacter jejuni*, which has been associated with multiple food borne outbreaks, on pieces of chicken [155, 156]. EAA was also investigated as a cheaper alternative to AAA and because it is the ethyl ester of AAA and has a similar chemical structure. AAA and EAA were both found to have an inhibitory effect on growth and biofilm amounts of *C. sakazakii, S. marcescens* and *Y. enterocolitica*. However, EAA was found to be more effective than AAA, reducing growth and biofilm amounts up to 3-logs [154].

The question arises whether it could be possible to use PEA and other small molecules as novel treatments of bacterial biofilms for a variety of applications in the medical and food processing industry. Not only could these small molecules be used in a liquid as a medical treatment or cleaning spray, but they could be integrated directly into materials that can then be

used to coat catheters, medical implants, and food processing equipment to prevent biofilm formation.

Objectives for this dissertation

Objective I investigate the effect of PEA on growth and biofilm amounts of bacterial pathogens.

Objective II investigate the effect of EAA, GP, and lyxose, as well as treatment combinations of these nutrients with PEA on growth and biofilm amounts of bacterial pathogens.

Objective III apply the use of small molecules/nutrients by integrating them directly in materials, using them in a liquid flush in catheters, and investigating their antimicrobial effect in a microfluidic system.

CHAPTER 2. β-PHENYLETHYLAMINE EXPERIMENTS[3,4]

Introduction

Bacterial biofilms are a group of bacteria that can attach or adhere to a surface. Biofilms can form on a variety of surfaces and can occur in many natural, environmental, clinical, and food processing settings [1]. Environmental biofilms can be found in natural waters, water-treatment plants, and chlorinated distribution networks, as well as in the soil, and on plants [157]. In the case of food processing, bacteria associated with foodborne illness (eg. *E. coli* O157:H7) form biofilms directly on food products, equipment, surfaces, and containers used in different stages of food processing [158-160]. It is estimated that 48 million people annually ingest microbe contaminated food products in the United States alone, which costs billions a year in medical and workers compensation expenses [161]. In clinical settings, 65 to 80 % of bacterial infections in humans have been attributed to biofilms. Examples of biofilm associated infectious diseases include catheter-associated urinary tract infections [33], periodontitis [34], and otitis [35], as well as *P. aeruginosa* infections of cystic fibrosis patients [36]. *P. aeruginosa* and MRSA are examples of pathogens linked with chronic biofilm associated infections [162, 163].

As bacterial biofilms mature the bacteria excrete EPS, which makes biofilms 100-1000 times harder to treat with antibiotics, chemical disinfectants, and physical cleaning [11, 164]. This unfortunately makes eradication of biofilms with conventional methods much harder. This

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[4] RFT-559. **Schroeder, M.,** S.M. Horne, S.J. Stafslien, and B.M. Prüβ. Biofilm inhibitor and method for inhibiting biofilm. Non-provisional patent application under 37 C.F.R. 1.53 (b).

problem is compounded with the increasing emergence of antibiotic resistant bacteria [165, 166]. As our technology has advanced, new biofilm preventing technologies have been developed, such as silver coatings or embedded silver nanoparticles [167, 168]. However, these materials are accompanied by high production costs, limited lifetimes, the emergence of resistant strains, and have even been shown to increase antibiotic resistance in some bacterial strains [74-76]. Wet heat, dry heat, pasteurization, and super-heated steam have all been used as sterilization methods to reduce biofilm formation in the food processing industry, as well as to sterilize instruments in the medical industry [82, 83]. However, any strategies that are aimed at killing the bacteria encourage microbial resistances.

Researchers are trying to address both of these problem by focusing research on signal transduction pathways, in order to disrupt cellular communication by introducing or modulating external signals to regulate bacterial phenotypes [87]. Two major signal transduction pathways currently being investigated include quorum sensing and two-component systems. Quorum sensing is a form of cell to cell communication that is responsive to changes in cellular density. In order to communicate bacteria produce chemical signal molecules, called AI, that are released into the external environment to be taken up by surrounding bacteria[91-93]. One study found that quorum sensing molecules enhanced attachment and biofilm formation of the strain *A. citrulli* [99]. Two-component systems are another way bacteria sense and respond to the environment around them. Two-component systems work by the binding of an external signaling molecule to a histidine kinase receptor protein that causes a phosphorylation cascade event which signals a response [101, 102]. Gene expression can be regulated through two-component systems in bacteria, which has been shown to increase virulence of bacterial strains [103]. One example of utilizing two-component signaling as a mechanism to control biofilm amounts is the regulation of the

FlhD/FlhC complex by the phosphorylated forms of OmpR and RcsB in *E. coli K-12* [118]. FlhD/FlhC, a flagella regulator, was found to reduce cell division, biofilm amount, and virulence of *E. coli* O157:H7 grown on meat [169].

Our research has focused on the use of small chemicals that present as nutrient sources for the bacteria, as an alternative treatment approach to using antibiotics. In high concentrations these nutrients exhibit antimicrobial effects by inhibiting planktonic growth and biofilm. Previous research in our lab identified a number of nutrients to have antimicrobial effects on growth, planktonic bacterial counts, and biofilm amounts of *E. coli* O157:H7 from a screen of 95 carbon and 95 nitrogen sources. PEA was found to have the greatest inhibitory effect on *E. coli* O157:H7's planktonic growth, bacterial counts, and biofilm amounts. This effect of PEA was also seen when pieces of beef were treated with PEA prior to inoculation with *E. coli* O157:H7 [153].

The research presented in this chapter builds upon our previous research by investigating the effect of PEA supplied in the liquid bacterial growth medium on planktonic growth and several parameters of biofilm for a variety of bacterial pathogens. This study had three major objectives: 1) examine the effect of PEA as a prevention method on a variety of bacterial strains 2) examine the effect of PEA as a treatment on a non-pathogenic *E. coli* K-12 strain, and 3) investigate the mode of action of PEA through five chemoreceptors and the degradation of PEA. We were able to demonstrate a reduction of all growth and biofilm parameters tested, when PEA was supplied at the time of inoculation and later during biofilm development. This is an indication that PEA could be used as both a prevention and a treatment method. We also observed that it was the molecule PEA that was having the inhibitory effect on growth and biofilm amounts of an *E. coli* K-12 strain, not PEA's metabolic degradation production. Finally,

we investigated the five chemoreceptors found in *E. coli*, which play a role in chemotaxis and biofilm formation. Overall, the use of nutrients as an antimicrobial agent is a novel approach to the prevention and treatment of biofilm formation, which could lead to possible applications in the medical and food processing industries.

Materials and methods

Bacterial strains

Bacterial strains used in this dissertation are summarized in Table 3 and include a non-pathogenic *E. coli* K-12 strain, wild type *E. coli* K-12 strain, seven mutant *E. coli* K-12 strains, and ten bacterial pathogens. The bacterial strains used in this chapter include a non-pathogenic *E. coli* K-12 strain, wild type *E. coli* K-12 strain, and five bacterial pathogens.

Table 3. Bacterial strains used for this study.

	Bacterial species	Designation	Characteristics	Source	Chapter		
					II.	III.	IV.
Wild-type	*E. coli* K-12	AJW678	*thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL136* Δ*laxX74*	[170]	X	X	X
Mutants	*E. coli* K-12	JW1381-1	BW25311 Δ*tynA750(del)::kan*	[171]	X		
	E. coli K-12	BP1593	AJW678 Δ*tynA750(del)::kan*	This study	X		
	E. coli K-12	JW1380-1	BW25311 Δ*feaB749(del)::kan*	[171]	X		
	E. coli K-12	BP1592	AJW678 Δ*feaB749(del)::kan*	This study	X		
	E. coli K-12	JW1875-5	BW25311 *Δtar739(del)::kan*	[171]	X		
	E. coli K-12	BP1596	AJW678 *Δtar739(del)::kan*	This study	X		
	E. coli K-12	JW1874-1	BW25311 Δ*tap738(del)::kan*	[171]	X		
	E. coli K-12	BP1597	AJW678 Δ*tap738(del)::kan*	This study	X		
	E. coli K-12	JW3043-1	BW25311 Δ*aer762(del)::kan*	[171]	X		
	E. coli K-12	BP1598	AJW678 Δ*aer762(del)::kan*	This study	X		
	E. coli K-12	JW4318-1	BW25311 Δ*tsr753(del)::kan*	[171]	X		
	E. coli K-12	BP1599	AJW678 Δ*tsr753(del)::kan*	This study	X		
	E. coli K-12	JW1417-1	BW25311 Δ*trg782(del)::kan*	[171]	X		
	E. coli K-12	BP1600	AJW678 Δ*trg782(del)::kan*	This study	X		
Pathogens	*P. aeruginosa*	ATCC 15442	Hospital isolate	Beloian, A.	X	X	X
	S. aureus	ATCC 25923	Control Strain	Rosenbach	X	X	X
	E. coli EHEC	ATCC 43894	EDL932 O157:H7	[172]	X	X	X
	E. coli UPEC	BAA-1161	O17:K52:H18	[173]	X	X	X
	E. coli UPEC	ATCC 700928	CFT073 O6:H1	[174]	X	X	X
	Y. enterocolitica 8081c		O8	[175]		X	X
	L. monocytogenes 10403S		1/2a	[176]		X	X
	C. sakazakii	BAA-894	Powdered infant formula	[177]		X	X
	S. marcescens	BAA-632	Urine isolate	[178]		X	X
	S. aureus	NR-48850	pFP650-R	[179-181]			X

The *E. coli* K-12 strain used was AJW678, which was previously described as a good former of biofilm[170]. The seven *E. coli* mutant strains were purchased from the KEIO collection (The Coli Genetic Stock Center)[171]. The kanamycin resistance conferring mutations were moved from the BM25113 parent strain into the *E. coli* K-12 strain AJW678 by P1 transduction [182]. Two mutations affected catabolism of PEA; we used the *ΔtynA750::kan* (*tynA*, copper-containing amine oxidase) mutation from strain JW1381-1 and the *ΔfeaB749::kan* (*feaB,* phenylacetaldehyde dehydrogenase) mutation from strain JW1380. These mutations in AJW678 were designated BP1593 and 1592, respectively. The other five mutations affect the five chemoreceptors found in *E. coli* that facilitate chemotaxis and motility [183-185]. We used the *Δtar739::kan* mutation from strain JW1875-5 (*tar*; aspartate receptor), the *Δtap738::kan* mutation from strain JW1874-1(*tap*; dipeptide receptor), the *Δaer762::kan* mutation from strain JW3043-1 (*aer*; oxygen sensor), the *Δtsr753::kan* mutation from strain JW4318-1 (*tsr*; serine receptor), and *Δtrg782::kan* mutation from strain JW1417-1 (*trg*; ribose/galactose receptor).

Pathogenic bacterial strains include *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 25923), the enterohemolytic *E. coli* (EHEC) EDL932 [172], and two uropathogenic *E. coli* (UPEC), UMN026 [173] and CFT073[174]. Bacterial strains were maintained as freezer stocks at -80ºC in 8 % dimethyl sulfoxide (DMSO). Prior to each experiment, the *E. coli* strains were plated onto Luria Bertani (LB; 10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) agar plates. *P. aeruginosa* and *S. aureus* were plated onto tryptic soy agar (TSA; 15 g/l tryptone, 5 g/l soytone, 5 g/l NaCl, 15 g/l agar) plates. Plates were incubated over night at 37°C. Media used in this study are summarized in Table 4.

Table 4. Composition of the bacterial growth media.

Name	Abbreviation	Composition	Brand
Luria Bertani Broth	LB	10 g/l tyrptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0	Difco BD
Tryptic Soy Broth	TSB	17 g/l pancreatic digest of casein, 3 g/l papaic digest of soy bean, 5 g/l NaCl, 2.5 g/l K_2HPO_4, 2.5 g/l glucose, pH 7.3	Difco BD
Luria Bertani Agar	LB agar	10 g/l tyrptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar, pH 7.0	Difco BD
Tryptic Soy Agar	TSA	15 g/l pancreatic digest of casein, 5 g/l papaic digest of soy bean, 5 g/l NaCl, 15 agar, pH 7.3	Difco BD
Beef Broth	BB	3 % beef extract, 5 % peptone	
Biofilm Growth Media	BGM	10.22 g/l Na_2HPO_4, 3.81 g/l KH_2PO_4, 1.01 g/l KCl, 0.793 g/l $(NH_4)_2SO_4$, 0.06 g/l $MgSO_4$, 0.001 g/l thiamine, 0.0005 g/l biotin, and 0.5 g/l dextrose	

Formation of the biofilms

Liquid overnight cultures in tryptone soy broth (TSB; 17 g/l tryptone, 3 g/l soytone, 2.5 g/l glucose, 5 g/l NaCl, 2.5 g/l K_2HPO_4) were centrifuged at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of 1 x phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.42 g/l Na_2PO_4, 0.24 g/l KH_2PO_4). Cultures were adjusted with PBS until their optical density values at 600 nm (OD_{600}) were 1 ± 0.05. The final inocula were prepared by diluting the PBS cultures 1:10 in 2 x TSB or 2 x beef broth (BB; 3 % beef extract, 5 % peptone). Dilutions of β-phenylethylamine hydrochloride (PEA; Alfa, Aesar, MA) were prepared in PBS; PEA concentrations ranged from 0 mg/ml to 100 mg/ml. A 1:1 dilution of final inocula and PEA solutions were used to inoculate individual wells of a 24-well with 1 ml/well (200 µl/well for 96-well polystyrene plate). This yields an inoculation OD_{600} of approximately 0.05 in 1 x TSB or 1 x BB/0.5 x PBS and a PEA concentration range from 0 to 50 mg/ml. The PEA solutions were added to the biofilm at the time of the initial inoculation (0 h) or at 2, 3, 4, 6, 8, 12, 18, 24, 48 h post-inoculation. The plates were then incubated at 37ºC for 16 or 24 h, at 25ºC for two days, and at 10ºC for four days. The experiments were done on three replicated plates (biological

replicates from independent overnight cultures) and two replicates per plate (technical replicates).

Determination of bacterial growth and biofilm amounts

Planktonic growth was determined as end point growth by using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), which recorded the OD_{600} at the end of the incubation period. Averages and standard deviations were determined over six replicate experiments.

To characterize the biofilm at the end of the incubation period, two analyses were used: the crystal violet (CV) assay that stains live and dead biomass, as well as exopolysaccharide and the adenosine triphosphate (ATP) assay that measures the energy content of live bacteria [186, 187]. Prior to the biofilm assays, we rinsed each well three times with 1.0 ml (200 µl). When biofilms of *E. coli* O157:H7 were processed, the wells were only rinsed twice because this strain only forms a small amount of biofilm. The plates were then inverted, tapped against an absorbent pad, and allowed to dry at ambient laboratory conditions for at least 1 h. For the CV assay, 1.0 ml (200 µl) of 0.1 % CV in ddH_2O was added to each well of the 24-well (96-well) plates and incubated at room temperature for 15 min, then rinsed three times with 1.0 ml (200 µl) of PBS. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for at least 1 h. The CV was extracted by adding 500 µl (200 µl) of 20 % acetone 80 % ethanol acetic acid to each well, followed by 15 min of incubation. 0.15 ml of each extract was then transferred into one well of a 96 well polystyrene plate and the OD_{600} measurements were determined with the Synergy H1 Hybrid Reader. Averages and standard deviations were determined across 6 replicates.

For the ATP assay, the BacTiter Glo^{TM} kit (Promega, Madison, WI) was used. Reagents were prepared per manufacturer instructions. To each well, we added identical amounts of PBS and BacTiter-Glo reagent. For 24 well plates, this was 250 µl. For 96 well plates, we added 100 µl of each solution. We covered the plates with tinfoil and thoroughly mixed solutions by placing the plate on a rotating shaker for 5 min. After this incubation, we transferred 150 µl from each well to an opaque white 96-well plate and read luminescence using the Synergy H1 Hybrid Reader. Averages and standard deviations of luminescence values were determined over 6 replicates

Data analysis

Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA) was used to find the best curve fit for both assays; the software utilizes a four or five parameter logistics curve fitting model [188]. The equation for the four parameter logistics was calculated as $F(x) = A + (D/(1+(X/C)^B))$, where A is the value for the minimum asymptote, B is the slope, C is the concentration at inflection point, and D is the value for the maximum asymptote. The curve fit with the five parameter logistics was calculated as $F(x) = A + (D/(1+(X/C)^B)^E)$, where the variables are the same as in the four parameter logistics equation plus E, the asymmetry factor. From the curve fit analysis software IC_{50} values were calculated for growth and biofilm amounts of each bacterial strain [189, 190]. IC_{50} values tell us the concentration at which the bacterial growth and biofilm amounts are reduced to 50 %. The IC_{50} values are calculated as $x = C((2^{(1/E)} – 1)^{(1/B)})$ and are given in mg/ml. Coefficient of determination values (R^2) are calculated with the software as a quantitative way to show how well the curve actually fits. The coefficient of determination (R^2) is the ratio of the explained variance to the total variance. Higher R^2 values indicate confidence that the curve fits and the

IC_{50} values are accurate. Statistical analysis software (SAS) 9.3 was used to analyze this data using Fisher's Least Squared Difference (LSD) to determine the statistical significance of the difference between the growth means for each concentration when compared to the mean growth at the concentration 0 mg/ml. Student's *t*-tests were also performed to determine the statistical significance of the difference between the growth means for each concentration when compared to the mean growth at the concentration 0 mg/ml. Differences were considered significant when the *p*-value was below 0.05.

Results

β-phenylethylamine reduces planktonic growth and biofilm amounts of AJW678

In a first experiment, the *E. coli* K-12 strain AJW678 was grown on 24-well polystyrene plates in TSB supplemented with a range of concentrations of PEA (0-50 mg/ml), that were supplied to the liquid growth medium at the beginning of biofilm development. Fig.2A shows the planktonic growth measured after 24 h of incubation at 37°C. PEA started to reduce growth at a concentration of 1 mg/ml and caused a 50 % reduction at approximately 3-4 mg/ml. Almost complete abolishment of growth was observed at 20 mg/ml. Fig.2B shows the absorbance data from the CV assay that was performed on the biofilm. There was a modest reduction in biofilm biomass up to 5 mg/ml, followed by a steep decline between 5 and 10 mg/ml of PEA. Fig. 2C shows the bioluminescence data from the ATP assay that was performed to further characterize the biofilm. PEA started to reduce the ATP content of the biofilm at a concentration of 0.25 mg/ml and caused a 50 % reduction at approximately1 mg/ml. Almost complete abolishment of ATP was observed at 3 mg/ml.

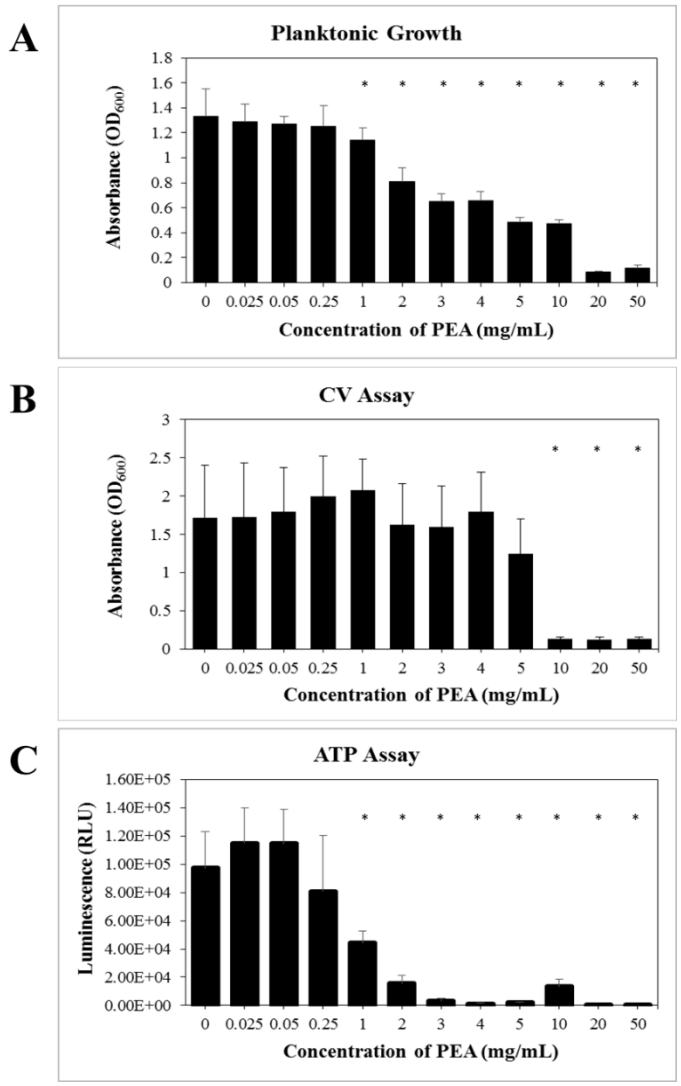

Figure 2. Effectiveness of PEA on AJW678 planktonic growth, biofilm biomass, and ATP content. AJW678 was grown in the presence of varying concentrations of PEA, shown on the x-axis. Averages and standard deviations were computed across all 6 replicates. Asterisks indicate a statistically significant difference between the mean growth, biofilm biomass (CV), or energy content (ATP) of the biofilm at the respective PEA concentration in comparison to the mean at a PEA concentration of 0 mg/ml.

The asterisks in Fig. 2 show at which concentrations of PEA the mean was found to be statistically significantly different in comparison to 0 mg/ml of PEA for planktonic growth, biofilm biomass, and ATP content of the biofilm. The mean planktonic growth starting at the concentrations of 1 mg/ml - 50 mg/ml of PEA was found to be statistically significant in comparison to the mean planktonic growth at a concentration of 0 mg/ml of PEA (p-value = 0.04

at 1 mg/ml of PEA). The mean biofilm biomass starting at the concentrations of 10 mg/ml - 50 mg/ml of PEA was found to be statistically significant in comparison to the mean biofilm biomass at a concentration of 0 mg/ml of PEA (p-value = 0.001 at 10 mg/ml of PEA). The mean ATP content of biofilm starting at the concentrations of 1 mg/ml - 50 mg/ml of PEA was found to be statistically significant in comparison to the mean ATP content of the biofilm at a concentration of 0 mg/ml of PEA (p-value = 0.001 at 1 mg/ml of PEA).

Addition of β-phenylethylamine at different time points during biofilm formation reduces planktonic growth and biofilm amounts of AJW678

For the next experiment, the *E. coli* K-12 strain AJW678 was grown on 24-well polystyrene plates in TSB or BB. PEA was added in different concentrations and at different time points. Incubation temperatures were either 10^{o}C (Fig. 3), 25^{o}C (Fig. 4), or 37^{o}C (Fig. 5). The time of incubation varied with the temperature.

Fig. 3A shows that PEA reduced planktonic growth over the 96-h incubation period at 10^{o}C in TSB when added at any time. However, the concentration of PEA that was needed to achieve 50 % reduction was dependent upon the time point of addition. Introducing PEA at earlier time points was more effective than at later time points. For example, a 50 % reduction in growth was observed at about 2-3 mg/ml when PEA was added at time 0 h (circles, pink solid line). In comparison, when PEA was added at 24 h (triangle, blue dashed line) and 48 h (square, blue dashed line), it took between 5-10 mg/ml and 10-20 mg/ml, respectively, to achieve an approximate 50 % reduction in planktonic growth.

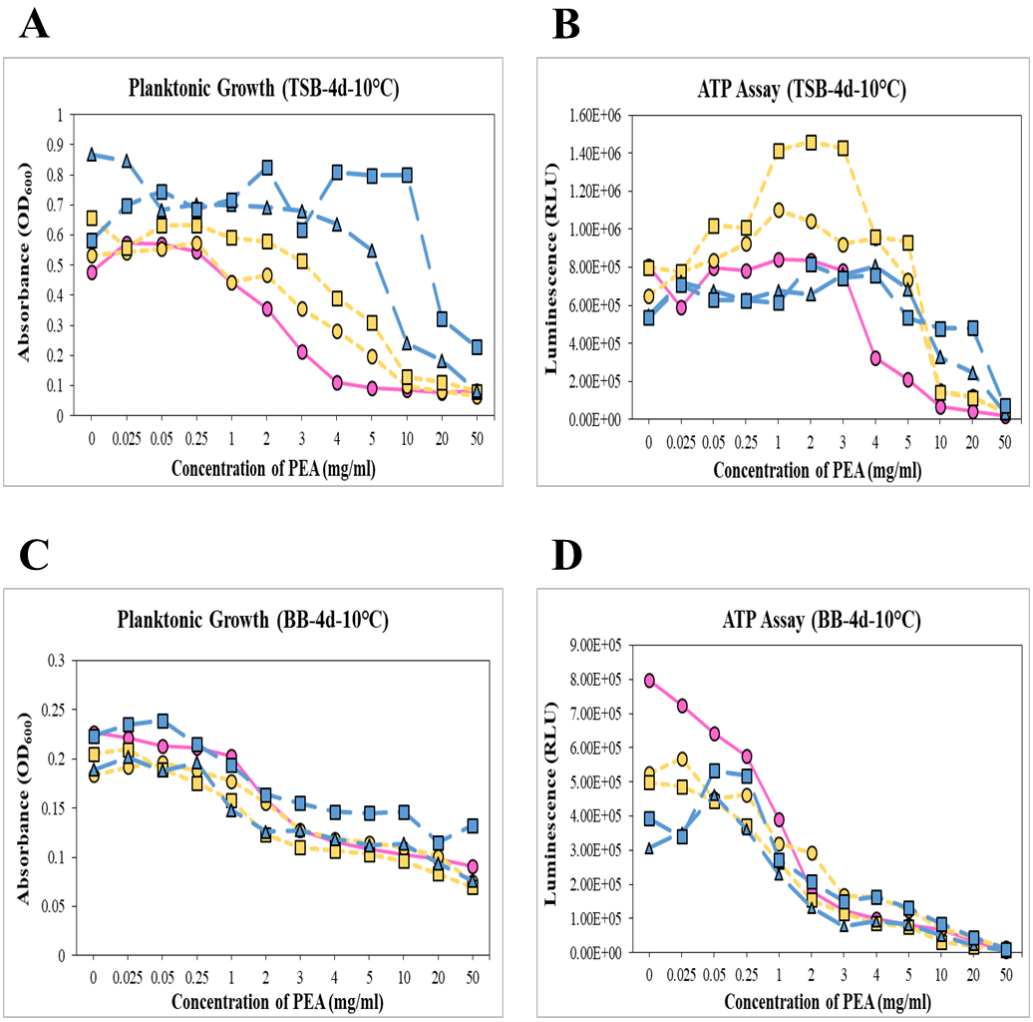

Figure 3. Effectiveness of PEA on AJW678 growth and ATP content when added at different time points over 96 h; 10°C experiment. Reduction of AJW678 planktonic growth and luminescence from the ATP assay that was done on the biofilm when PEA was added to the liquid growth medium (TSB or BB) at different time points during incubation. Growth (Panel A and C) and luminescence from the ATP assay (Panel B and D) of AJW678 were determined after 96 h at 10°C. PEA was added at 0 h (circle, pink solid line), 6 h (circle, yellow dashed line), 12 h (square, yellow dashed line), 24 h (triangle, blue dashed line), and 48 h (diamond, blue dashed line).

Fig.3B shows the bioluminescence data from the ATP assay that was performed on the biofilms. This trend was very similar to that observed for planktonic growth; PEA reduced ATP content when added at any time point but occurred at different concentrations depending upon the time point of addition. For example, a 50 % reduction was observed at about 4 mg/ml when

PEA was added at time 0 h. However, when PEA was added after at 24 h, a 50 % reduction was observed between 5 and 10 mg/ml. Complete abolishment of ATP production was observed at 50 mg/ml of PEA for all time points. It is interesting to note that the addition of PEA at 12 h (squares, yellow dashed lines) resulted in elevated levels of measurable ATP between 1 and 4 mg/ml before a reduction in ATP content could be noted.

Figs. 3C and 3D shows the same experiment when biofilms were grown in BB. Once again, PEA reduced both planktonic growth (Fig. 3C) and the ATP content in viable cells of the biofilm (Fig. 3D) when added at any time point. Similar to the results observed in TSB, differences in the inhibitory concentration of PEA were dependent upon the time point at which PEA was added. For growth (Fig. 3C), a 50 % reduction was observed at about 3-5 mg/ml of PEA for most of the time points evaluated. For ATP content (Fig. 3D), the inhibitory concentration of PEA was more dependent upon the time point of the PEA addition than was observed for planktonic growth. For example, a 50 % reduction was observed at about 1 mg/ml when PEA was added at time 0 h. However, when PEA was added after 24 h, a 50 % reduction was observed between 3 mg/ml.

Fig. 4 shows the results from the 25°C experiment. Once again, PEA reduced both planktonic growth (Fig. 4A and C) and the ATP content in viable cells of the biofilm (Fig. 4B and D) when added at any time point. Similar to the results observed in Fig. 3, differences in the inhibitory concentration of PEA were dependent upon the time point at which PEA was added. Interestingly, for growth (Fig. 4A and C), a 50 % reduction was observed at about 10-50 mg/ml of PEA in TSB and about 3-5 mg/ml of PEA in BB for most or all of the time points evaluated. For ATP content (Fig. 4B and D), a 50 % reduction was observed at about 10-50 mg/ml of PEA

in TSB and about 1-3 mg/ml of PEA in BB for most or all of the time points evaluated. ATP production was greatly reduced at 50 mg/ml of PEA for all time points.

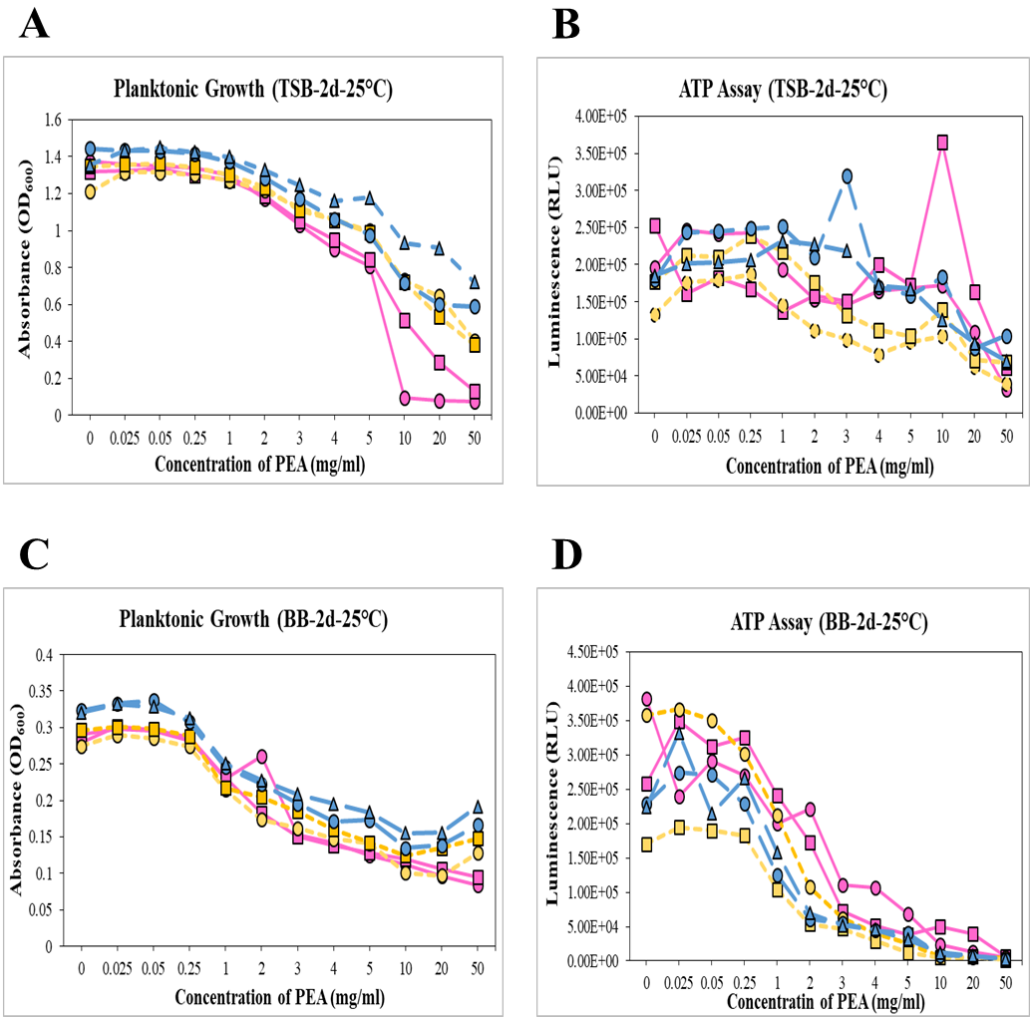

Figure 4. Effectiveness of PEA on AJW678 growth and ATP content when added at different time points over 48 h; 25ºC experiment. Reduction of AJW678 planktonic growth and luminescence from the ATP assay that was done on the biofilm when PEA was added to the liquid growth medium (TSB or BB) at different time points during incubation. Growth (Panel A and C) and luminescence from the ATP assay (Panel B and D) of AJW678 were determined after 48 h at 25°C. PEA was added at 0 h (circle, pink solid line), 3h (square, pink solid line), 6 h (circle, yellow dashed line), 12 h (square, yellow dashed line), 18h (circle, blue dashed line), and 24 h (triangle, blue dashed line).

Fig. 5 shows the results from the 37°C experiment. Once again, PEA reduced both planktonic growth (Fig. 5A and C) and the ATP content in viable cells of the biofilm (Fig. 5B

and D) when added at any time point. Differences in the inhibitory concentration of PEA were dependent upon the time point at which PEA was added. For growth (Fig. 5A and C), a 50 % reduction was observed at about 3-5 mg/ml of PEA in TSB and about 1-3 mg/ml of PEA in BB when it was added earlier (0, 2, and 4h). When the PEA was added later (6, 8, and 12h), it took higher concentrations of PEA to get a 50 % reduction. Interestingly, when PEA was added at 12 h in TSB, we were unable to get a 50 % reduction in growth. Similarly, when PEA was added at 6, 8, and 12 h in BB, we were unable to achieve a 50 % reduction in growth. For ATP (Fig. 5B and D), a 50 % reduction was observed at about 10-50 mg/ml of PEA in TSB and about 1-3 mg/ml of PEA in BB. Complete abolishment of ATP production was observed at 50 mg/ml of PEA in TSB and 5 mg/ml of PEA in BB for all time points. It is interesting to note that the addition of PEA in TSB resulted in elevated levels of measurable ATP between 1 and 5 mg/ml before a reduction in ATP content could be noted.

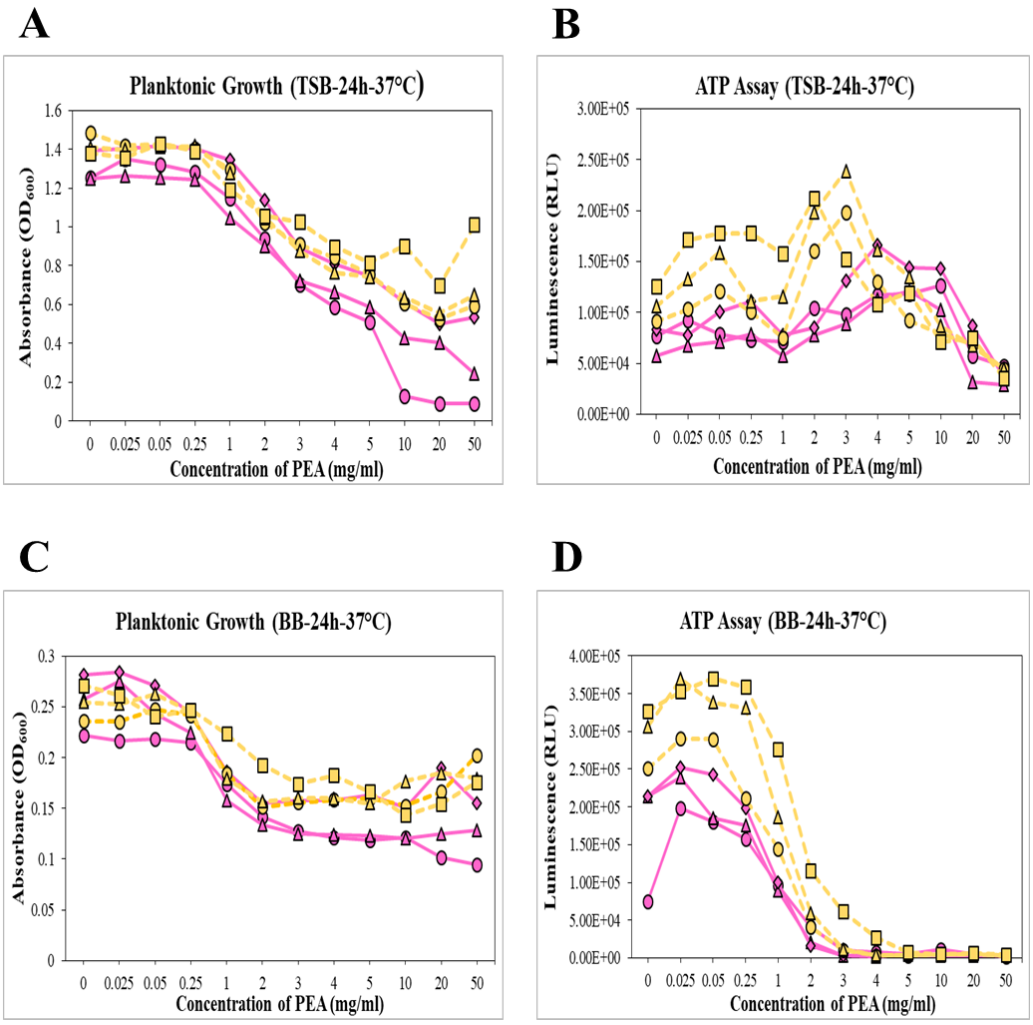

Figure 5. Effectiveness of PEA on AJW678 growth and ATP content when added at different time points over 24 h; 37ºC experiment. Reduction of AJW678 planktonic growth and luminescence from the ATP assay that was done on the biofilm when PEA was added to the liquid growth medium (TSB or BB) at different time points during incubation. Growth (Panel A and C) and luminescence from the ATP assay (Panel B and D) of AJW678 were determined after 24 h at 37°C. PEA was added at 0 h (circle, pink solid line), 2h (triangle, pink solid line), 4h (diamond, pink solid line), 6 h (circle, yellow dashed line), 8 h (triangle, yellow dashed line), and 12 h (square, yellow dashed line).

Calculation of IC_{50} values for biofilm amounts and growth of AJW678

IC_{50} values were calculated for the inhibitory effect of PEA on biofilms amounts and growth of AJW678 (Table 5). These were calculated using Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA), which was used to find the best

curve fit for both assays; the software utilizes a five or four parameter logistics curve fitting model [188]. The output from the Master Plex® Reader Fit analysis shows the best fit curve for biofilm amounts and growth, which helps to determine the IC_{50} and R^2 values (Fig. 6).

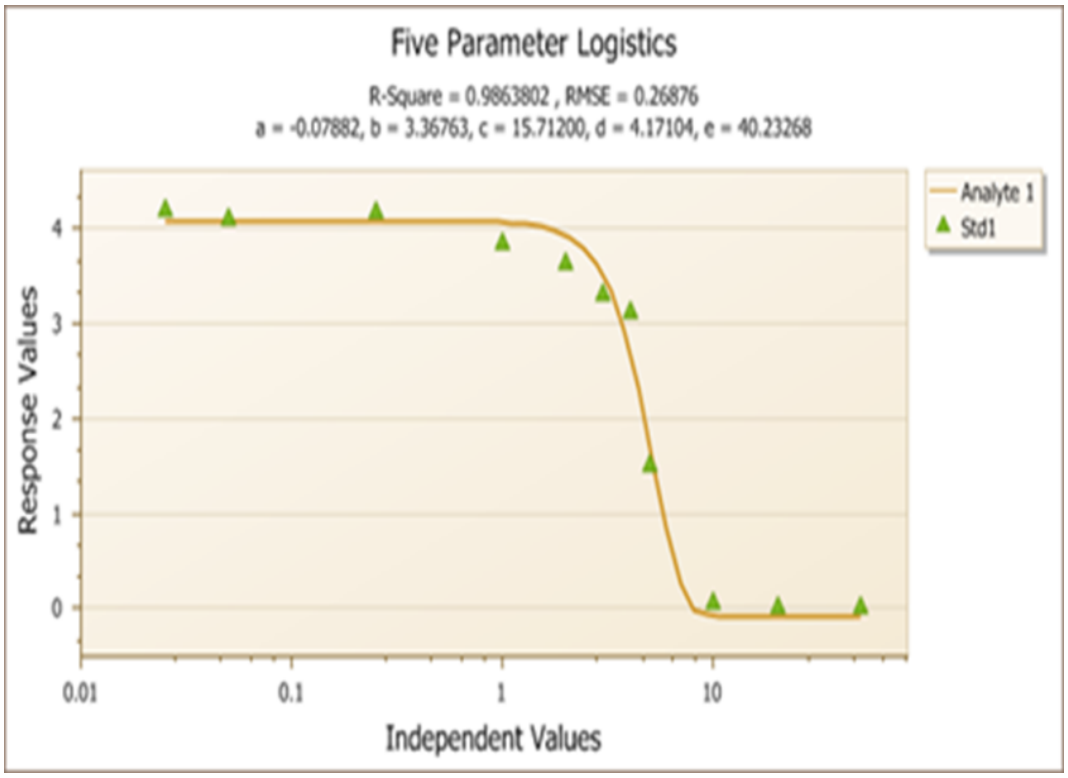

Figure 6. Example of an output from Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA). The half maximal inhibitory concentration (IC_{50}) and coefficient of determination (R^2) values are displayed at the top of the graph. The graph displays the average growth of AJW678 and the best curve was calculated to best fit the average.

Table 5. IC_{50}/R^2 values for AJW678 planktonic growth and ATP content of the biofilm.

Expt. Conditions			Growth		Biofilm		*t*-tests[5]	
Media	Temp. (°C)	Time (h)	R^2	IC_{50}	R^2	IC_{50}	Growth (mg/ml)	Biofilm (mg/ml)
TSB	37	0	0.9978	3.45	0.4888	33.55	1	20
		2	0.9951	4.56	0.5726	15.68	1	20
		4	0.9947	2.86	-	-	2	50
		6	0.9867	2.24	-	-	1	20
		8	0.9948	2.19	-	-	1	10
		12	0.7666	2.06	0.9134	6.92	1	10
	25	0	0.9902	5.25	0.8541	12.03	2	2
		3	0.9996	7.27	-	-	2	2
		6	0.9946	15.61	0.9378	4.76	2	3
		12	0.9988	7.49	0.9160	6.35	2	3
		18	0.9981	4.58	0.7457	12.54	1	5
		24	0.9873	53.02	0.9657	14.16	3	10
	10	0	0.9940	2.37	0.9555	3.86	3	4
		6	0.9794	3.66	0.9008	5.16	3	10
		12	0.9764	5.44	-	-	4	10
		24	0.9585	8.34	-	-	10	10
		48	0.8982	18.67	-	-	20	50
BB	37	0	0.9876	1.32	0.6794	1.41	1	2
		2	0.9829	0.50	0.9835	0.75	0.25	0.25
		4	0.9477	0.38	0.9856	0.89	0.05	1
		6	0.8585	0.96	0.9788	1.02	1	1
		8	0.9398	0.51	0.9851	1.14	1	1
		12	0.8873	0.93	0.9930	1.61	1	1
	25	0	0.9423	2.59	0.9377	2.85	1	0.025
		3	0.9859	3.90	0.9723	2.04	1	2
		6	0.9717	1.64	0.9983	1.18	1	0.25
		12	0.9722	1.35	0.9875	1.22	1	1
		18	0.9729	1.40	0.9808	0.99	0.25	1
		24	0.9649	1.34	0.9281	1.21	1	1
	10	0	0.9828	1.89	0.9848	0.67	0.05	0.05
		6	0.9733	14.86	0.9738	1.82	2	1
		12	0.9818	1.51	0.9970	0.93	0.05	0.25
		24	0.9591	8.13	0.9411	1.32	1	1
		48	0.9532	1.60	0.9048	1.53	1	1

[5] A Student's *t*-test was performed to determine the statistical significance of the difference between the means of growth or biofilm amounts at the respective PEA concentration and the means of growth or biofilm amounts at 0 mg/ml PEA. The lowest concentration at which the first statistically significant difference was observed is shown in this column.

We were able to calculate IC_{50} values for growth at all of the time points and in both TSB and BB. However, we were unable to calculate some IC_{50} values for biofilm when PEA was added to TSB at all the temperatures. PEA was found to have different IC_{50} values with R^2 values > 0.9 depending on when the PEA was added to the liquid growth media and what growth conditions were used.

When PEA was supplemented to BB all of the IC_{50} values calculated were found to be below 10 mg/ml at R^2 values > 0.8 for AJW678 growth and ATP content, except when PEA was added at 6 h at 10°C in BB. When PEA was supplemented to TSB most of the IC_{50} values calculated were found to be below 10 mg/ml at R^2 values > 0.8 for AJW678 growth and ATP content. A few IC_{50} values calculated were found to be higher than 10 mg/ml of PEA, but all of these IC_{50} values were associated with later additions of PEA. Interestingly, in general the IC_{50} values calculated for ATP content were higher than the IC_{50} values calculated for growth at the same time point. This is an indication that PEA was more effective at reducing the planktonic growth than reducing the ATP content of the biofilm. The IC_{50} values are indicative of an inhibitory effect of PEA on growth and ATP content of AJW678. Large R^2 values indicate that the curves fit, which gives us more confidence in the IC_{50} value.

β-phenylethylamine reduces growth and the ATP content in biofilms of pathogens

The bacterial pathogens from Table 3 including, *S. aureus*, *P. aeruginosa*, *E. coli* UMN026, *E, coli* CFT073, and *E. coli* EDL932 were grown on 96-well polystyrene plates in TSB or BB, supplemented by a range of concentrations of PEA (0-50 mg/ml). Fig. 7 shows the results from the 10^{o}C experiment, Fig. 8 shows the 37^{o}C data. Table 6 lists the corresponding IC_{50} values for both temperatures.

Table 6. IC_{50}/R^2 values for pathogen planktonic growth and ATP content of the biofilm.

Microorganisms	Expt. Conditions		Growth		Biofilm		*t*-test[6]	
	Media	Temp. (°C) & Time	R^2	IC_{50}	R^2	IC_{50}	Growth	Biofilm
E. coli O157:H7 (EDL932)	BB	10; 4 days	0.9377	5.64	0.8111	4.67	4	5
		37; 16 hrs	0.9816	2.89	0.9353	2.12	1	0.025
	TSB	10; 4 days	0.9761	5.69	0.6719	7.91	1	10
		37; 16 hrs	0.9713	11.73	0.9767	7.90	1	4
S. aureus (ATCC 25923)	BB	10; 4 days	-	-	-	-	-	-
		37; 16 hrs	-	-	-	-	-	-
	TSB	10; 4 days	-	-	-	-	50	50
		37; 16 hrs	0.9211	21.57	0.9771	12.10	1	10
P. aeruginosa (ATCC 15442)	BB	10; 4 days	0.9948	1.53	0.9197	3.39	1	1
		37; 16 hrs	0.9913	9.73	0.9875	3.19	2	3
	TSB	10; 4 days	0.9179	4.31	0.9657	4.62	2	4
		37; 16 hrs	0.8432	4.13	0.9359	3.20	5	2
E. coli O6:H1 (CFT073)	BB	10; 4 days	0.9664	4.11	0.9832	5.93	2	4
		37; 16 hrs	0.9657	21.19	0.8847	3.30	2	0.05
	TSB	10; 4 days	0.9764	8.64	0.6116	20.28	2	20*
		37; 16 hrs	0.9587	11.73	0.9862	5.24	4	10
E. coli O17:K52:H18 (UMN026)	BB	10; 4 days	0.9925	4.75	-	-	3	10
		37; 16 hrs	0. 0.9848	2.94	0.9788	3.83	1	2
	TSB	10; 4 days	0.9824	5.91	0.954	12.15	1	10
		37; 16 hrs	0.9761	14.55	0.9089	6.28	2	10

Fig. 7A shows planktonic growth measured after 96 h of incubation at 10°C in TSB. PEA started to reduce growth for *P. aeruginosa* (circles, solid line) at a concentration of 2 mg/ml with an IC_{50} value of 4 mg/ml. Planktonic growth of *S. aureus* (squares, solid line) was low at all concentrations of PEA and an IC_{50} could not be calculated. PEA started to reduce *E. coli* EDL932 (diamonds, solid line), *E. coli* UMN026 (triangle, solid line), and *E. coli* CFT073 (triangle, dashed line) at 1 mg/ml (remaining lines) with corresponding IC_{50} values of 5.69 mg/ml, 6 mg/ml, and 9 mg/ml, respectively. Almost complete abolishment of planktonic growth was observed at 20 mg/ml for all bacterial strains, except *S. aureus*.

[6] A Student's t-test was performed to determine the statistical significance of the difference between the means of growth or biofilm amounts at the respective PEA concentration and the means of growth or biofilm amounts at 0 mg/ml PEA. The lowest concentration at which the first statistically significant difference was observed is shown in this column.

Fig. 7B shows the ATP content of viable cells in the biofilm of the bacterial pathogens from the same experiment. Of all the pathogens tested, *P. aeruginosa* started out with the highest amount of ATP content and PEA began to reduce *P. aeruginosa* (circles, solid line) ATP content at a concentration of 2 mg/ml with an IC_{50} of 5 mg/ml. For *S. aureus* (squares, solid line) and *E. coli* EDL932 (diamonds, solid line), ATP content was very low to start out with. PEA further reduced ATP content of *S. aureus* biofilm between 20-50 mg/ml. For *E. coli* EDL932, ATP reduction by PEA started at 5 mg/ml with an IC_{50} value of 8 mg/ml. PEA started to reduce ATP content by *E. coli* UMN026 (triangles, solid line) and *E. coli* CFT073 (triangles, dashed line) at 5-10 mg/ml with IC_{50} values for these two strains of 12 mg/ml and 20 mg/ml, respectively. Complete abolishment of measurable metabolic activity was observed at 50 mg/ml for all tested strains.

Fig. 7C shows planktonic growth measured after 96 h incubation at 10°C in BB. PEA started to reduce growth for *P. aeruginosa* (circles, solid line) at a concentration of 1 mg/ml with an IC_{50} value of 2 mg/ml. Planktonic growth of *S. aureus* (squares, solid line) was low at all concentrations of PEA and an IC_{50} could not be calculated. PEA started to steadily reduce growth for *E. coli* EDL923 (diamonds, solid line) and *E. coli* UMN026 (triangles, solid line) at 1 mg/ml with IC_{50} values of 6 mg/ml and 5 mg/ml, respectively. For *E. coli* CFT073 (triangles, dashed line) at 0.05 mg/ml with IC_{50} value of 4 mg/ml. Almost complete abolishment of planktonic growth was observed at 50 mg/ml.

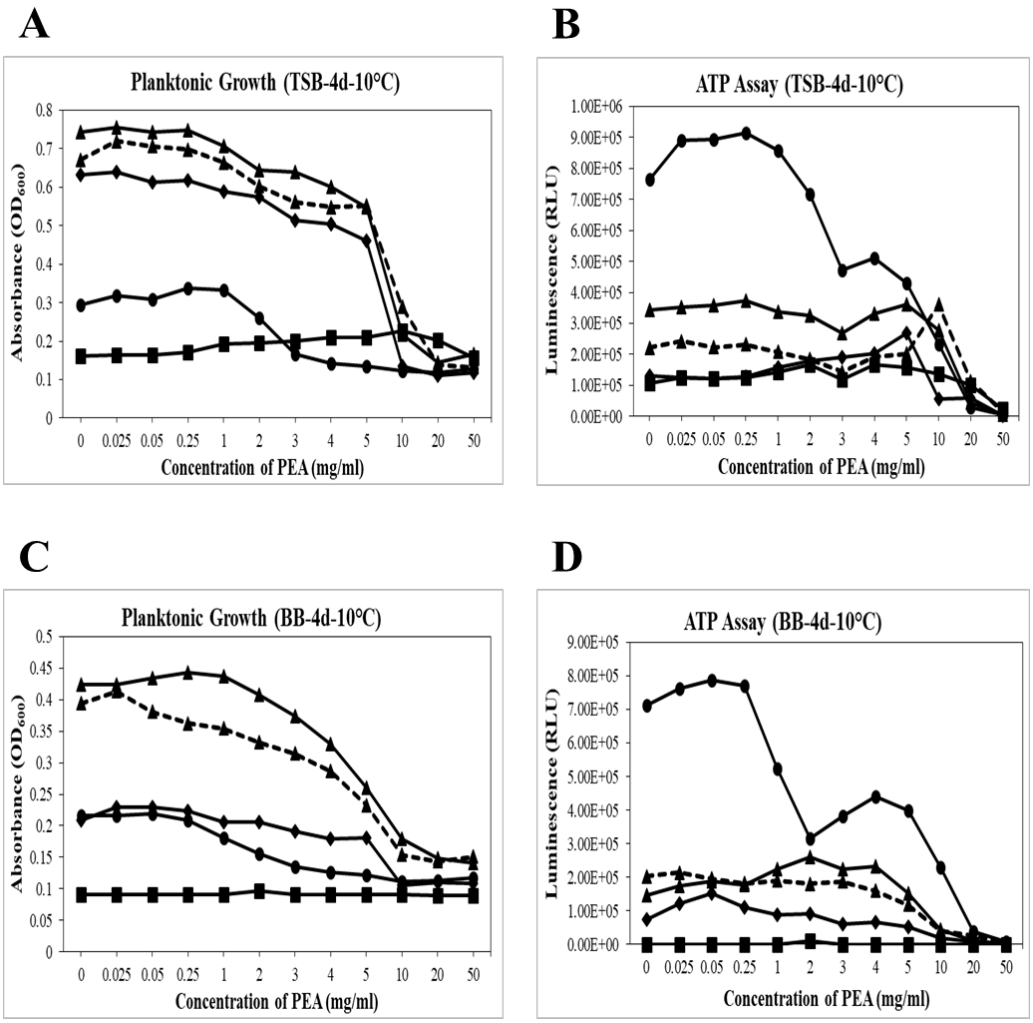

Figure 7. Effectiveness of PEA on pathogens growth and ATP content at 10°C for 96 h. Reduction of planktonic growth and luminescence from the ATP assay for bacterial pathogens, when PEA was supplemented to the liquid growth medium (TSB or BB) at inoculation. Growth (Panel A and C) and luminescence from the ATP assay that was performed on the biofilm (Panel B and D) of *P. aeruginosa* (circle, solid line), *S. aureus* (square, solid line), *E. coli* EDL932 (diamond, solid line), *E. coli* UMN026 (triangle, solid line), and *E. coli* CFT073 (triangle, dashed line) were determined after 96 h at 10°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

Fig. 7D shows ATP content of viable cells in the biofilm of the bacterial pathogens from the same experiment. At 4ºC in BB, the pathogen *P. aeruginosa* exhibited the highest levels of ATP content. PEA started to reduce ATP content for *P. aeruginosa* (circles, solid line) at a concentration of 1 mg/ml and the IC_{50} was 3 mg/ml. Interestingly, the ATP content started to

increase slightly between 2-5 mg/ml before continuing to decline. ATP content of *S. aureus* (squares, solid line) was low at all concentrations of PEA and an IC_{50} could not be calculated. PEA started to reduce ATP content for *E. coli* EDL932 (diamonds, solid line) at 0.25 mg/ml and for *E. coli* UMN026 (triangles, solid line) at 4 mg/ml with IC_{50} values of 5 mg/ml and 6 mg/ml, respectively. For *E. coli* CFT073 (triangles, dashed line) was not significantly reduced and we were unable to calculate an IC_{50} value. Similar to the experiments at 10°C, there was a great reduction in measurable metabolic activity at 50 mg/ml.

Fig. 8A shows planktonic growth measured after 16 h incubation at 37°C in TSB. PEA started to reduce growth for *P. aeruginosa* (circles, solid line) at a concentration of 0.25 mg/ml with an IC_{50} value of 4 mg/ml. *S. aureus* (squares, solid line) planktonic growth declined steadily between 0 and 10 mg/ml of PEA and dropped more drastically towards 20 mg/ml. The IC_{50} was 22 mg/ml. PEA started to reduce growth for *E. coli* EDL923 (diamonds, solid line) at 0.25 mg/ml, for *E. coli* UMN026 (triangles, solid line) at 1 mg/ml, and for *E. coli* CFT073 (triangles, dashed line) at 0.025 mg/ml with IC_{50} values of 12 mg/ml, 15 mg/ml, and 12 mg/ml, respectively. Almost complete abolishment of planktonic growth was observed at 50 mg/ml.

Fig. 8B shows ATP content of viable cells in the biofilm of the bacterial pathogens from the same experiment. At 37°C in TSB, the pathogens *S. aureus* and *P. aeruginosa* exhibited the highest levels of ATP content. PEA started to reduce ATP content for *P. aeruginosa* (circles, solid line) at a concentration of 0.05 mg/ml and the IC_{50} was 3 mg/ml. For *S. aureus* (squares, solid line), PEA started to reduce ATP content at 5 mg/ml and the IC_{50} was 12 mg/ml. PEA started to reduce ATP content for *E. coli* EDL932 (diamonds, solid line) at 1 mg/ml, for *E. coli* UMN026 (triangles, solid line) at 4 mg/ml, and for *E. coli* CFT073 (triangles, dashed line) at 2 mg/ml with IC_{50} values of 8 mg/ml, 6 mg/ml, and 5 mg/ml, respectively. Similar to the

experiments at 10°C, complete abolishment of measurable metabolic activity was observed at 50 mg/ml. Interestingly, for *E. coli* UMN026 (triangles, solid line) the ATP content of the biofilm increased from 0.05 mg/ml to 3 mg/ml of PEA then had a steep decline in ATP content of the biofilm from 4 mg/ml to 10 mg/ml of PEA.

Fig. 8C shows planktonic growth measured after 16 h incubation at 37°C in BB. PEA started to reduce growth for *P. aeruginosa* (circles, solid line) at a concentration of 0.25 mg/ml with an IC_{50} value of 10 mg/ml. Planktonic Growth of *S. aureus* (squares, solid line) was low at all concentrations of PEA and an IC_{50} could not be calculated. PEA started to reduce growth for *E. coli* UMN026 (triangles, solid line) at 0.25 mg/ml and for *E. coli* CFT073 (triangles, dashed line) at 0.025 mg/ml with IC_{50} values of 3 mg/ml and 21 mg/ml, respectively. There was a moderate reduction in growth for *E. coli* EDL923 (diamonds, solid line) starting at 1 mg/ml with an IC_{50} values of 3 mg/ml. Almost complete abolishment of planktonic growth was observed at 50 mg/ml.

Fig. 8D shows ATP content of viable cells in the biofilm of the bacterial pathogens from the same experiment. At 37^{o}C in BB, the pathogen *P. aeruginosa* exhibited the highest levels of ATP content. PEA started to reduce ATP content for *P. aeruginosa* (circles, solid line) at a concentration of 3 mg/ml and the IC_{50} was 3 mg/ml. The ATP content of *S. aureus* (squares, solid line) was low at all concentrations of PEA and an IC_{50} could not be calculated. PEA started to reduce ATP content for *E. coli* EDL932 (diamonds, solid line), for *E. coli* UMN026 (triangles, solid line), and *E. coli* CFT073 (triangles, dashed line) at 0.05 mg/ml with IC_{50} values of 2 mg/ml, 4 mg/ml, and 3 mg/ml, respectively. Similar to the experiments at 10°C, complete abolishment of measurable metabolic activity was observed at 50 mg/ml.

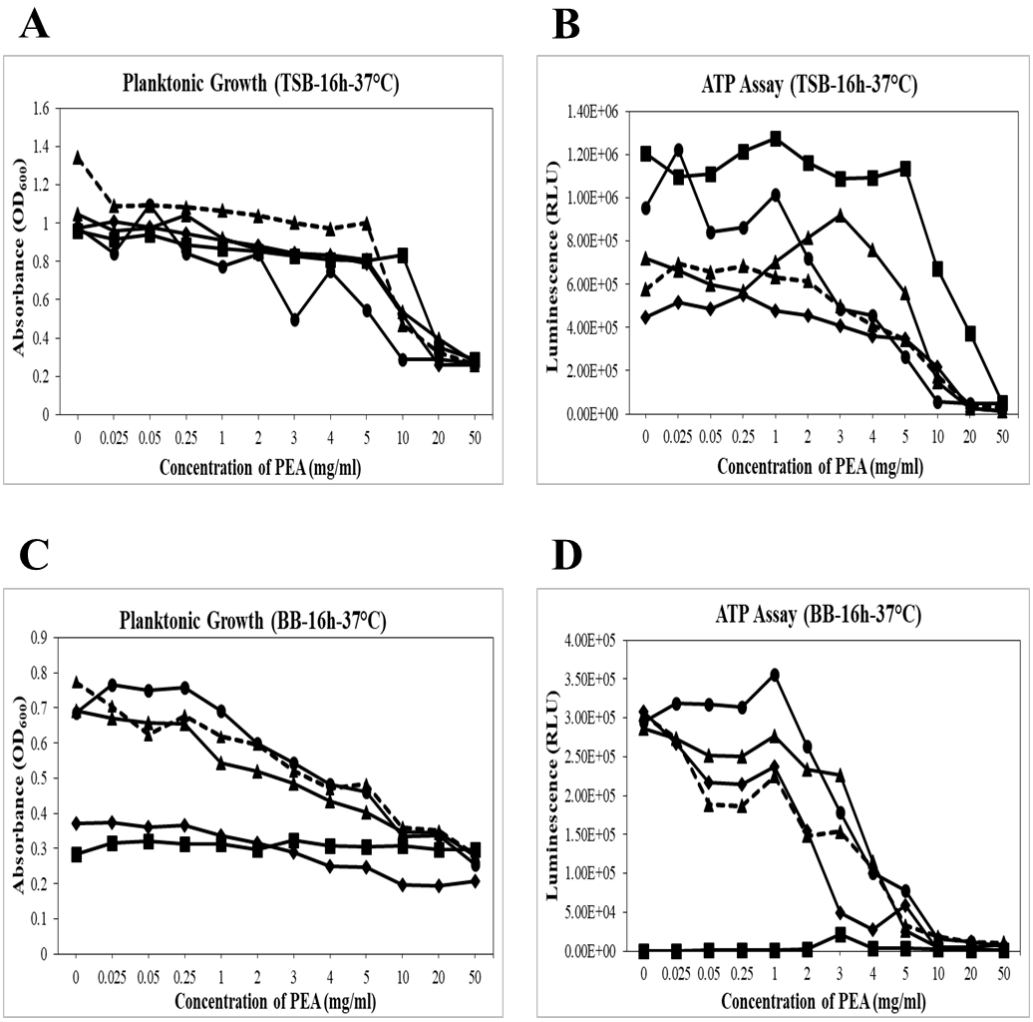

Figure 8. Effectiveness of PEA on pathogen growth and ATP content at 37°C for 16 h. Reduction of planktonic growth and luminescence from the ATP assay for bacterial pathogens, when PEA was supplemented to the liquid growth medium (TSB or BB) at inoculation. Growth (Panel A and C) and luminescence from the ATP assay that was performed on the biofilm (Panel B and D) of *P. aeruginosa* (circle, solid line), *S. aureus* (square, solid line), *E. coli* EDL932 (diamond, solid line), *E. coli* UMN026 (triangle, solid line), and *E. coli* CFT073 (triangle, dashed line) were determined after 16 h at 37°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

Mutations in E. coli K-12 β-phenylethylamine catabolism and chemoreceptors result in reduction of planktonic growth and biofilm biomass similar to the wild type strain AJW678

To determine whether the PEA effect was due to PEA itself or one of its degradation products phenylacetaldehyde or phenylacetic acid, we determined planktonic growth and biofilm

biomass in knock-out mutants of AJW678 that were deficient in the enzymes catalyzing these two reactions (TynA and FeaB). The *E. coli* K-12 strain AJW678 (circle), the *tynA* mutant strain (square), and the *feaB* mutant (diamond) were grown on 96-well polystyrene plates in TSB or BB supplemented with a range of concentrations of PEA (0-50 mg/ml), that were supplied to the liquid growth medium at the time of inoculation. Fig. 9A shows the planktonic growth measured after 96 h of incubation at 10°C in BB. PEA started to reduce growth of all the strains at a concentration of 0.05 mg/ml and caused a 50 % reduction at approximately 3-4 mg/ml. There was no significant differences in growth between the mutant strains and the wild type strain. Fig. 9B shows the absorbance data from the CV assay that was performed on the biofilm. All strains had a slight decline in biofilm biomass amounts from 0.025 to 50 mg/ml. There were no significant differences between the mutant strains and the wild type strain in biofilm amounts, except at 4 mg/ml of PEA where we observed a significant difference between the biofilm biomass amounts between the *feaB* (diamond) mutant and the wild type strain (p-value = 0.03).

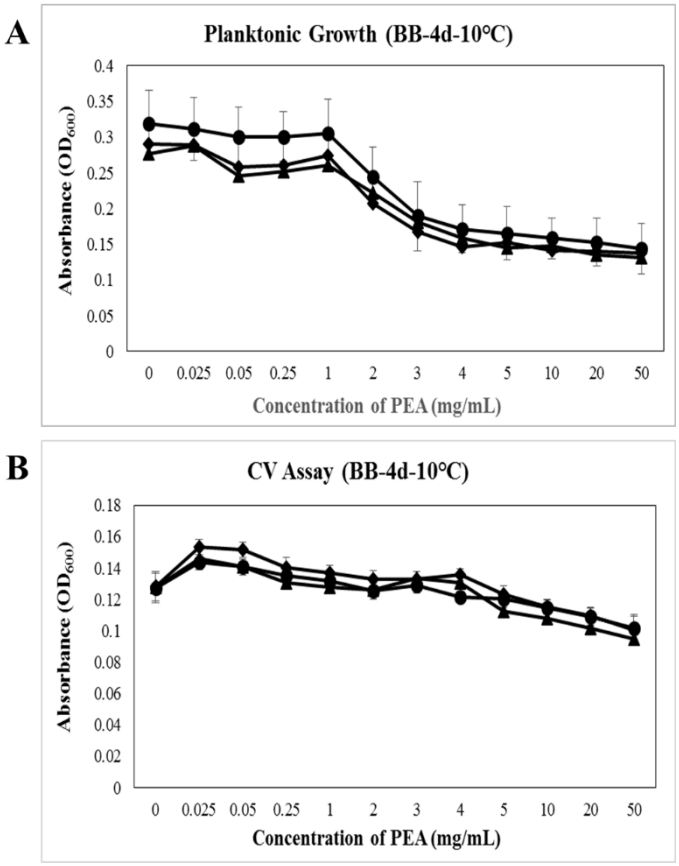

Figure 9. Effectiveness of PEA on growth and biofilm biomass amounts of the wild type and PEA catabolism mutants at 10°C in BB. Reduction of planktonic growth and biofilm biomass amounts of AJW678, when PEA was supplemented to the liquid growth medium (BB) at inoculation. Growth (Panel A) and biofilm biomass amounts (Panel B), of AJW678 (circle), *tynA* mutant (square), and *feaB* mutant (diamond), were determined after 96 h at 10°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

Fig. 10A shows the planktonic growth measured after 24 h of incubation at 37°C in TSB. PEA started to reduce growth of all the strains at a concentration of 1 mg/ml and caused a 50 % reduction at approximately 3-4 mg/ml. We observed a significant difference in growth between the *tynA* mutant (square) and the wild type strain (circle) at 0, 0.025, and 1 mg/ml of PEA with p-values of 0.01, 0.01, and 0.03 respectively. For the *feaB* mutant (diamond) we observed a significant difference in growth in comparison to the wild type strain at all of the concentrations of PEA except for 3, 5, and 10 mg/ml of PEA with p-values of 0.01, 0.03, and 0.06 respectively. Fig. 10B shows the absorbance data from the CV assay that was performed on the biofilm. All

strains had a slight increase in biofilm biomass amounts from 0 to 0.05 mg/ml and then began to decline from 0.25 to 50 mg/ml. There were no significant differences between the *tynA* mutant strain (square) and the wild type strain (circle) in biofilm amounts, while we observed a significant difference in biofilm biomass amounts between the *feaB* mutant (diamond) and the wild type strain (circle) 0, 0.025, 0.05, and 2 mg/ml of PEA with p-values of 0.001, 0.01, 0.01, and 0.01 respectively. For all the assays it appeared as the concentration of PEA increased and the values began to decline the difference between the mutant strains and the wild type became insignificant.

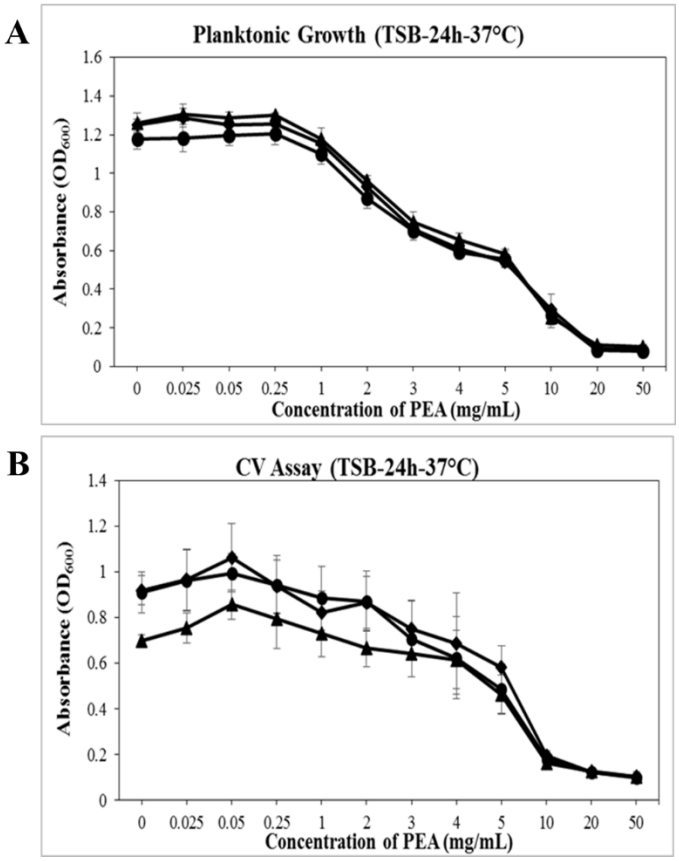

Figure 10. Effectiveness of PEA on growth and biofilm biomass amounts of the wild type and PEA catabolism mutants at 37°C in TSB. Reduction of planktonic growth and biofilm biomass amounts of AJW678, when PEA was supplemented to the liquid growth medium (BB) at inoculation. Growth (Panel A) and biofilm biomass amounts (Panel B), of AJW678 (circle), *tynA* mutant (square), and *feaB* mutant (diamond), were determined after 96 h at 37°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

To determine whether the antimicrobial action of PEA required one of *E. coli*'s chemoreceptors (Tar, Tap, Aer, Tsr, or Trg) we determined planktonic growth (Fig. 11A) and biofilm biomass (Fig. 11B) in knock-out mutants of AJW678 that were deficient in one of the five chemoreceptors Tar, Tap, Aer, Tsr, and Trg. The *E. coli* K-12 strain AJW678 (circle, solid line), the *tar* (square, dashed line), *tap* (square, solid line), *aer* (diamond, solid line), *tsr* (triangle, solid line), and *trg* (triangle, dashed line) mutant strains were grown on 96-well polystyrene plates in TSB or BB supplemented with a range of concentrations of PEA (0-50 mg/ml), that were supplied to the liquid growth medium at the time of inoculation. The *tar* mutant was not able to grow at all at 10°C in BB and is not included in Fig. 10.

Fig. 11A shows the planktonic growth measured after 96 h of incubation at 10°C in BB. Planktonic growth was seen for the mutant strains and wild type strain at all the concentrations of PEA, including 0 mg/ml of PEA. However, in general there was a decline in planktonic growth of the mutant strains and the wild type strain as the concentration of PEA increased. Fig. 11B shows the absorbance data from the CV assay that was performed on the biofilm. Less biofilm biomass was seen for the mutant strains in comparison to the biofilm biomass of the wild-type and there was no significant difference in biofilm biomass at all of the concentrations of PEA in comparison to 0 mg/ml of PEA. The wild type strain did make a little biofilm biomass and a small decrease in biofilm biomass was observed as the concentration of PEA increased. A statistically significant difference in biofilm biomass between the mutants and the wild type strain was observed at the concentrations of PEA from 0 mg/ml to 5 mg/ml. No statistically significant difference in biofilm biomass was observed for all of the mutants at all concentrations of PEA.

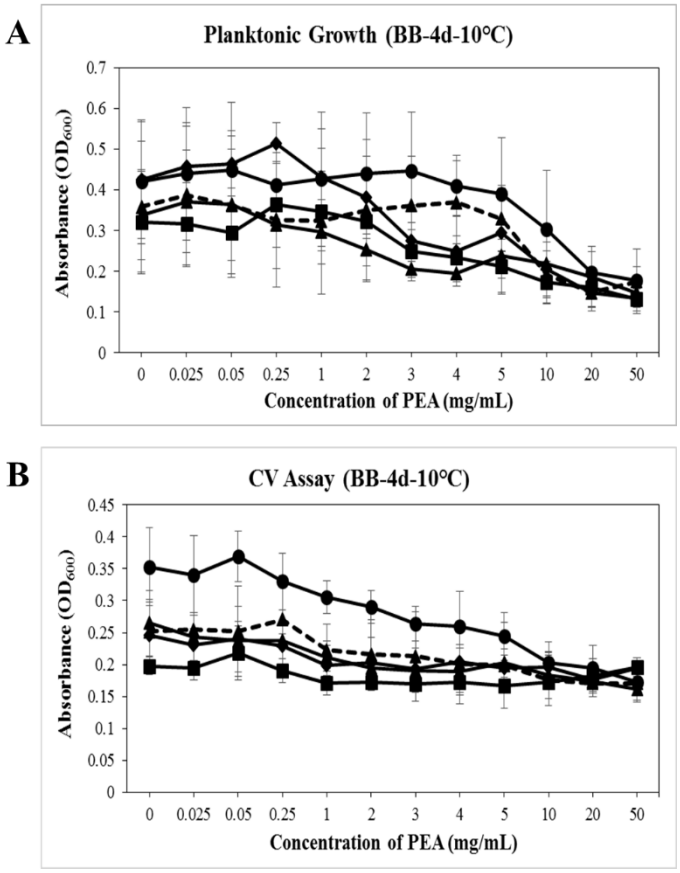

Figure 11. Effectiveness of PEA on growth and biofilm biomass amounts of the wild type and chemoreceptor mutants at 10°C in BB. Reduction of planktonic growth and biofilm biomass amounts of AJW678, when PEA was supplemented to the liquid growth medium (BB) at inoculation. Growth (Panel A) and biofilm biomass amounts (Panel B), of AJW678 (circle, solid line), the *tar* (square, dashed line), *tap* (square, solid line), *aer* (diamond, solid line), *tsr* (triangle, solid line), and *trg* (triangle, dashed line) mutant strains, were determined after 96 h at 10°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

Fig. 12A shows the planktonic growth measured after 24 h of incubation at 37°C in TSB. PEA started to reduce growth of all the strains at a concentration of 0.025 mg/ml and caused a 50 % reduction at approximately 5-10 mg/ml. We observed no significant difference in growth between all of the mutant and the wild type strain at all of the concentrations of PEA.

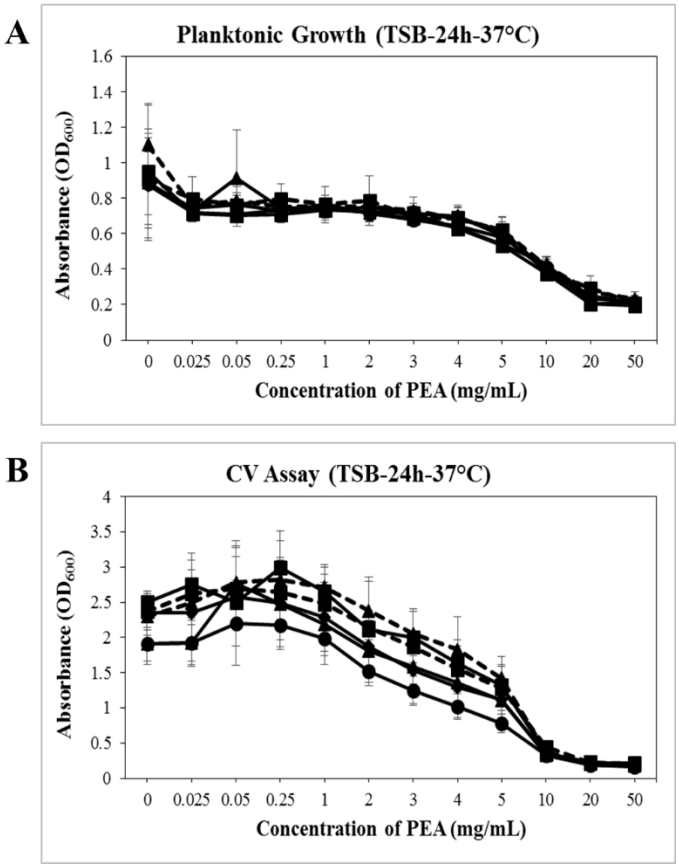

Figure 12. Effectiveness of PEA on growth and biofilm biomass amounts of the wild type and chemoreceptor mutants at 37°C in TSB. Reduction of planktonic growth and biofilm biomass amounts of AJW678, when PEA was supplemented to the liquid growth medium (TSB) at inoculation. Growth (Panel A) and biofilm biomass amounts (Panel B), of AJW678 (circle, solid line), the *tar* (square, dashed line), *tap* (square, solid line), *aer* (diamond, solid line), *tsr* (triangle, solid line), and *trg* (triangle, dashed line) mutant strains, were determined after 96 h at 37°C in the presence of varying concentrations of PEA. Averages are calculated across 6 replicates.

Fig. 12B shows the absorbance data from the CV assay that was performed on the biofilm. All strains had a slight increase in biofilm biomass amounts from 0 to 0.25 mg/ml and then began to decline from 1 to 50 mg/ml. In general, there was a statistically significant difference in biofilm biomass of the mutants in comparison to the wild type strain at the concentrations of PEA from 0 mg/ml to 5 mg/ml. There were no significant differences between biofilm biomass of the mutants and wild type strain at concentrations of PEA from 10 mg/ml to 100 mg/ml. For all the assays in general it appeared as the concentration of PEA increased and

the values began to decline the difference between the mutant strains and the wild type became insignificant.

Discussion

PEA had an inhibitory effect on planktonic growth, biofilm biomass amounts, and ATP energy content of AJW678 as we increased the concentration of PEA. We saw a significant reduction in growth and ATP energy content of the biofilm at low concentrations of PEA, while higher concentrations of PEA were necessary to completely abolish the biofilm biomass. This can be attributed to the differences in the assays used to characterize the biofilms (for a review, see [191]). The CV assay stains negatively charged surface molecules, including live bacteria, dead bacteria, and the EPS [192]. This assay has been used to assess biofilm in many studies, including our own work [154, 193]. Whereas, the ATP bioluminescence assay is used to quantify the metabolic activity or energy content of live bacteria. Luminescence occurs through the enzymatic reaction where luciferin and ATP are converted to oxyluciferin and AMP when the luciferase enzyme cleaves off ADP causing an emission of light. The ATP content of bacteria does not vary with the growth rate, which is essential for using ATP bioluminescence to assess cell viability [194]. Previous research in our lab using the BacTiter Glo™ kit from Promega, demonstrated a linear correlation between the luminescence signal and the number of viable bacteria in planktonic culture [195]. However, the correlation between the ATP signal and viable bacteria is less clear in biofilm bound bacteria than in planktonic bacteria. One explanation for this difference between biofilm biomass and biofilm ATP content could be that there are no energetically active cells (measured by the ATP assay) within the biofilm at lower concentrations of PEA, but the EPS of the biofilm is still left behind and gets stained with the CV assay. We have observed a decrease in viable bacteria as the ATP signal decreases with increasing

concentrations of PEA, which has been seen in multiple experimental settings (for data see Chapter 3). This supports the hypothesis that there is a correlation between ATP signal and viable bacteria.

Expanding upon the observation that PEA could prevent the formation of biofilm in *E. coli*, we then determined that PEA could be used as a treatment for biofilms. We tested whether the addition of PEA after inoculation, was able to reduce planktonic growth and ATP energy content of *E. coli* AJW678 biofilm. It was observed that PEA was able to reduce the planktonic growth and ATP energy content with increasing concentrations, regardless of the environmental conditions or the time at which the PEA was added. However, supplementing PEA to the bacterial growth media at later time points during biofilm development decreased the percent reduction that could be accomplished by adding PEA to the bacterial growth medium. Interestingly, under all the environmental conditions, when PEA was supplemented to TSB at later time points, an increase in ATP content occurred before we began to observe a decline in ATP content. The later PEA was supplemented to the media, the longer this increase in ATP content of the biofilm extended to higher concentrations of PEA. One explanation for this could be that those concentrations contain sub-lethal levels of PEA and instead of inhibiting are actually enhancing the growth of the bacteria and promoting biofilm formation. Similar results have shown that exposure of bacteria to sub-lethal doses of antibiotics can promote resistance and biofilm formation. One study found increased biofilm formation of *P. aeruginosa* when exposed to sub-lethal doses of cefotaxim, amoxicillin, and azithromycin in the presence of chlorohexidine [196]. Our data are in agreement with the idea that PEA added at the beginning of the experiment may serve as a biofilm prevention technique, whereas PEA added later would act as a treatment of the biofilm.

Since we had success reducing the growth, biofilm biomass, and ATP content of a non-pathogenic *E. coli* strain, we next wanted to investigate the effect of PEA on five clinically relevant bacterial pathogens. PEA reduced planktonic growth and ATP content in the biofilms of all bacterial strains tested and under all of the tested conditions. However, the extent of these reductions was dependent upon the bacterial strain and the conditions under which these biofilms had been cultured. PEA was able to reduce growth and ATP content of the four Gram-negative and one Gram-positive strain that we tested. However, higher concentrations of PEA were required to get a complete reduction in ATP content of *S. aureus* when PEA was supplemented TSB at 37°C. This could be due to the fact the optimal growth temperature for *S. aureus* is body temperature (~37°C). *P. aeruginosa* also required higher concentrations of PEA to get a complete reduction in ATP of when PEA was supplemented to the growth media at 10°C. The clinical strains of *S. aureus* and *P. aeruginosa* have been found to have more resistance to antibiotics and antimicrobials in recent years [55, 197]. *Pseudomonas spp.* can tolerate refrigeration temperatures and have been associated with contamination of raw milk, which may explain why higher concentrations of PEA were necessary to reduce the ATP content in BB [198]. Our data indicates that PEA could have antimicrobial effects on a broad spectrum of bacterial strains, both gram negative and gram positive.

Interestingly, we observed that in *E. coli* (Fig. 7, *E. coli* UMN026) at moderate PEA concentrations (~3 mg/ml) there was an increase in the ATP content of the biofilm before decreasing at higher concentrations of PEA (>10 mg/ml). We offer two possible explanations for this phenomenon: first, it is possible that the activity of the PEA's degrading enzymes TynA and FeaB that convert PEA to phenylacetaldehyde and phenylacetic acid [199] enable *E. coli* to utilize PEA as a carbon source. In fact, aromatic amino acids and amines are thought to be a

major ‘food’ component for *E. coli* living in the intestine (for a review, see [200]). It is possible that the observed increases in the ATP content of the biofilm at moderate PEA concentrations may be due to PEA acting as a nutrient. Second, it is possible that sub-lethal concentrations of PEA promote biofilm formation due to this phenotype’s enhanced resistance to toxic substances. The fact that we only saw this increase in the data from the ATP assay that was performed on the biofilm, and not in the data on growth of planktonic bacteria, supports this idea.

In a recent work, researchers found that PEA was shown to mimic overexpression of DisA, phenylalanine decarboxylase, from *Proteus mirabilis*, when transformed into *E. coli.* Over expression of DisA and exposure to PEA both resulted in decreased motility, flagellar gene expression, and biofilm amounts in *E. coli*. This lead to the hypothesis that PEA or biproducts of DisA decarboxylation may affect transcription of the FlhD/FlhC complex [201]. We observed that PEA reduced planktonic growth and biofilm biomass of both knockout mutants and the wild type strain under all of the tested conditions. This supports the hypothesis that the PEA effect is due to PEA itself and not one of its degradation products.

Previous work has shown that growth of *E. coli* K-12 with NE induces the transcription of the periplasmic tyramine oxidase, TynA, and the aromatic aldehyde dehydrogenase, FeaB, which converts NE to 3,4-dihydroxymandelic acid (DHMA) [119, 120]. Chemotaxis occurred when DHMA bound to the serine chemoreceptor Tsr [122]. NE is able to induce transcription of TynA and FeaB through the two-component signaling pathway QseB/QseC, where QseC is the membrane-bound quorum-sensing histidine protein kinase and QseB is the associated response regulator [121]. Thus, the chemotaxis response to NE that was observed occurs indirectly [122]. Direct binding of NE to QseC has been shown to play a role in the NE-induced expression of virulence and motility genes, whose products are involved in adherence, motility, and

pathogenesis [115, 116]. For example, it has been shown in EHEC that QseB/QseC transcriptionally regulates the master flagella regulator, FlhD/FlhC, which is responsible for swimming motility [117]. Our own research has shown a correlation between flagella and chemotaxis gene expression, which impacts biofilm formation [118]. The similarities between PEA and NE led us to the hypothesis that PEA may be affecting biofilm formation through serine receptor Tsr or one of the other chemoreceptors found in *E. coli*. This hypothesis is supported by the observation that NE is converted to DHMA by TynA and FeaB, resulting in the binding of DHMA to the Tsr receptor. The antimicrobial action of PEA could be occurring through this same pathway because PEA has a similar chemical structure as NE (PEA lacks the 3 hydroxyl groups found on NE) and the degradation of PEA also require TynA and FeaB. In addition, NE was shown to impact biofilm formation through QseC/QseB. In general, we observed that PEA reduced planktonic growth and biofilm biomass of all knockout mutants and the wild type strain under all of the tested conditions. However, little growth was observed for the knockout mutants and wildtype strain when they were grown in BB at 10°C, which resulted in little biofilm formation. This could be attributed to the growth media used and the environmental conditions. Overall, the results we observed do not indicate that PEA is reducing growth and biofilm amounts by impacting chemotaxis through the chemoreceptors. Further research needs to be done to determine the exact mechanism by which PEA is acting to reduce growth and biofilm amounts. Overall, PEA was able to prevent the growth and biofilm formation of *E.* coli, which makes it a good alternative to the use of traditional antibiotics for prevention of biofilm formation.

Conclusion

The WHO has declared antibiotic resistance as one of the top three threats to global public health [32]. More than 2 million Americans suffer from an antibiotic-resistant infection, imposing an enormous economic burden (~$20 billion in direct healthcare costs) on the country's healthcare systems [30]. Unfortunately, the emergence of antibiotic resistant strains of bacteria is outpacing the discovery and development of new antibiotics, which makes finding alternative treatment option imperative [32]. We have identified the nutrient PEA to have antimicrobial effects on growth and biofilm of a variety of clinically relevant bacterial pathogens. PEA effectively reduced growth and biofilm of a non-pathogenic *E. coli* K-12 strain with increasing concentrations of PEA, regardless of whether PEA was supplemented to the media at the time of inoculation or during the different stages of biofilm development. We were able to determine that it was the molecule PEA itself that caused the antimicrobial effects on growth and biofilm, not the degradation products of PEA metabolism. It is envisioned that this nutrient can be used in the future as an alternative to traditional antibiotics to prevent and treat bacterial biofilms. This could help reduce the emergence of antibiotic resistant bacteria and help prolong the existing antibiotics that are last resort treatments for severe bacterial infections. Hopefully, this will lead to the use of PEA in a variety of applications, including coating of food processing equipment or medical devices, a spray to prevent or treat biofilm formation on food products or food processing equipment, or as a treatment flush to reduce catheter associated bacterial infections.

CHAPTER 3. ETHYL ACETOACETATE EXPERIMENTS[7,8]

Introduction

The CDC estimates that 48 million Americans annually ingest bacteria contaminated food that results in illness and costs $77.7 billion in health care costs, workers compensation, and loss of product. Food contact surfaces, equipment, and processing environments that aren't properly cleaned have led to foodborne outbreaks, particularly with *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella spp.* [202]. During food processing, pathogenic microbes can be spread to beef and poultry from the equipment, surfaces, and containers used in different stages of the process. Infected animals that are not properly disinfected prior to slaughter can transmit bacterial pathogens to equipment as well [57]. This spread of bacteria to previously uninfected surfaces can be facilitated by the formation of biofilms, leading to outbreaks of infectious disease. Biofilms are defined as communities of bacteria that attach or adhere on to a variety of surfaces and are very hard to treat with antibiotics, chemical disinfectants, and physical cleaning. Many bacterial pathogens are associated with foodborne illnesses, such as *Pseudomonas spp., S. marcescens, E. coli spp.*, and *S. simulans*. These bacteria strains have been identified as bacteria that can attach to the polyurethane and polyvinylchloride surfaces that line the conveyor belts used during different stages in food processing [58].

[7] Horne, S.M., **Schroeder, M.,** Murphy, J., and Prüβ, B.M. *Acetoacetate and ethyl acetoacetate as novel inhibitor of bacterial biofilm*. Letters in Applied Microbiology. 2018. 66(4): p. 329-339. The initial observation that EAA was a novel biofilm inhibitor on growth and biofilm by *Y. enterocolitica*, *S. marcescens*, and *C. sakazakii* was published. The material in this chapter was co-authored by Meredith Schroeder and Birgit M Prüβ. Meredith Schroeder had primary responsibility for preforming the experiments, collecting data, and analyzing the data presented below. Meredith Schroeder was instrumental in the development of the conclusions that are advanced here. Meredith Schroeder also drafted and revised all versions of this chapter. Birgit M Prüβ served as proofreader and checked the math in the statistical analysis conducted by Meredith Schroeder.

[8] RFT-559. **Schroeder, M.,** S.M. Horne, S.J. Stafslien, and B.M. Prüβ. Biofilm inhibitor and method for inhibiting biofilm. Non-provisional patent application under 37 C.F.R. 1.53 (b).

A variety of methods and safety controls have been introduced over the years by the food processing industry to control biofilm formation and reduce foodborne outbreaks. Mechanical and manual cleaning of food products and equipment to remove debris that may contain bacteria or could promote bacterial growth has been used to reduce biofilm formation. SHS has been shown to be effective at reducing *E. coli* O157:H7, *S. enterica* serovar Typhimurium, *S. Enteritidis* phage type 30, and *L. monocytogenes* on almonds and pistachios [82]. Chemical cleaning using halogens, peroxygens, acids, and quaternary ammonium compounds has been another strategy to reduce bacterial biofilms in food processing. Chlorine has been used in food processing due to its oxidizing and disinfecting power, but recently has been shown to cause resistance in biofilms of *L. monocytogenes* with repeated expose [67, 203]. Treatments of lactic acid and peracetic acid have been shown to effectively reduce *Salmonella spp.* on pieces of chicken [68]. Quaternary ammonium salts effectively kill fungi, gram positive bacteria, and gram negative bacteria and have been used to modify surface materials used in food packaging [80]. Despite all these efforts, outbreaks of food-borne infectious diseases persist and an urgent need for novel biofilm treatments is evident.

The research in this study constitutes a different approach toward the development of novel biofilm treatments by utilizing nutrients bacteria need to grow. Previous research in our lab screened 95 carbon and 95 nitrogen sources and found AAA to have inhibitory effects on the growth, biofilm amounts, and cell counts of *E. coli* O157: H7 grown in liquid BB at refrigerator temperature (10°C) [204]. AAA is a derivative of acetic acid, which has been used in the food industry as a cleaning technique. Acetic acid has been shown to be effective at reducing *C. jejuni*, which has been associated with multiple foodborne outbreaks, on pieces of chicken [155, 156]. AAA and EAA, a homolog of AAA, were found to have an inhibitory effect on growth and

biofilm amounts of *C. sakazakii*, *S. marcescens* and *Y. enterocolitica*. However, EAA was found to be more effective than AAA, reducing growth and biofilm amounts up to 3-logs [154].

This study expands on these previous observations by testing the effect of EAA supplied in the liquid bacterial growth medium on non-pathogenic *E. coli* K-12, as well as a selection of pathogens relevant to food safety and clinical settings. This study had two major objectives: 1) examine the antimicrobial effect of EAA, GP, and lyxose on a variety of bacterial strains and 2) investigate synergistic antimicrobial effects of combinations of PEA with EAA, GP, and lyxose. We were able to observe a reduction of ATP content and biofilm biomass of some of the bacterial strains tested when treated with EAA. Only *L. monocytogenes* had a reduction of ATP content and biofilm biomass when treated with GP. However, lyxose was unable to reduce ATP content and biofilm biomass of any of the bacterial pathogens tested. In addition, combinations of PEA with EAA, GP, or lyxose were less effective at reducing the ATP content and biofilm biomass of the bacterial pathogens in comparison to when they were treated alone with PEA, EAA, GP, or lyxose. This is an indication that only some nutrients may be able to be used to reduce ATP content and biofilm biomass of bacterial pathogens. Unfortunately, EAA and GP were only able to reduce the ATP content and biofilm biomass of some pathogens making their use pathogen specific. Overall, the use of nutrients as an antimicrobial agent is a novel approach to the prevention and treatment of biofilm formation, which could lead to possible applications in the medical and food processing industries.

Materials and methods

Bacterial strains

Bacterial strains used in this study are summarized in Table 3 and include the non-pathogenic, wild type *E. coli* K-12 strain and nine bacterial pathogens. The *E. coli* K-12 strain

used was AJW678, which was previously described as a good former of biofilm [170]. Pathogenic bacterial strains include *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 25923), EHEC EDL932 [172], and two UPECs, UMN026 [173] and CFT073 [174], *Y. enterocolitica* 8081c (ATCC 9610) [175], *L. monocytogenes* 10403S [176], *C. sakazakii* (BAA-894) [177], and *S. marcescens* (BAA-632) [178]. Bacterial strains were maintained as freezer stocks at -80ºC in 8 % DMSO. Prior to each experiment, the *E. coli* strains were plated onto LB (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) agar plates. *P. aeruginosa* and *S. aureus* were plated onto TSA (15 g/l tryptone, 5 g/l soytone, 5 g/l NaCl, 15 g/l agar) plates. Plates were incubated over night at 37°C.

Formation of the biofilms

Liquid overnight cultures in TSB (17 g/l tryptone, 3 g/l soytone, 2.5 g/l glucose, 5 g/l NaCl, 2.5 g/l K_2HPO_4) were pelleted by centrifuging at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of 1 x PBS. Cultures were adjusted with PBS until their optical density values at 600 nm (OD_{600}) was 1 $\pm$ 0.05. The final inocula were prepared by diluting the PBS cultures 1:10 in 2 x TSB. Dilutions of ethyl acetoacetate (EAA; Alfa Aesar, Ward Hill, MA), DL-α-glycerol phosphate (GP; Sigma Aldrich, St. Louis, MO), and lyxose (Alfa Aesar, Ward Hill, MA) were prepared in PBS at concentrations of 0, 2, 10, and 20 mg/ml. Supplements were chosen based on the cost and the antimicrobial effect of the nutrient observed in previous studies and performed in our lab [154, 204]. Combinations of GP, EAA, or lyxose with PEA were prepared in PBS at concentrations of 0 mg/ml of supplement to 0 mg/ml of PEA, 2 mg/ml of supplement to 2 mg/ml of PEA, 10 mg/ml of supplement to 10 mg/ml of PEA, and 20 mg/ml of supplement to 20 mg/ml of PEA. A 1:1 dilution of final inocula and supplement solution was used to inoculate individual wells of a 24-well with 1 ml/well (200 µl/well for 96-well polystyrene plate). This yields an inoculation OD_{600} of ~0.05 in 1 x TSB/0.5 x PBS and supplement

concentrations of 0, 1, 5, and 10 mg/ml. The plates were incubated at 37°C for 24 h. The experiments were done on two replicate plates with independently grown overnight cultures. Each plate contained two replicates from the same overnight culture.

Determination of bacterial growth and biofilm amounts

Planktonic growth was determined as end point growth by using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), which recorded the OD_{600} at the end of the incubation period. Averages and standard deviations were determined over 4 replicates.

To characterize the biofilm at the end of the incubation period, two analyses were used: the CV assay that stains live and dead biomass, as well as exopolysaccharide and the ATP assay that measures the energy content of live bacteria [186, 187]. Prior to the biofilm assays, we rinsed each well three times with 1.0 ml (200 µl). When biofilms of *E. coli* O157:H7 were processed, the wells were only rinsed twice because this strain only forms a small amount of biofilm. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for at least 1 h. For the CV assay, 1.0 ml (200 µl) of 0.1 % CV in ddH_2O was added to each well of the 24-well (96-well) plates and incubated at room temperature for 15 min, then rinsed three times with 1.0 ml (200 µl) of ddH_2O. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for at least 1 h. The CV was extracted by adding 500 µl (200 µl) of 20 % acetone 80 % ethanol to each well, followed by 15 min of incubation. 0.15 ml of each extract was then transferred into one well of a 96 well polystyrene plate and the OD_{600} measurements were determined with the Synergy H1 Hybrid Reader. Averages and standard deviations were determined across 4 replicates.

For the ATP assay, the BacTiter Glo™ kit (Promega, Madison, WI) was used. Reagents were prepared per manufacturer instructions. To each well, we added identical amounts of PBS

and BacTiter-Glo reagent. For 24 well plates, this was 250 µl. For 96 well plates, we added 100 µl of each solution. We covered the plates with tinfoil and thoroughly mixed solutions by placing the plate on a rotating shaker for 5 min. After this incubation, we transferred 150 µl from each well to an opaque white 96-well plate and read luminescence using the Synergy H1 Hybrid Reader.

Data analysis

Averages and standard deviations of luminescence values were determined over 6 replicates. Student's *t*-test were ran to determine the statistical significance of the difference between the growth means for each concentration when compared to the mean growth of the concentration 0 mg/ml.

Results

Ethyl acetoacetate reduces energy content and biomass of biofilm for P. aeruginosa and E. coli K-12

In this experiment, 10 bacterial pathogens including *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enterocolitica, L. monocytogenes, S. marcescens*, and *C. sakazakii*, were grown in TSB supplemented with EAA at concentrations of 0, 1, 5, and 10 mg/ml that were supplied to the liquid growth medium at the time of inoculation. Only the bacterial pathogens where EAA reduced the ATP content of the biofilm or the total biofilm biomass are shown in Fig. 13. EAA was able to reduce the energy content of the biofilm and biofilm biomass of some of the bacterial strains tested. Fig.13A shows the bioluminescence data for *E. coli* K-12, *P. aeruginosa, L. monocytogenes, C. sakazakii*, and *S. marcescens* from the ATP assay that was performed from the 24 h old biofilm. EAA began to reduce ATP content of the biofilms formed by all of the bacterial strains at a concentration of 1

mg/ml. We observed a 50 % reduction of ATP content in *E. coli* K-12 biofilm between 1 mg/ml and 5 mg/ml. The ATP content in *E. coli* K-12 biofilm measured at the concentrations of 1, 5, and 10 mg/ml of EAA were found to be statistically significantly different in comparison to the ATP content measured at 0 mg/ml of EAA with p-values of 0.04, 0.001, and 0.002 respectively. A 50 % reduction of ATP content of *P. aeruginosa* biofilm was observed at approximately 5 mg/ml. The ATP contents in *P. aeruginosa* biofilm measured at the concentrations of 5 and 10 mg/ml of EAA were found be statistically significantly different in comparison to the ATP content measured at 0 mg/ml of EAA with a p-values of 0.01. A 50 % reduction of ATP content of *L. monocytogenes* biofilm was seen at approximately 10 mg/ml. The ATP content in *L. monocytogenes* biofilm measured at the all of the concentrations of EAA were found not to be statistically significantly different from the ATP content measured at 0 mg/ml of EAA. *C. sakazakii* did not grow very much and thus had low ATP values even at 0 mg/ml of EAA. Complete abolishment of ATP was seen at approximately 1 mg/ml. The ATP content in *C. sakazakii* biofilm measured at the concentrations of 1, 5, and 10 mg/ml of EAA were found be statistically significantly different in comparison to the ATP content measured at 0 mg/ml of EAA with p-values of 0.0003, 0.0003, and 0.0002 respectively. The ATP content of *S. marcescens* biofilm was only slightly reduced at 10 mg/ml. The ATP contents in *S. marcescens* biofilm measured at the concentrations of 5 and 10 mg/ml of EAA were found be statistically significantly different in comparison to the ATP content measured at 0 mg/ml of EAA with a p-values of 0.02 and 0.0008 respectively. The ATP content of the biofilm formed by most of the strains was greatly reduced at 10 mg/ml.

Fig.13B shows the absorbance data from the CV assay that was performed on the biofilm. EAA began to reduce biofilm biomass of all of the bacterial strains, except *L. monocytogenes*

and *S. marcescens*, at a concentration of 1 mg/ml. We observed a 50 % reduction of *E. coli* K-12 biofilm biomass at approximately 5 mg/ml. The biofilm biomass of *E. coli* K-12 measured at the concentrations of 5 and 10 mg/ml of EAA were found be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA with p-values of 0.01 and 0.002 respectively. A 50 % reduction of biofilm biomass of *P. aeruginosa* was observed at approximately 5 mg/ml. The biofilm biomass of *P. aeruginosa* measured at the concentration of 10 mg/ml of EAA was found be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA with a p-value of 0.05. An increase in biofilm formation was observed for *L. monocytogenes* at 1 and 5 mg/ml of EAA, while the values at the 10 mg/ml EAA concentration was almost identical to the 0 mg/ml.

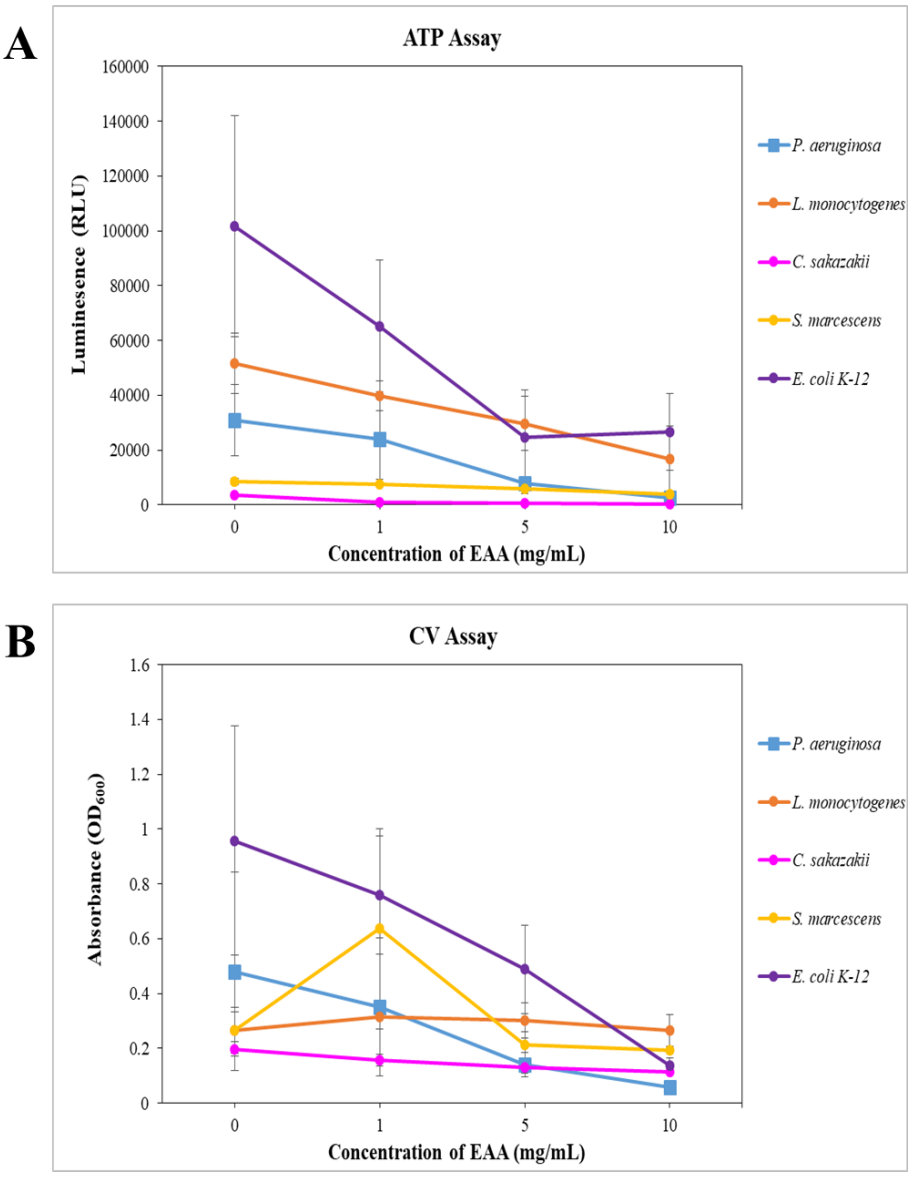

Figure 13. Effectiveness of EAA. Luminescence from the ATP assay that was performed on the biofilm (Panel A) and absorbance from the CV assay (Panel B) of *E. coli* K-12 (purple), *P. aeruginosa* (blue), *L. monocytogenes* (orange), *C. sakazakii* (pink), and *S. marcescens* (yellow) were determined after 24 h at 37°C in the presence of varying concentrations of EAA. Averages are calculated across 4 replicates.

The biofilm biomass of *L. monocytogenes* measured at all the concentrations of EAA were found not to be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA. We observed for *C. sakazakii* very low ATP content and biofilm mass, even at 0 mg/ml of EAA. The biofilm biomass of *C. sakazakii* measured at all the

concentrations of EAA were found not to be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA. Interestingly, a large increase in biofilm biomass was observed at 1 mg/ml of EAA for *S. marcescens*. Biomass decreased again at 5 mg/ml of EAA to values similar to those measured at 0 mg/ml of EAA. Biofilm biomass only decreased a little more when the concentration of EAA was increased to 10 mg/ml. The biofilm biomass of *S. marcescens* measured at all the concentrations of EAA were found not to be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA. Almost complete abolishment of biomass could be seen for most of the strains at 10 mg/ml.

DL-α-glycerol phosphate reduces energy content and biomass of Listeria monocytogenes

In a second experiment, 10 bacterial pathogens including *S. aureus*, *P. aeruginosa*, *E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enterocolitica*, *L. monocytogenes*, *S. marcescens*, and *C. sakazakii*, were grown in TSB supplemented with GP at concentrations of 0, 1, 5, and 10 mg/ml that were supplied to the liquid growth medium at the time of inoculation. Only the bacterial pathogens where GP reduced the ATP content of the biofilm or the total biofilm biomass are shown in Fig. 14. GP was able to reduce the energy content and biomass of only one of the pathogens tested, *L. monocytogenes*. Fig.14 shows the bioluminescence data of *L. monocytogenes* from the ATP assay and the absorbance data from the CV assay that was performed on the 24h old biofilm. GP started to reduce ATP (orange line) of *L. monocytogenes* at a concentration of 1 mg/ml and we observed a 50 % reduction of at approximately 10 mg/ml of GP. The ATP content in *L. monocytogenes* biofilm measured at the concentrations of 5 and 10 mg/ml of EAA were found to be statistically significantly different in comparison to the ATP content measured at 0 mg/ml of EAA. Interestingly, a small increase in

biomass was observed at 1 mg/ml of GP for *L. monocytogenes*. Then a decrease in biomass was observed at 5 mg/ml of EAA, but a slight increase could be seen at 10 mg/ml of EAA. The biofilm biomass of *L. monocytogenes* measured at all of the concentrations of EAA were found not to be statistically significantly different in comparison to the biofilm biomass measured at 0 mg/ml of EAA.

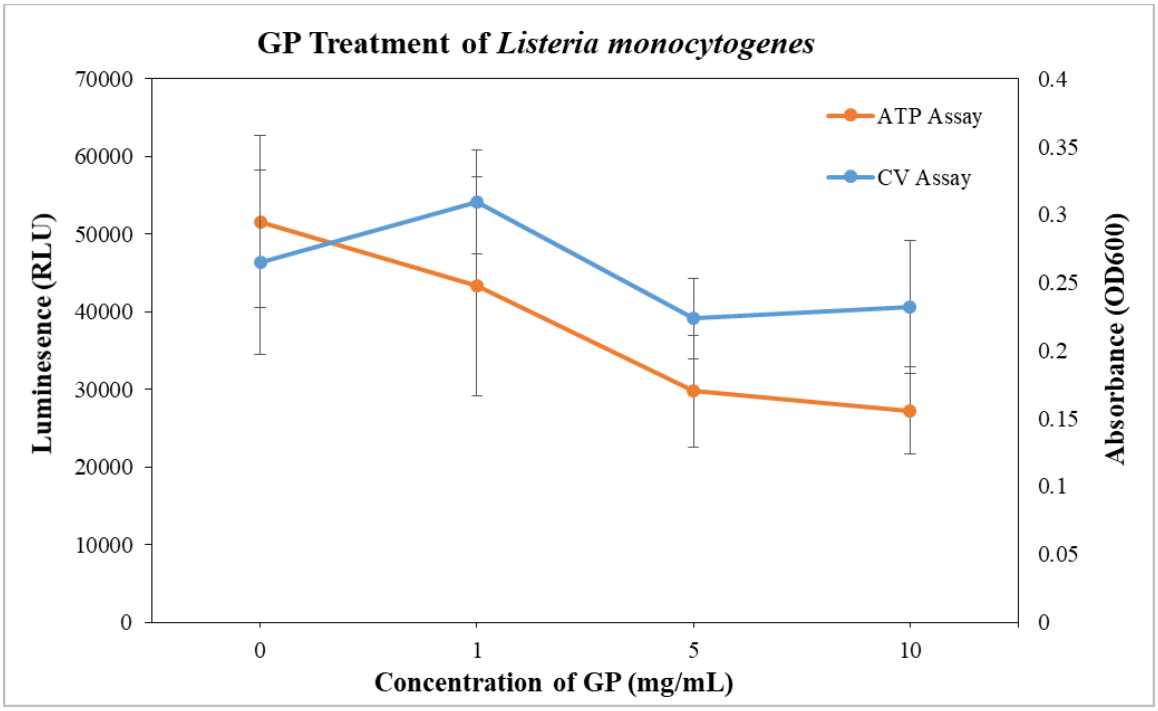

Figure 14. Effectiveness of GP on *L. monocytogenes* ATP content and biomass at 37°C for 24 h. Reduction of luminescence from the ATP assay and absorbance from the CV assay for *L. monocytogenes*, when GP was supplemented to TSB at inoculation. Luminescence from the ATP assay that was performed on the biofilm (orange line) and absorbance from the CV assay (blue line) *L. monocytogenes* were determined after 24 h at 37°C in the presence of varying concentrations of GP. Averages are calculated across 4 replicates.

The nutrient lyxose was also tested against all of the pathogens to see the effectiveness at reducing ATP content and biomass. However, no reduction was observed in ATP content or biomass for any of the pathogens tested and it actually increased the biofilm formation of many of the pathogens tested (data not shown).

Combinations of β-phenylethylamine and ethyl acetoacetate reduces energy content and biofilm biomass of P. aeruginosa and Y. enterocolitica

In another experiment, 10 bacterial pathogens including *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enterocolitica, L. monocytogenes, S. marcescens*, and *C. sakazakii*, were grown in TSB supplemented with equal amounts of PEA and EAA. Only the bacterial pathogens where combination treatments of PEA and EAA reduced the ATP content of the biofilm or the total biofilm biomass are shown in Fig. 15. Combination treatments of PEA and EAA were only able to reduce the energy content and biomass of some of the pathogens tested. Fig.15A shows the bioluminescence data of *E. coli* K-12, *P. aeruginosa, Y. enterocolitica*, and *L. monocytogenes* from the ATP assay after 24 h. For all the pathogens, except *Y. enterocolitica*, ATP content increased at 1 mg/ml of PEA and 1 mg/ml of EAA. ATP content then started to steadily decrease at 5 mg/ml of PEA and 5 mg/ml of EAA for *P. aeruginosa* and *L. monocytogenes*. However, we observed an increase in ATP content for *E. coli* K-12 at the concentration of 1 mg/ml of PEA and 1 mg/ml of EAA, as well as the concentration of 5 mg/ml of PEA and 5 mg/ml of EAA before. We only observed a decrease in ATP content for *E. coli* K-12 at the concentration of 10 mg/ml of PEA and 10 mg/ml of EAA. The ATP content of *Y. enterocolitica* steadily decreased as the concentrations of PEA and EAA were increased. None of the ATP values were found to be statistically significant for *E. coli* K-12 and *L. monocytogenes*. However, the ATP values at 5 mg/ml of PEA and 5 mg/ml of EAA, as well as the values at 10 mg/ml of PEA and 10 mg/ml of EAA were found to be statistically significant for *P. aeruginosa* and *Y. enterocolitica*.

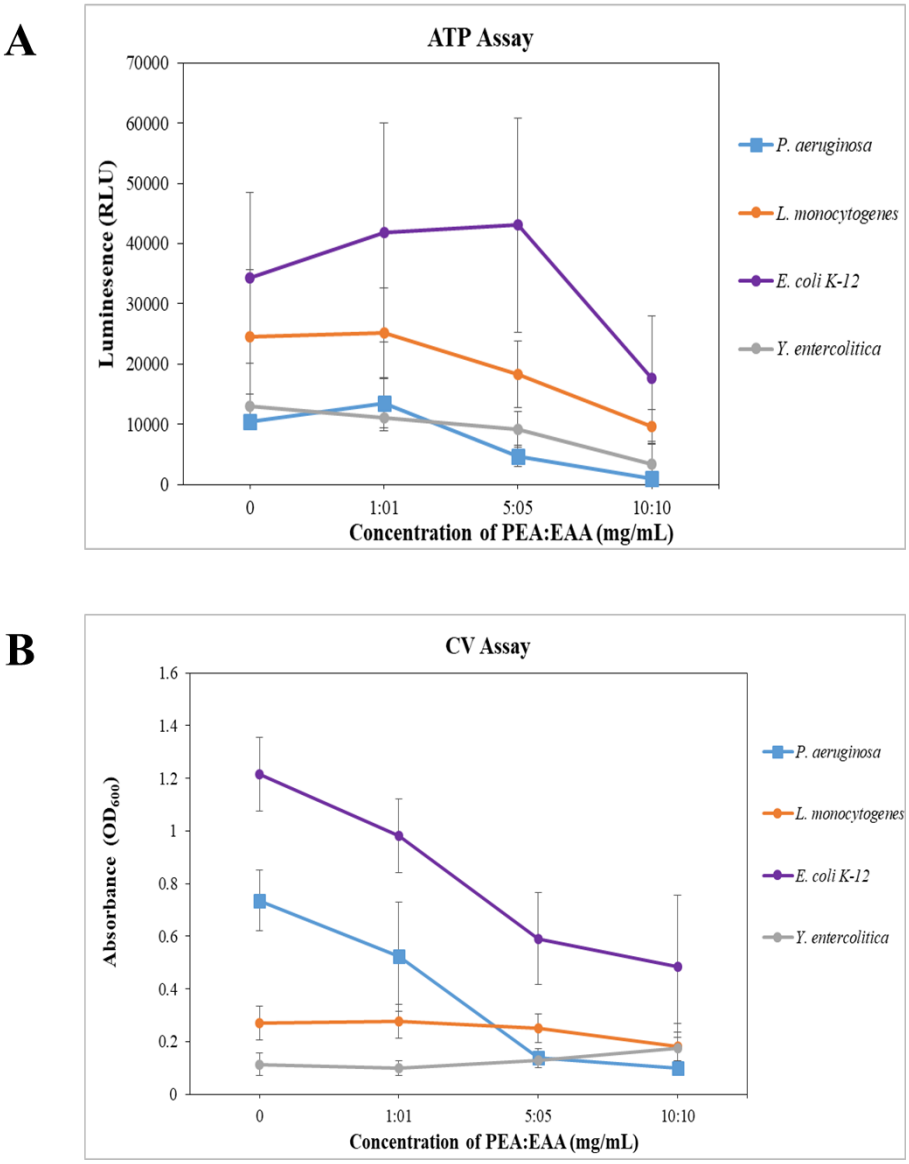

Figure 15. Effectiveness of combinations of PEA and EAA on pathogens ATP content and biomass at 37°C for 24 h. Reduction of luminescence from the ATP assay and absorbance from the CV assay for bacterial pathogens, when combinations of PEA and EAA were supplemented to TSB at inoculation. Luminescence from the ATP assay that was performed on the biofilm (Panel A) and absorbance from the CV assay (Panel B) of *E. coli* K-12 (purple), *P. aeruginosa* (blue), *L. monocytogenes* (orange), and *Y. enterocolitica* (grey) were determined after 24 h at 37°C. Averages are calculated across 4 replicates.

Fig.15B shows the absorbance data from the CV assay that was performed on the biofilm. Biomass started to decrease at 1 mg/ml of PEA and 1 mg/ml of EAA for *E. coli* K-12 and *P. aeruginosa*. We observed a 50 % reduction of *E. coli* K-12 and *P. aeruginosa* at approximately 5

mg/ml of PEA and 5 mg/ml of EAA. The absorbance values found at 5mg/ml and 10mg/ml of PEA/EAA were found to be statistically significant in comparison to the zero for *P. aeruginosa*, but not for *E. coli* K-12. No change in biomass amounts from the 0 to 10 mg/ml of PEA and 10 mg/ml of EAA were observed for *Y. enterocolitica* or *L. monocytogenes*.

Combinations of β-phenylethylamine and DL-α-glycerol-phosphate reduces energy content and biomass of some pathogens

In this experiment, 10 bacterial pathogens including *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enterocolitica, L. monocytogenes, S. marcescens,* and *C. sakazakii* were grown in TSB supplemented with equal amounts of PEA and GP and were supplied to the liquid growth medium at the beginning of biofilm development. Only the bacterial pathogens where combination treatments of PEA and GP reduced the ATP content of the biofilm or the total biofilm biomass are shown in Fig. 16. Combination treatments of PEA and GP were only able to reduce the energy content and biomass of some of the pathogens tested. Fig.16A shows the bioluminescence data of *P. aeruginosa* and *L. monocytogenes* from the ATP assay after 24 h of incubation at 37°C. At 1 mg/ml of PEA and 1 mg/ml of GP, we observed the ATP content of *L. monocytogenes* decrease. A 50 % reduction in ATP was seen between 1-5 mg/ml of PEA and GP. The ATP values observed at 5 and 10 mg/ml of PEA and GP were found to be statistically significant in comparison to the zero. For *P. aeruginosa* the ATP content increased at 1 mg/ml of PEA and 1 mg/ml of GP and then the ATP content stayed about the same at 5 mg/ml of PEA and 5 mg/ml of GP. There was a slight decrease in the ATP content at 10 mg/ml of PEA and 10 mg/ml of GP, however none of the values were found to be statistically significant in comparison to the zero.

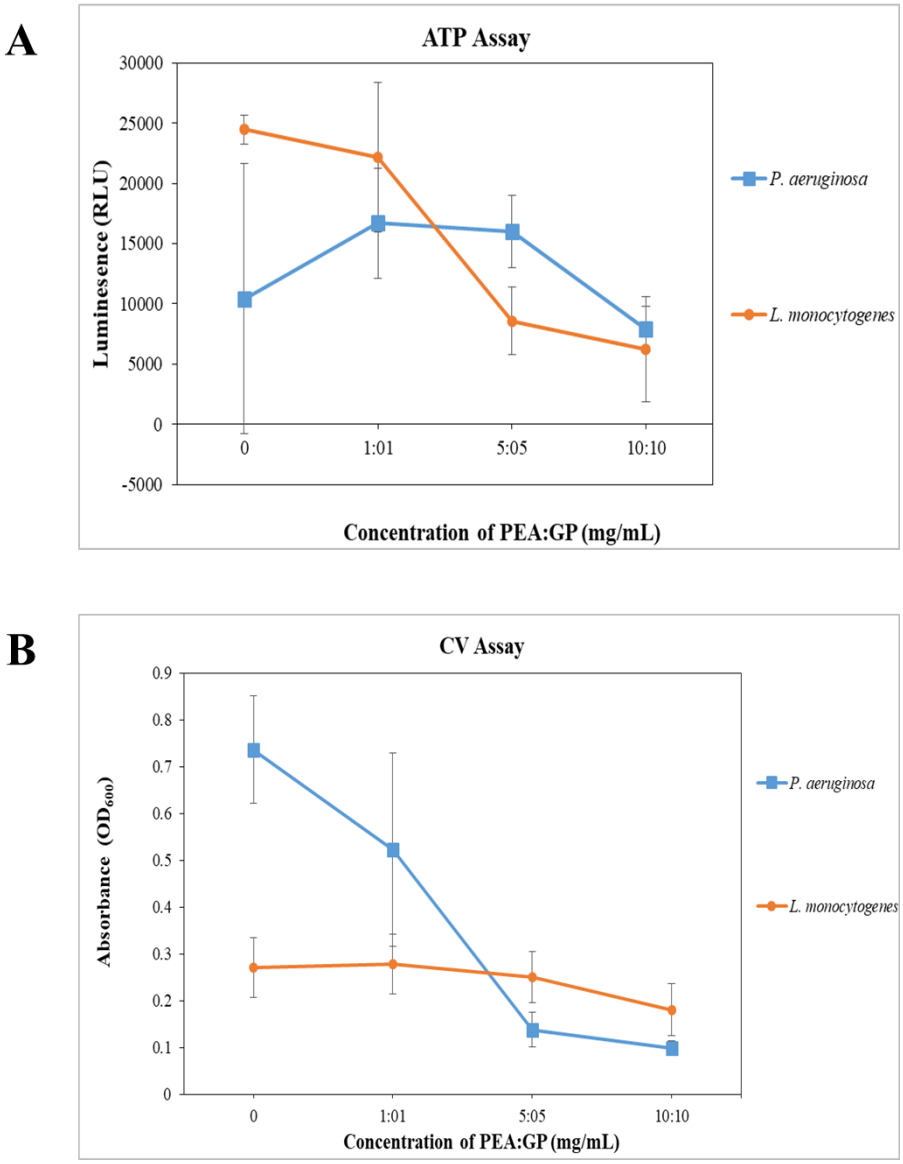

Figure 16. Effectiveness of combinations of PEA and GP on pathogens ATP content and biomass at 37°C for 24 h. Reduction of luminescence from the ATP assay and absorbance from the CV assay for bacterial pathogens, when combinations of PEA and GP were supplemented to TSB at inoculation. Luminescence from the ATP assay that was performed on the biofilm (Panel A) and absorbance from the CV assay (Panel B) of *P. aeruginosa* (blue) and *L. monocytogenes* (orange) were determined after 24 h at 37°C. Averages are calculated across 4 replicates.

Fig.16B shows the absorbance data from the CV assay that was performed on the biofilm. Interestingly, the decrease in biomass was observed for *P. aeruginosa*, while there was only a very small decrease in biomass for *L. monocytogenes.* Biomass started to decrease at 1 mg/ml of PEA and 1 mg/ml of GP for *P. aeruginosa* and a 50 % reduction could be seen between 1-5

mg/ml of PEA and GP. The absorbance values found at 5mg/ml and 10mg/ml of PEA/GP were found to be statistically significant in comparison to the zero for *P. aeruginosa*. No change in biomass amounts from the 0 to 10 mg/ml of PEA and 10 mg/ml of GP were observed for *L. monocytogenes*. No significant difference in the ATP content of *L. monocytogenes* biofilm were seen at all concentrations of PEA:GP in comparison to the ATP content of *L. monocytogenes* biofilm at 0 mg/ml of PEA and 0 mg/ml of GP.

Combinations of β-phenylethylamine and lyxose reduces energy content and biomass of some pathogens

Finally, 10 bacterial pathogens including *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enterocolitica, L. monocytogenes, S. marcescens,* and *C. sakazakii* were grown in TSB supplemented with equal amounts of PEA and lyxose for concentrations of 0 mg/ml of PEA and 0 mg/ml of lyxose, 1 mg/ml of PEA and 1 mg/ml of lyxose, 5 mg/ml of PEA and 5 mg/ml of lyxose, and 10 mg/ml of PEA and 10 mg/ml of lyxose that were supplied to the liquid growth medium at the beginning of biofilm development. Only the bacterial pathogens where combination treatments of PEA and lyxose reduced the ATP content of the biofilm or the total biofilm biomass are shown in Fig. 17. Combination treatments of PEA and lyxose were only able to reduce the energy content and biomass of some of the pathogens tested. Fig.17A shows the bioluminescence data of *P. aeruginosa, Y. enterocolitica*, and *L. monocytogenes* from the ATP assay after 24 h of incubation at 37°C. At 1 mg/ml of PEA and 1 mg/ml of lyxose, we observed the ATP content of *Y. enterocolitica* and *L. monocytogenes* start to decrease. A 50 % reduction in ATP was seen at 5 mg/ml of PEA and lyxose for *Y. enterocolitica*, however a 50 % reduction was not achieved for *L. monocytogenes*.

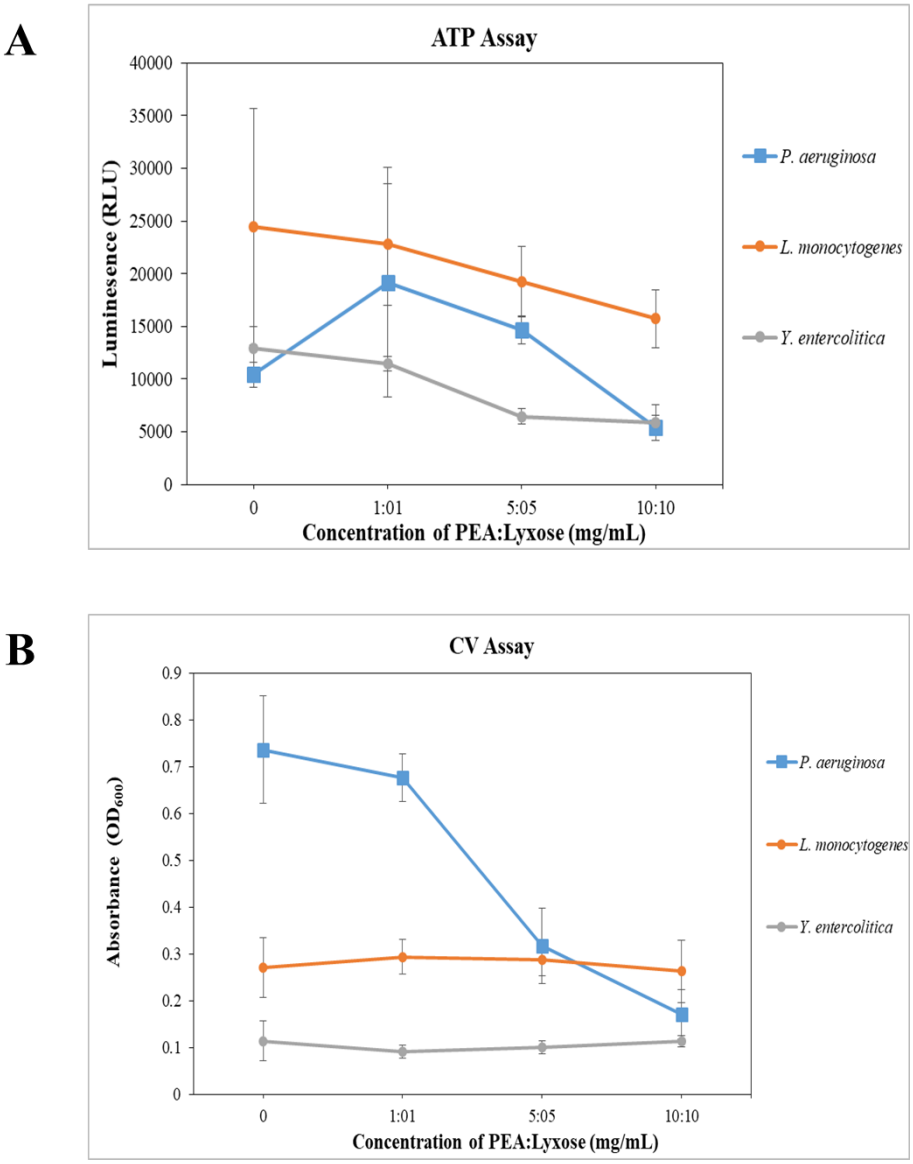

Figure 17. Effectiveness of combinations of PEA and lyxose on pathogens ATP content and biomass at 37°C for 24 h. Reduction of luminescence from the ATP assay and absorbance from the CV assay for bacterial pathogens, when combinations of PEA and lyxose were supplemented to TSB at inoculation. Luminescence from the ATP assay that was performed on the biofilm (Panel A) and absorbance from the CV assay (Panel B) of *E. coli* K-12 (purple), *P. aeruginosa* (blue), *L. monocytogenes* (orange), and *Y. enterocolitica* (grey) were determined after 24 h at 37°C. Averages are calculated across 4 replicates.

Thus, the ATP values observed at 5 and 10 mg/ml of PEA and GP were found to be statistically significant in comparison to the zero for *Y. enterocolitica*, but not for *L. monocytogenes*. For *P. aeruginosa* the ATP content increased at 1 mg/ml of PEA and 1 mg/ml of

lyxose but started to decrease at 5 mg/ml of PEA/lyxose. Overall, there was only a slight decrease in the ATP content at 10 mg/ml of PEA/lyxose in comparison to the zero, but the values observed at 10 mg/ml of PEA/lyxose were found to be significantly different in comparison to the zero.

Fig.17B shows the absorbance data from the CV assay that was performed on the biofilm. Interestingly, the decrease in biomass was observed for *P. aeruginosa,* while little to no change in biomass for *Y. enterocolitica* or *L. monocytogenes.* Biomass started to decrease at 1 mg/ml of PEA and 1 mg/ml of lyxose for *P. aeruginosa* and a 50 % reduction could be seen between 1-5 mg/ml of PEA/lyxose. The absorbance values found at 5mg/ml and 10mg/ml of PEA/GP were found to be statistically significant in comparison to the zero for *P. aeruginosa*. Little to no change in biomass amounts was observed at any of the combinations of PEA and lyxose in comparison to the zero for *Y. enterocolitica* and *L. monocytogenes*. None of the ATP values were found to be significant.

Discussion

EAA was found to be effective at reducing the ATP content and biofilm biomass of *P. aeruginosa* and *E. coli* K-12 AJW678 of the bacterial pathogens tested. Interestingly, EAA was found to inhibit biofilm formation of four gram-negative bacterial strains (*E. coli* K-12, *P. aeruginosa, C. sakazakii,* and *S. marcescens*) and one gram-positive bacterial strain (*L. monocytogenes*). This could be an indication that EAA has broad-spectrum inhibitory effects and could be used to treat both gram-positive and gram-negative infections. However, EAA did not have an inhibitory effect on all of the bacteria tested which included multiple gram positive (*S. aureus* and *L. monocytogenes*) and gram negative bacteria (*P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y, enterocolitica, S. marcescens* and *C.*

sakazakii), which may be an indication that EAA's antimicrobial effects are strain dependent. The similar chemical structure of acetic acid and EAA may be attributed to the antimicrobial effects seen on some pathogens. Acetic acid has been used as an antiseptic in medicine for over 6000 years and was found in a recent study to have bactericidal effects, particularly for *P. vulgaris*, *A. baumannii* or *P. aeruginosa*. However, like our study, it found that acetic acid was better in comparison to traditional antiseptics for some bacterial strains, but not for all [205]. Interestingly, it has also been shown that derivatives of 2-aryl EAAs have antifungal, antimalarial, and antibacterial effects [206-208]. Another interesting item to point out is unlike our studies with PEA we see a decrease in both ATP and biofilm biomass when our bacterial pathogens were treated with EAA.

In the second part of the study, we investigated other nutrients that had previously been identified to have antimicrobial effects and were cost effective for our material studies (Chapter 3) [204]. We observed that GP was only effective at reducing the ATP content and biofilm biomass of *L. monocytogenes*, while lyxose was found to have no inhibitory effect on the bacterial pathogens tested. We observed a decrease in ATP content, however there is very little reduction in biofilm biomass of *L. monocytogenes* as concentrations of GP are increased. This could be attributed to the two assays we used to assess biofilm, the CV and ATP assay. The CV assay stains everything that is negatively charged, while the ATP assay determines the metabolically active cells. This could mean that the number of energetically active cells are being reduce determined with the ATP assay, while the EPS remains and is being stained with the CV assay.

One explanation for why lyxose was unable to reduce ATP content and biofilm biomass is that not all bacterial strains can grow in the prescence of lyxose. For example, *E. coli* cannot

grow on L-lyxose, which supports the growth of other enteric bacteria, so we may not be able to determine an inhibitory effect due to little growth [209]. However, another reason could be that we are unable to dissolve enough lyxose in the media to achieve an inhibitory effect.

Increasing concentrations of PEA and EAA in combination were found to reduce ATP content and biofilm biomass of *P. aeruginosa* and *Y. enterocolitica* tested. However, we found that when we treated the biofilm only with PEA or EAA separately there was a greater inhibitory effect on ATP content and biofilm biomass in comparison to when biofilms were treated with combinations of PEA and EAA together. In fact, for *E. coli* K-12 we see an increase in ATP content between 1-5 mg/ml of PEA before we see a decrease at 10 mg/ml of EAA. One hypothesis for this phenomenon could be that PEA and EAA do not synergistically work to reduce biofilm formation. Thus, as one of the nutrients tries to inhibit growth, the bacteria utilize the other nutrient to grow and still form a biofilm. Overall, using both PEA and EAA in combination is not as effective of a treatment for bacterial biofilms in comparison to using PEA or EAA alone.

Increasing concentrations of PEA and GP in combination were found to reduce ATP content of *L. monocytogenes*, but almost no reduction in biofilm biomass was observed. This could be due to the fact that there was very little biofilm when no treatment was used, so we were unable to see an inhibitory effect. Interestingly, the inverse can be seen for *P. aeruginosa*. We observed that at increasing concentrations of PEA and GP in combination there was little reduction in ATP content of *P. aeruginosa*, but a reduction in biofilm biomass was observed. We actually see an increase in ATP content of *P. aeruginosa* from 1-5 mg/ml of PEA:GP and the decrease in ATP content at 10 mg/ml of PEA:GP was almost equal to the untreated biofilm. Again, one hypothesis for this phenomenon could be that PEA and GP do not synergistically

work to reduce biofilm formation. Thus, as one of the nutrients tries to inhibit growth, the bacteria utilize the other nutrient to grow and still form a biofilm. Overall, using both PEA and GP in combination is not as effective of a treatment for bacterial biofilms in comparison to using PEA or GP alone. However, it is worth noting that GP alone was only able to reduce ATP content and biofilm biomass *L. monocytogenes,* combinations of PEA and GP reduced ATP content and biofilm biomass of *L. monocytogenes* and *P. aeruginosa.* However, this increases the support for the hypothesis that PEA and GP are not working synergistically and that we see a reduction in *P. aeruginosa* because of the antimicrobial effect of PEA.

Increasing concentrations of PEA and lyxose in combination were found to reduce ATP content of *P. aeruginosa, L. monocytogenes,* and *Y. enterocolitica*. We observed no reduction in biofilm biomass of *L. monocytogenes,* and *Y. enterocolitica,* while a significant reduction in biofilm biomass was observed for *P. aeruginosa*. However, the reduction in ATP content at 10 mg/ml of PEA:lyxose was only slightly reduced in comparison to 0 mg/ml of PEA:lyxose. It was ineffective at reducing the biofilm amounts of *L. monocytogenes*, and *Y. enterocolitica*. Treatment with PEA by itself was observed to be more effective at reducing growth and biofilm amounts of these pathogens in comparison to treatment with PEA:lyxose. In addition, because we observed no reduction in growth and biofilm amounts with treatment of lyxose alone. This supports the hypothesis that PEA and lyxose do not work synergistically together.

Overall, of all the nutrients we tested in this chapter we found EAA to be the most effective at reducing growth and biofilm amounts of multiple pathogens. Treatments with EAA alone were found to be more effective then treatments of EAA in combination with PEA. Lyxose was the least effective at reducing the growth and biofilm amounts of pathogens. Combinations of EAA, GP, and lyxose with PEA were found to have antagonistic effects and could even

promote growth in some instances. However, all of the bacterial pathogens tested were found to be susceptible to treatments of PEA, EAA, GP, lyxose, or combinations of PEA with EAA, GP, or lyxose (Table 7). PEA and EAA would be the best options for treatments of bacterial biofilms, but further research needs to be done.

Table 7. Summary of nutrient treatments for bacterial biofilms. Treatments that reduced either biofilm biomass or ATP content of the biofilm of the bacterial pathogens are marked with an x.

Bacterial strians	PEA	EAA	GP	PEA+EAA	PEA+GP	PEA+lyxose
S. aureus	X					
P. aeruginosa	X	X		X	X	X
E. coli UMN026	X					
E. coli CFT073	X					
E. coli EDL932	X					
E. coli K-12	X	X				
Y. enterocolitica				X		X
L. monocytogenes			X		X	X

Conclusion

Foodborne outbreaks, particularly with *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella spp.* [202], are still a serious problem in the united states. This costs billions of dollars in health care cost, workers compensation, and lost product and kills millions of people annually. During food processing, bacteria can form biofilms on surfaces and spread to beef and poultry from the equipment, surfaces, and containers used in different stages of the process. A variety of methods and safety controls, such as mechanical cleaning, chemical cleaning, and developing anti-biofilm biomaterials. Unfortunately, these efforts have been ineffective and have caused an increase in antimicrobial resistant bacteria. We have identified the nutrients EAA and GP to have antimicrobial effects on growth and biofilm of a variety of clinically relevant bacterial pathogens. EAA was found to be the most effective of the nutrients tested at reducing growth and biofilm amounts of bacterial pathogens. However, when EAA was used in combination with PEA it was less effective at reducing growth and biofilm amounts of bacteria pathogens in

comparison to when EAA was used alone. The other nutrients GP and lyxose were determined to be almost completely ineffective at reducing growth and biofilm amounts of bacterial pathogens. When GP and lyxose were used in combination with PEA it shown to have antagonistic effects and PEA was determined to have actually reduced growth and biofilm amounts of a few pathogens. Further research with EAA needs to be done to investigate the efficacy of EAA on biofilm formation and to determine the mechanism of action. It is envisioned that EAA or other nutrients could be used in the future as an alternative to traditional antibiotics to prevent and treat bacterial biofilms. This could help reduce the emergence of antibiotic resistant bacteria and help prolong the existing antibiotics and antimicrobials for treatment of biofilms. Hopefully, this will lead to the use of EAA and other nutrients in a variety of applications including, coating of food processing equipment or medical devices, a spray to prevent or treat biofilm formation on food products or food processing equipment, or as a treatment flush to reduce catheter associated bacterial infections.

CHAPTER 4. APPLICATION EXPERIMENTS[9,10,11]

Development of a prototype, antimicrobial polyurethane integrated with β-phenylethylamine for use in food processing applications

Introduction

Livestock, such as cattle, poultry, pigs, and seafood make up large portion of the food we consume and are a huge part of the agriculture industry. Microorganisms including pathogens can be naturally found in the intestinal tract of livestock, such as *E. coli* O157:H7 which is considered part of the normal flora in the stomach of ruminants [56]. These pathogens are shed through the feces of the livestock, which can infect other livestock and could contaminate the soil and water in their environment. Infected animals that are not properly disinfected prior to slaughter can transmit bacterial pathogens to equipment, such as equipment, surfaces, and containers used in different stages of the process. [57]. This spread of bacteria to previously uninfected surfaces can be facilitated by the formation of biofilms, leading to outbreaks of infectious disease. Bacterial biofilms are a group of bacteria that can attach or adhere to a surface [210]. Many bacterial pathogens have been associated with foodborne illnesses, such as *Pseudomonas spp.*, *S. marcescens*, *E. coli spp.*, and *S. simulans*. These bacteria strains have been

[9] Hasan, R., **Schroeder, M.,** Nodland, J., Striker, R.,Vetter, A., Horne, S.M., Droel1, J., Brooks, B.D., Prüß, B.M., Ewert, D.L., and Brooks, A.E. *Development of a microfluidic system to deliver vancomycin to a mature S. aureus biofilm.* ISA. 2017. The material in this chapter was co-authored by Meredith Schroeder and Amanda E. Brooks. Meredith Schroeder had primary responsibility for designing and preforming the experiments, collecting data, and analyzing the data. Meredith Schroeder was instrumental in the development of the conclusions that are advanced here. Meredith Schroeder also drafted and revised all versions of this chapter. Amanda E. Brooks served as proofreader and checked the math in the statistical analysis conducted by Meredith Schroeder.

[10] **Schroeder, M.,** Horne, S.M., and Prüß, B.M. *Efficacy of β-phenylethylamine as a novel anti-microbial and application as a liquid catheter flush.* Journal of Medical Microbiology. 2018. 67(12):1778-1788. The material in this chapter was co-authored by Meredith Schroeder and Birgit M. Prüß. Meredith Schroeder had primary responsibility for preforming the experiments, collecting data, and analyzing the data. Meredith Schroeder was instrumental in the development of the conclusions that are advanced here. Meredith Schroeder also drafted and revised all versions of this chapter. Birgit M. Prüß served as proofreader and checked the math in the statistical analysis conducted by Meredith Schroeder..

[11] RFT-559. **Schroeder, M.,** S.M. Horne, S.J. Stafslien, and B.M. Prüβ. Biofilm inhibitor and method for inhibiting biofilm. Non-provisional patent application under 37 C.F.R. 1.53 (b).

identified as bacteria that can attach to the polyurethane and polyvinylchloride surfaces that line the conveyor belts used during different stages in food processing [58].

The CDC estimates that 48 million Americans annually ingest bacteria contaminated food that results in illness and costs $77.7 billion in health care costs, workers compensation, and loss of product. This also results in 300,000 hospitalizations and up to 5,000 deaths per year due to foodborne illness [59]. This could be attributed to the increased consumption of livestock and seafood over the years, which has greatly increased the likelihood for consumers to ingest food contaminated with bacterial pathogens. In addition, the demand for these foods, globalization, advancements in technology, and a competitive market have immensely changed the way we produce and process food [60]. The problem of foodborne outbreaks is compounded by the increasing emergence of antibiotic resistant bacteria due to the overuse of antibiotics, which can be added to the animal feed and have even been used in fisheries for prevention and treatment of diseases [61, 62]. More importantly sub therapeutic doses of antibiotics have been shown to enhance growth of the animals, making it an attractive option for farmers [63]. Use of sub therapeutic doses of antibiotics can reduce the normal flora of the animal, which usually outcompete more pathogenic microorganisms.

One solution to combat foodborne illness has been to develop materials embedded with silver nano-particles, which have known antimicrobial effects. However, the materials have very high production costs, limited lifetimes, and the development of silver resistant bacteria is an eminent threat [76, 211]. Another group of scientists have developed a rechargeable antimicrobial material integrated with halamines, which has been shown to be effective against *L. monocytogenes* and *E. coli* O157:H7, which could provide continuous sanitation of the surface.

Other strategies have focused on new treatment options for biofilms, like combinations of antibiotics with antimicrobial peptides and SHS [212-214].

Our research is focused on the development of a material that is able to prevent the formation of biofilms by the integration of small molecules with antimicrobial properties that will not rely on antibiotics. In high concentrations these nutrients exhibit antimicrobial effects by inhibiting planktonic growth and biofilm. Previous research in our lab screened 95 carbon and 95 nitrogen sources and identified antimicrobial properties of PEA, a nitrogen source found in chocolate, weight loss products, and mood elevators [204]. PEA was found to have the greatest inhibitory effects on growth, planktonic bacterial counts, and biofilm amounts of *E. coli* O157:H7. This effect of PEA was also seen when pieces of beef were treated with PEA prior to inoculation with *E. coli* O157:H7 [153].

The research presented in this chapter builds upon our previous research by investigating the effect of PEA physically integrated with polyurethane. This study had two major objectives: 1) to develop a polyurethane integrated with PEA and 2) examine the effect of our polyurethane-PEA (PU-PEA) material as an anti-biofilm biomaterial on a non-pathogenic *E. coli.* We used heat melt extrusion to melt down food grade polyurethane and physically integrated a PEA powder, which was then pressed into sheets. Our material was able to achieve at least a 25 % reduction in biofilm amounts of all of the bacterial strains tested. We observed a 50 to 80 % reduction in bacterial biofilm for *E. coli* K-12 and *S. aureus* and a 20 to 30 % reduction for *E. coli* O157:H7 and *P. aeruginosa*.

Materials and methods

Bacterial strains

Bacterial strains used in this study are summarized in Table 3 and include one non-pathogenic *E. coli* K-12 strain and five bacterial pathogens. The *E. coli* K-12 strain used was AJW678, which was previously described as a good former of biofilm [170]. Pathogenic bacterial strains include *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 25923), EHEC EDL932 [172], and two UPECs, UMN026 [173] and CFT073 [174]. Bacterial strains were maintained as freezer stocks at -80ºC in 8 % DMSO. Prior to each experiment, the *E. coli* strains were plated onto LB (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) agar plates. *P. aeruginosa* and *S. aureus* were plated onto TSA (15 g/l tryptone, 5 g/l soytone, 5 g/l NaCl, 15 g/l agar) plates. Plates were incubated over night at 37°C.

Biofilm experiments with PEA hot-melt blended into polyurethane

The production of the PEA-polyurethane (PEA-PU) blends was done by melting, blending and extrusion on a twin-screw extruder with gravimetric and volumetric feeders (Thermo-Haake Polylab OS) (Fig. 18).

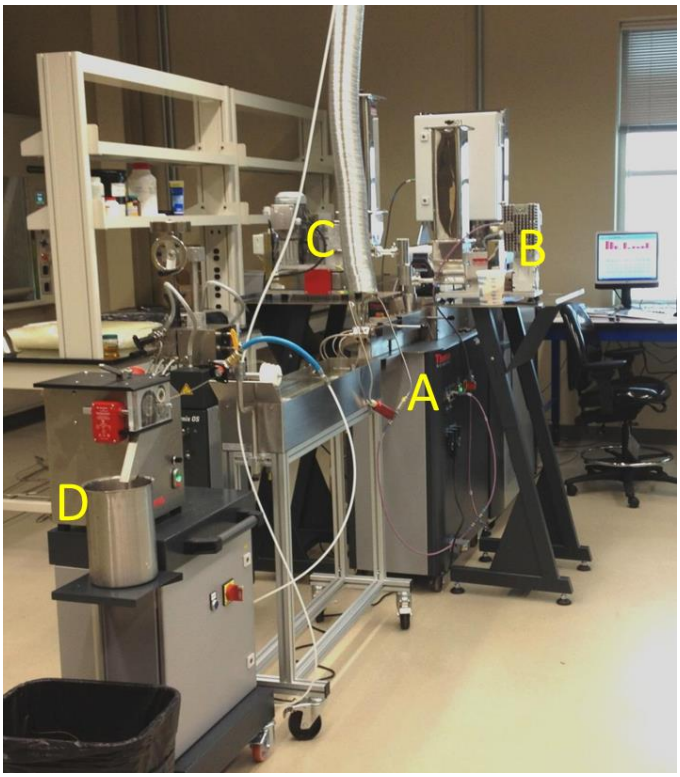

Figure 18. The production of our PEA-PU material. The melting, blending and extrusion of the PEA-PU was performed on a twin-screw extruder (A) with polymer bead (B) and powder feeders (C). Samples of the molten extrudate where collected in aluminum pans (D).

Prior to feeding the polyurethane beads (Zythane 7085A^{TM}), the twin screw extruder (A) was heated to 215°C. The polyurethane beads were then fed into the extruder using a volumetric polymer bead feeder (B) at a rate of 20 g/min for 20 min in order to purge the system. The PEA powder was co-fed into the extruder with the polyurethane beads at a rate of 0.95, 0.46 and 0.31 g/min using a gravimetric powder feeder (C) to generate a final PEA concentration of 10.0, 5.0 and 2.5 wt. %, respectively. Each PEA feed rate was held constant for 20 min after which time the torque required to drive the twin screw extruder dropped off due to the lower melting point of the PEA-PU blends. At this time, the temperature of the twin screw extruder was lowered to 195°C to minimize any thermal degradation that may have occurred to the PEA. This condition was held constant for 20 min until a steady state condition was achieved. Samples of the molten extrudate (~20g each) where then collected in aluminum pans at 1 min intervals and allowed to

cool to room temperature (D). Once cooled, the PEA-PU blends where then hot pressed at 75°C to a thickness of 1.5 mm and then allowed to cool to room temperature before removal from the press plates. The flat sheets of PEA-PU blends were then die punched into 15 mm discs and adhered to the bottom of 24-well plates for biofilm growth studies as described previously [215].

Samples (15 mm discs) of pure polyurethane and the 2.5, 5 and 10 % PEA-PU blends were subjected to bright-field microscopy. Samples were adhered to glass microscope slides using a double-sided adhesive tape. The glass slide-mounted samples were then placed onto the stage of an inverted bright-field microscope (VistaVision) outfitted with a charge-coupled device camera to capture images at 250 x total magnification to qualitatively examine the distribution of PEA in the polyurethane bulk material. Plates containing the inserts of our PEA-PU material used for testing were provided by Shane Stafslien and were produced in Research I at NDSU.

To test the PEA-PU blends with bacteria, liquid overnight cultures (10 ml) in TSB were pelleted by centrifuging at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of PBS, centrifugation and resuspension was repeated two more times. Cultures were adjusted with PBS until a specific OD_{600} was attained that depended on the strain; *E. coli* was diluted to an OD_{600} of 0.5 ± 0.05, *P. aeruginosa* was diluted to an OD_{600} of 0.4 ± 0.05, and *S. aureus* was diluted to an OD_{600} of 0.6 ± 0.05. *E. coli* and *P. aeruginosa* strains were further diluted 10-fold into TSB. *S. aureus* was diluted 10-fold into biofilm growth media (BGM; 10.22 g/l Na_2HPO_4, 3.81 g/l KH_2PO_4, 1.01 g/l KCl, 0.793 g/l $(NH_4)_2SO_4$, 0.06 g/l $MgSO_4$, 0.001 g/l thiamine, 0.0005 g/l biotin, and 0.5 g/l dextrose). One milliliter of these final inocula was pipetted into individual wells of two 24-well polystyrene plates; one plate contained discs of pure polyurethane, while an analogous plate consisted of 5 % PEA-PU. For the initial experiments examining the effect of PEA concentration on biofilm growth of AJW678, additional plates of 2.5 and 10 % PEA-PU

were prepared. The plates were then incubated at 37°C for 24 h for all the strains, except *S. aureus* which was incubated for 72 h. *S. aureus* was incubated for longer because it was grown in minimal media. Replicates varied across the experiments.

To characterize the biofilm at the end of the incubation period, two analyses were used; the CV assay that stains live and dead biomass, as well as exopolysaccharide and the ATP assay that measures the energy content of live bacteria [186, 187] Prior to the biofilm assays, we rinsed each well three times with 1.0 ml. The plates were then inverted, tapped against an absorbent pad, and allowed to dry at ambient laboratory conditions for at least 1 h. For the CV assay, 1.0 ml (200 µl) of 0.1 % CV in ddH_2O was added to each well of the 24-well (96-well) plates and incubated at room temperature for 15 min, then rinsed three times with 1.0 ml of ddH_2O. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for at least 1 h. The CV was extracted by adding 500 µl of 20 % acetone 80 % ethanol to each well, followed by 15 min of incubation. 0.15 ml of each extract was then transferred into one well of a 96 well polystyrene plate and the OD_{600} measurements were determined with the Synergy H1 Hybrid Reader.

For the ATP assay, the BacTiter Glo™ kit (Promega, Madison, WI) was used. Reagents were prepared per manufacturer instructions. To each well, we added identical amounts of PBS and BacTiter-Glo reagent. For 24 well plates, this was 250 µl. We covered the plates with tinfoil and thoroughly mixed solutions by placing the plate on a rotating shaker for 5 min. After this incubation, we transferred 150 µl from each well to an opaque white 96-well plate and read luminescence using the Synergy H1 Hybrid Reader.

The data obtained from the CV and ATP assays to characterize the biofilm on the pure polyurethane and PEA containing blends were analyzed differently dependent on the experiment.

For the experiment with the *E. coli* K-12 strain, absolute values are presented. For the experiment with the bacterial pathogens, a reduction between the 0 % PEA-PU material and 5% PEA-PU material was calculated for both assays and expressed as a ratio reduction. Absorbance reduction (CV assay) by the PEA-PU blends were determined as follows for each bacterial strain: % reduction = $(1-(OD_{600}X\%/\ OD_{600}0\%))$, where $OD_{600}X\%$ is the absorbance value of the wells of the PEA-PU blend (with PEA at a concentration of X%) and OD_{600} 0% the absorbance value for the wells of the pure polyurethane plates. Luminescence reduction (ATP assay) was calculated accordingly, replacing OD_{600} values by luminescence values. Averages and standard deviations of the ratio reductions were determined over up to 12 replicates (four replicates per plate, three plates). Student's *t*-test were performed to determine the statistical significance of the difference between the ratio reduction means of the PEA-PU material means when compared to the mean of the ratio reduction of the pure polyurethane material. *p*-values below 0.05 were considered to be statistically significant.

The viability of biofilm bound bacteria in the absence and presence of PEA was compared by plating serial dilutions of bacteria recovered from biofilms grown on 5 % PEA-PU and plain polyurethane. After the 24 h incubation period, each of the wells of the 24-well was rinsed three times with 1 ml of PBS. Bacteria were recovered with nylon flocked swabs (Copan Diagnostics Inc., Murrieta, CA), which were immediately transferred into a 15 ml test tube containing either 10 ml of PBS. The tubes with the flocked swab were vortexed vigorously for 1 min to thoroughly mix the recovered cells into solution. Serial dilutions were plated in three replicates onto LB agar plates, colony forming units (CFU) were reported as average and standard deviations across those three replicates. Student's *t*-test was run to determine the

statistical significance of the difference between the values obtained from the plain polyurethane and the PEA-PU plates.

To determine whether the antibacterial effect of PEA in the 5 % PEA-PU was due to leaching of PEA from the material, we used an indirect approach where medium was pre-incubated on pure polyurethane or 5 % PEA-PU and then used as the growth medium for bacterial biofilm formation. Specifically, 1 ml of TSB was pipetted into half of the wells of two 24-well polystyrene plates; one plate contained discs of pure polyurethane, while an analogous plate contained discs of 5 % PEA-PU and the plates were incubated on the shaker at 37°C. After 24 h, 190 µl were removed from each well and transferred to a 96 well plate. Liquid overnight cultures (10 ml) in TSB of AJW678 were prepared as described above, adjusted to an OD_{600} of 1.0 ± 0.05. 10 µl of this inoculum was then added to each 190 µl of pre-incubated growth medium on the 96-well plate. For negative controls, one row of the 96-well plate was inoculated with 200 µl of TSB. The 96-well plate was then incubated for 24 h at 37°C. Following the incubation period, CV and ATP assays were performed on the biofilms as described above. Averages and standard deviations were determined across six replicates for the CV assay and three replicates for the ATP assay. Student's *t*-test was run to determine the statistical significance of the difference between the values obtained from the plain polyurethane and the PEA-PU plates.

Results

Polyurethane integrated with PEA reduced planktonic growth and ATP content in biofilms

Using the extruder setup shown in Fig.18, PEA was physically blended into food grade polyurethane at a concentration ranging from 0-10 percent by weight. Fig.19 displays representative bright field microscopy images of the PEA-PU surfaces where large macroscale

aggregates/agglomerations of PEA crystals can clearly be distinguished within the bulk polyurethane matrix for the 10 % material as areas of refracted light. Aggregation of PEA can also be observed for the 5 % material but are qualitatively smaller in size and more homogenously dispersed throughout the bulk matrix.

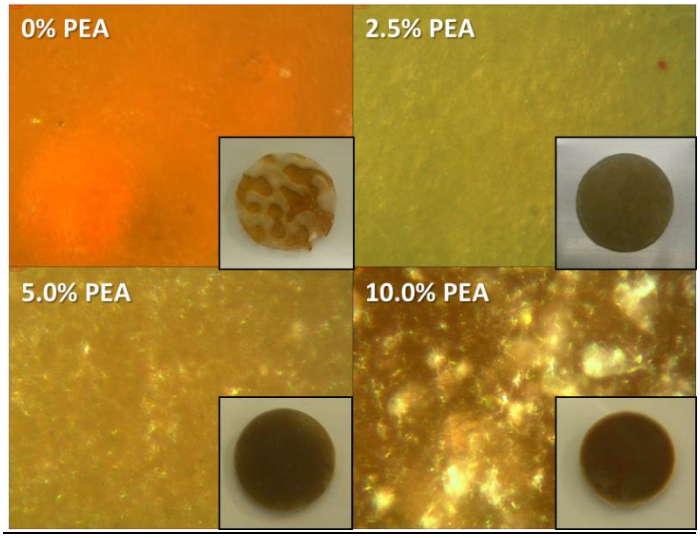

Figure 19. Bright-field microscopy imaging of PEA-PU blends. Images were taken with an inverted bright-field microscope of pure polyurethane and the 2.5, 5 and 10 wt. % PEA-PU blends at 250 x magnification. The inserts show the 15 mm discs that were cut out of the materials and adhered to the bottoms of 24 well plates for characterization of bacterial biofilm growth.

To test the effectiveness of the new material, AJW678 was cultured on the surface of PEA-PU samples adhered in 24-well plates, where each well contained a 15 mm disc of either pure polyurethane or PEA-PU at a range of concentrations (Fig. 20). The addition of PEA to the polyurethane caused a reduction of absorbance from the CV assay of approximately 40 % for the concentration of 2.5 %, 5.0 %, and 10 % PEA-PU (Fig. 20A). The ATP content of viable cells in the biofilms, however, was reduced at both the 5 % (83 % reduction) and 10 % (>90 % reduction) PEA concentrations, while 2.5 % PEA-PU resulted in only a 35 % reduction (Fig. 20B). Since i) 5 % PEA caused reductions in both biofilm biomass and ATP content for AJW678 and ii) PEA formed large, macroscale aggregates in the polyurethane bulk matrix at a

concentration of 10% (Fig. 19), we selected 5 % PEA-PU as our prototype material for further experimentation.

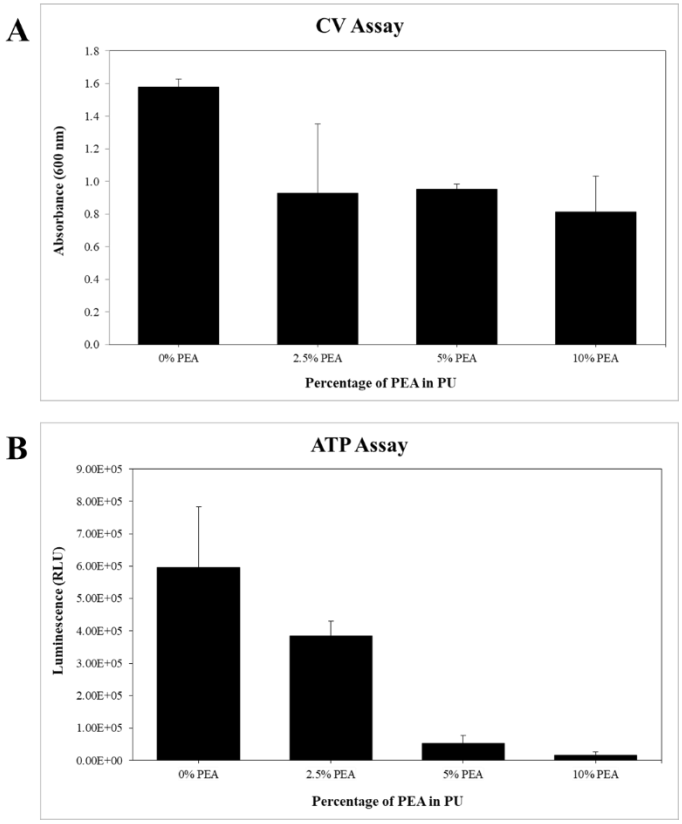

Figure 20. Reduction of absorbance from the CV assay and luminescence from the ATP assay when PEA was physically blended into polyurethane at 0, 2.5, 5.0 and 10.0 wt. percent. Panel A shows absorbance data from the CV assay that was done on biofilm formed by AJW678. Panel B shows the luminescence data from the ATP assay for the same strain. Averages and standard deviations were calculated across 9 replicates. Error bars are 2 s.

To explain the differences of the PEA effect on biofilm biomass (live plus dead bacteria, plus exopolysaccharide) and on ATP content of live bacteria that was seen in Fig. 20, bacteria were recovered from biofilm that was grown on 0 % PEA-PU and 5 % PEA-PU and the cell count for viable bacteria was compared (Fig. 21). CFUs that are indicative of viable bacteria were reduced by 5 % PEA to approximately 50 %. The cell counts from our 5 % PEA-PU material were found to be statistically significantly different than the cells counts from the 0% PEA-PU material with a p-value of 0.005. It seems like the biofilm that forms on PEA-PU does

indeed contain a smaller number of viable bacteria than the biofilm that forms on pure polyurethane. Interestingly, there was only 50 % reduction in viable cells, while there was closer to an 83 % reduction in the ATP content of the biofilm (Fig. 20).

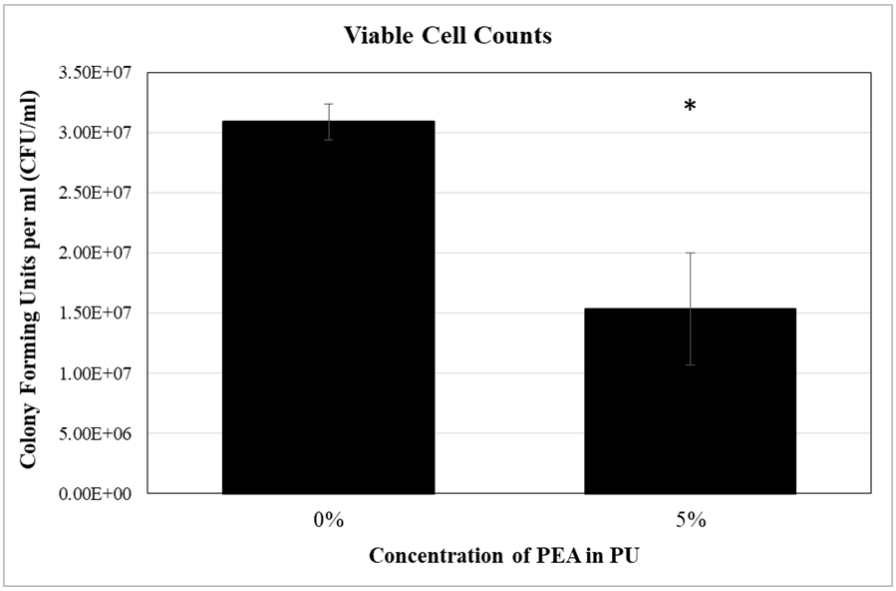

Figure 21. Reduction of viable cell counts by 5 % PEA, physically blended into polyurethane. CFUs were obtained from serial dilutions, with a lower limit of detection of 999 CFU/ml. Averages and standard deviations were calculated across 9 replicates.

To determine whether the reduction of biofilm biomass, ATP content, and viable bacteria on the PEA-PU material relative to pure polyurethane was due to PEA that had leached from the material, an indirect approach was used where TSB was pre-incubated on the 0 % and 5 % PEA-PU material, removed, and inoculated with AJW678 (Fig. 22). Planktonic growth (not shown), biofilm biomass as estimated by the CV assay (Fig. 22B), and ATP content (Fig. 22A) showed no difference between the two materials tested. Statistical analysis with the Student's *t*-test and an alpha set at 0.05 yielded *p*-values above 0.05 for all comparisons, confirming the lack of a significant difference between the two materials. These results indicate that any PEA that may have potentially leached out from the PEA-PU materials during the biofilm growth experiments

conducted in 24-well plates accumulated below the concentration threshold that was needed to be effective for AJW678.

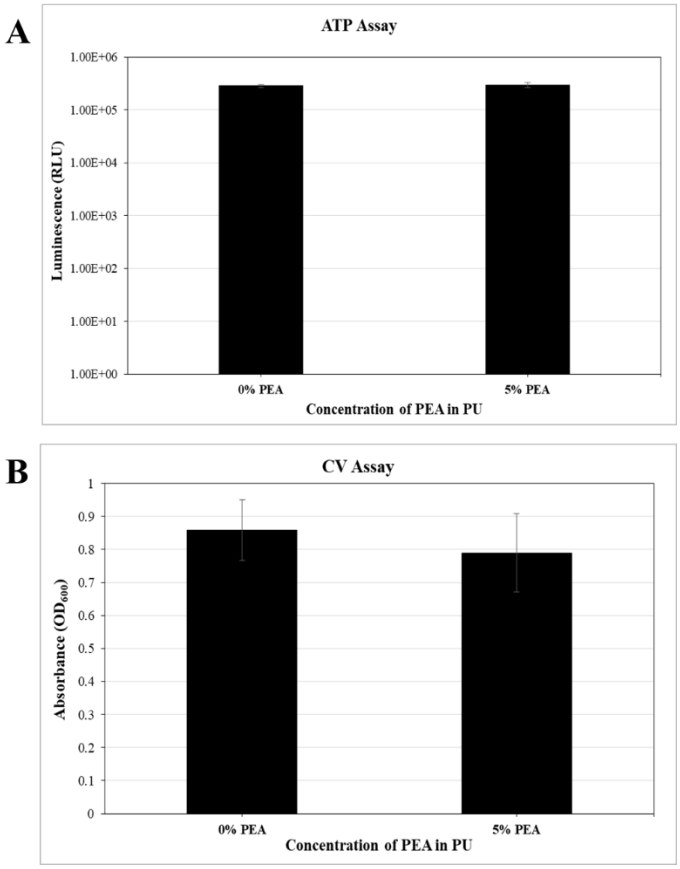

Figure 22. Reduction of absorbance from the CV assay and log reduction of luminescence from the ATP assay of AJW678 incubated with leachate from the 0 and 5 % PEA-PU. Panel A shows luminescence data from the ATP assay that was done on biofilm formed by AJW678. Panel B shows the absorbance data from the CV assay for the same strain. Averages and standard deviations were calculated across 6 replicates.

In another experiment, 5 % PEA-PU was tested for its effectiveness against six bacterial strains. AJW678 and five bacterial pathogens from Table 3 were cultured in 24-well polystyrene plates which contained 15 mm discs of pure polyurethane or 5 % PEA-PU. Fig. 23A shows the absorbance data from the CV assay (relative ratio of absorbance on 5 % PEA divided by absorbance on 0 % PEA) that was carried out on the biofilms.

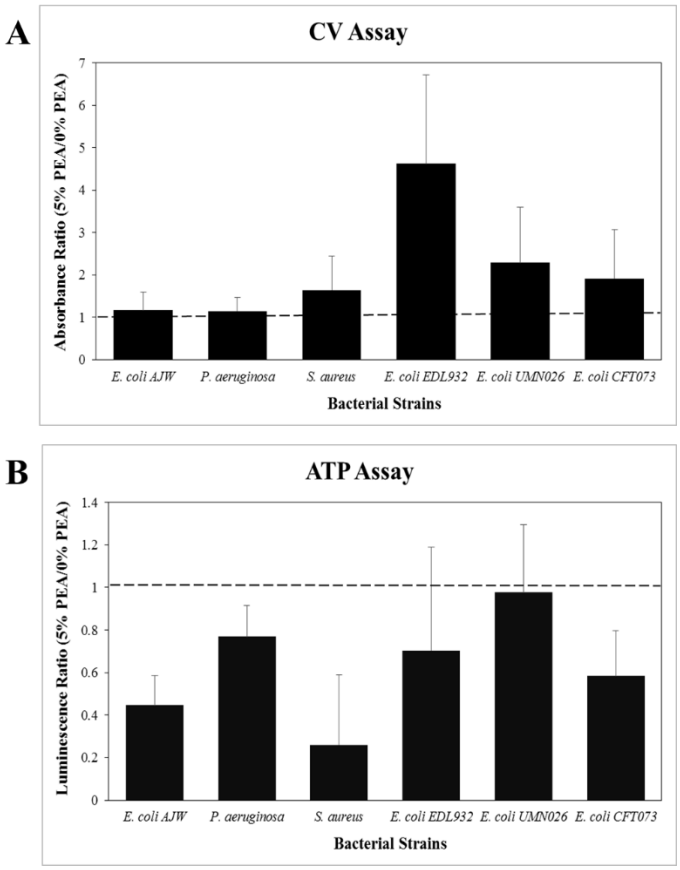

Figure 23. Reduction of absorbance from the CV assay and luminescence from the ATP assay when PEA was physically blended into polyurethane at 5 %. Panel A shows absorbance data from the CV assay that was done on biofilm formed by six bacterial strains. Absorbance ratios were calculated as absorbance on 5 % PEA divided by absorbance on 0 % PEA. Panel B shows the luminescence data from the ATP assay for the same strains. Luminescence ratios were calculated as luminescence on 5 % PEA divided by luminescence on 0 % PEA. Averages and standard deviations were calculated across 12 replicates.

The 5 % PEA in the polyurethane was unable to reduce the biofilm biomass for any of the tested strains when compared to the pure polyurethane control. In fact, *E. coli* EDL932 produced four times higher OD_{600} from this assay. Fig. 23B shows the bioluminescence data from the ATP assay that was done on the biofilms. PEA-PU exhibited a decreased ATP content in the biofilms of five of the bacterial strains tested, relative to pure polyurethane. There was at least a 20 % reduction in ATP content on PEA-PU for *P. aeruginosa*, *E. coli* EDL932, and *E. coli* CFT073. Reductions in ATP content of the biofilms for AJW678 and *S. aureus* were 60 %

and 80 %, respectively. Altogether, PEA hot-melted into polyurethane at a concentration of 5 % reduced the ATP content in biofilms between 20 and 83 % for five of the six bacterial strains tested but was ineffective at mitigating biofilm biomass as quantified with the CV assay.

Discussion

One of the most important findings of this study was the fact that we were able to physically blend PEA into polyurethane using a hot-melt extrusion process while retaining some of the antimicrobial effectiveness of the PEA. As we increased the concentration of PEA in the polyurethane material we observed a substantial decrease in ATP content of AJW678. However, for the CV assay we observed a 40 % reduction in total biomass on our 2.5 %, 5 %, and 10 % PEA-PU materials. We also observed large macroscale aggregation of the PEA within the polyurethane (Fig. 19) at this highest concentration and therefore decided to perform subsequent experiments at 5 % PEA only, as the PEA was more homogenously distributed through the bulk polyurethane and yet retained a comparable antimicrobial effectiveness (*i.e.*, >90 % reduction in ATP content and 10 % reduction in biofilm biomass). In addition, this is important to note because the increasing aggregation creates pockets in the polyurethane material. This impacts the structural integrity of the material and limits us to how much PEA can physically be incorporated into the material.

One explanation for the discrepancies between the ATP content and the total biofilm biomass could be due to the differences in the assays that were used to characterize the biofilms on the surface of PEA-PU materials. The CV colorimetric assay is one of the most widely used techniques to quantify total biofilm biomass because it stains all negatively charged molecules, including both live bacteria, dead bacteria, and the EPS matrix [192, 216]. One disadvantage of using the CV assay to assess biofilm is that it doesn't distinguish between live and dead bacteria

within the biofilm. We hypothesized that the reason we fail to see an increased reduction in the total biomass is because the PEA is killing the bacterial cells within the biofilm, but the EPS is still left behind.

In order to distinguish between live and dead bacteria in biofilms, we performed a live/dead assay on the biofilms using a green fluorescence dye, Syto 9, which stains live and dead bacteria. A red fluorescent die, propidium iodide, which only penetrates damaged membranes of dead bacteria was used to stain dead cells to allow for calculation of the total live cells within the biofilm [18]. However, the PEA-PU materials were shown to possess an inherent, high-intensity fluorescent signal which interfered with our ability to adequately distinguish between live and dead cells residing on the PEA-PU material surfaces using this technique (data not shown). As a result, we decided to use viable cell counts to determine the number of live and energetically active cells. We observed that PEA reduced ATP content and the number of viable AJW678 bacteria when hot-melted into polyurethane at a concentration of 5 % about half a log. Initially our hypothesis was that the bacterial cells are being killed and the EPS remains, which would explain the discrepancies in the CV and ATP assay. However, we saw about a 50 % reduction in cell counts on our 5 % PEA-PU material in comparison to our 0 % PEA-PU material, which is similar to the 50 % reduction in total biofilm biomass seen on the 5 % PEA-PU material. This maybe an indication that the bacteria are just metabolically dormant.

Antimicrobial materials, such as coatings or additives to polymer material can be differentiated into leaching and non-leaching antimicrobial materials. This is an important distinction for applications in the medical or food processing industry [217]. Materials that leach antimicrobials have been shown to increase the likelihood for antibiotic resistance due to repeated exposure to sub-inhibitory concentrations of antibiotics from the leach material [218, 219].

However, we observed that PEA was not being leached out from the material and the reduction in ATP content and biofilm biomass occurs when the bacteria come in contact with the surface of our material.

For the five pathogenic strains, 5 % PEA-PU reduced ATP content in the biofilms when compared to the pure polyurethane (Fig. 23B). The largest ATP reduction was seen for by *E. coli* AJW678 at about 83 %, followed by *S. aureus* at about 75 %, then *E. coli* CFT073 at around 40 %, *E. coli* EDL932 at about 30 %, *P. aeruginosa* at a little over 20 % and *E. coli* UMN026 at less than 5 %. It is important to note here that the antimicrobial activity of the 5 % PEA-PU material was limited exclusively to reductions in ATP content (*i.e.* cell viability/metabolic activity), while biofilm growth was essentially unadulterated (Fig. 23A). In fact, a considerably higher amount of biofilm growth was observed for the three *E. coli* strains. This could be due to the fact that PEA is a nutrient that bacteria utilize to grow and not enough PEA is present to cause inhibition of biofilm biomass and may actually induce EPS production [220]. One potential explanation for the seemingly disparate results between the ATP and CV assays may be due to bacterial cell death as they come in direct contact with PEA at the surface of the PEA-PU material. Since the PEA does not leach out of our material, the other bacteria that didn't come into contact could form a biofilm and would explain the higher values for the CV assay. This supports the conclusion that the antimicrobial effect of our PEA-PU material is due to bacterial contact with the surface resulting in cell death, which would result in the reduction of the ATP content of the biofilm.

In conclusion, we have demonstrated that our PEA-PU material can effectively reduce ATP content of a variety of bacterial pathogens. However, how much ATP content is reduced is dependent on the cell. Unfortunately, our material wasn't able to reduce the total biofilm biomass

of any of our bacterial pathogens tested. Since PEA is a nutrient, higher concentrations may be needed to completely abolish biofilm formation. Thus, the amount of PEA needed to achieve this may be unattainable due to physical limitations of the material that impact structural stability. We believe that 5 % PEA-PU has the potential to be used in multiple applications in the food processing industry to prevent or minimize the microbial contamination of food products. Examples of these applications could include conveyor belts, chiller tanks, scalding tanks, and cutting tools [57, 58].

Overall, our material might help to reduce antibiotic use, by using a nitrogen source that bacteria typically use for food as a novel approach to the prevention and treatment of biofilm formation. In the future, our material could be used to coat food processing equipment to reduce the outbreak of foodborne illness.

Development of a liquid flush to reduce bacterial biofilm in a catheter

Introduction

Currently in the United State about 150 million intravascular catheters are used every year for critically ill patients, such as patients with chronic renal failure, cancer patients, ICU patients, and patients with long-term illness [46]. An intravascular catheter or central line can be used for hemodynamic monitoring, renal replacement therapy, nutritional support, or medication administration. A central line catheter is a long, flexible tube usually made of polytetrafluoroethylene (Teflon), polyurethane, or silicone. This requires surgical implantation, where the catheter is threaded into the jugular vein and guided into the superior vena cava. This however is not ideal because the patient becomes more vulnerable to developing a blood clot or contracting a central line-associated bloodstream infection (CLABSI). CLABSIs can be caused by bacterial biofilms, which are a group of bacteria that can attach or adhere to a surface. The

phenotypic changes that bacteria undergo as they form the biofilm community cause difficulties with standard chemical, physical, or biological removal techniques [26, 221, 222]. In addition, CLABSIs are even harder to treat with the emergence of antibiotic resistant strains, causing an increase in morbidity and treatment costs [223]. Last-resort antibiotics, such as methicillin and vancomycin, which were once very effective at treating CLABSIs are becoming insufficient due to overuse. CLABSIs caused by antibiotic resistant strains, such as MRSA and VRSA are becoming more prevalent (for a review see [55]).

Since the formation of bacterial biofilms on central venous catheters continues to cause problems, current research efforts have been aimed at mitigating this problem by incorporating traditional antibiotics and antimicrobials directly into the materials of biomedical devices. When central venous catheters were integrated with chlorhexidine-silver sulfadiazine or minocycline-rifampicin, bacterial colonization and CLABSI occurrence was shown to be reduced [224]. However, recent studies have shown that the antimicrobial effect of chlorhexidine and silver sulfadiazine coated catheters only lasted 48 h and may not be sufficient for reducing catheter related infections [69, 70]. Another strategy has been to use metal nanoparticles to coat or embed into the catheter material. Both silver and copper nanoparticles have been used due to their anti-microbial properties (for a review, please, see [71, 72]). In vitro, Ag/Cu-coated catheters have been shown to prevent the attachment of MRSA. The Ag/Cu coated catheter was also shown, using a rat animal model, to reduce the occurrence of catheter-related infections (though not significantly) and bacteremia in comparison to non-coated catheters [73]. However, these materials are accompanied by high production costs, limited lifetimes, the emergence of resistant bacterial strains, and have even been shown to increase antibiotic resistance in some bacterial strains [74-76]. In addition, high doses of silver can have toxic effects on the human body and have

been shown to accelerate thrombin formation and platelet activation when catheters were coated with silver nanoparticles [77, 78]. Another strategy to reduce catheter-related infections has been to use ALT, where high concentrations of antibiotic solutions are left to dwell in the catheter when the catheter is not in use. Traditional antibiotics like vancomycin, cefazolin, ceftazidime, ciprofloxacin, gentamicin, and ampicillin are typically used for ALT treatment. ALT treatment was shown to be effective at reducing CLABSI of cancer patients, however no significant conclusions could be drawn due to differences in administration and dwell time [64]. Unfortunately, ALT contributes to the overuse of antibiotics and could increase the emergence of antibiotic resistant bacterial strains. Despite all these efforts, incidence of catheter-related infections continues to occur and an urgent need for novel approaches to inhibit biofilm is evident.

The research presented in this study constitutes a different approach towards the development of novel biofilm inhibiting antimicrobials. Instead of using traditional antibiotics to inhibit biofilm formation, we based our study on a previous screen, where we used a library of bacterial nutrients and investigated their effect on bacterial cell number and biofilm biomass [204]. Among the 95 carbon and 95 nitrogen sources tested, PEA exhibited the most potent antimicrobial effect on *E. coli* O157: H7 grown in liquid BB at refrigerator temperature (4°C). This study expands on the previous observation by testing the effect of PEA supplied in the liquid bacterial growth medium on non-pathogenic *E. coli* K-12, as well as a selection of pathogens relevant to the clinical settings. In a first step towards our goal of developing a novel biofilm inhibiting antimicrobial flush, we inoculated hemodialysis fistula needles to simulate a catheter and mimicked ALT by leaving PEA solutions in the catheter to treat the bacterial biofilms formed in the catheter. The catheters were subsequently assessed for antimicrobial

effectiveness towards the same selection of bacterial strains and the highest concentrated PEA flush was shown to reduce ATP content by 100 %. Thus, PEA could be a better alternative to treat bacterial biofilms than traditional antibiotics and in the future could be used as an ALT.

Materials and methods

Bacterial strains

Bacterial strains used in this study are summarized in Table 3 and include the non-pathogenic, wild type *E. coli* K-12 strain and nine bacterial pathogens. The *E. coli* K-12 strain used was AJW678, which was previously described as a good former of biofilm [170]. Pathogenic bacterial strains include *P. aeruginosa* (ATCC 15442), *S. aureus* (ATCC 25923), EHEC EDL932 [172], and two UPECs, UMN026 [173] and CFT073 [174], *Y. enterocolitica* 8081c (ATCC 9610), *L. monocytogenes* 10403S, *C. sakazakii* (BAA-894), and *S. marcescens* (BAA-632). Bacterial strains were maintained as freezer stocks at -80ºC in 8 % DMSO. Prior to each experiment, the *E. coli* strains were plated onto LB (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) agar plates. *P. aeruginosa* and *S. aureus* were plated onto TSA (15 g/l tryptone, 5 g/l soytone, 5 g/l NaCl, 15 g/l agar) plates. Plates were incubated over night at 37°C.

Formation of biofilm in silicone tubing

Liquid overnight cultures (10 ml) in TSB were centrifuged at 4,500 *g* for 10 min, bacteria were resuspended in TSB to an OD_{600} of 0.05. An AV Fistula Needle 17 Ga. X 1-1/4" Single Pk FW with MasterGuard® anti-stick needle protector was purchased from Medisystems Corporation (Lawrence, MA). The key piece of this Hemodialysis Fistula Set is a 12-inch-long piece of silicone tubing with an inner diameter of 1/8 inch and an outer diameter of 3/8 inch. Prior to inoculating with bacteria, the tubing was prepared as described in Fig. 24. The needle guard was pulled over the needle and cut off. An alcohol swab was used to remove the cap at the

other end of the tubing to be used later as a cap on the end of the tubing where the needle was removed. Using a sterile syringe, the tubing was inoculated with 3 ml of inocula. The clamp on the tubing was then closed and the syringe was left attached to the needle. Finally, the cap that was saved earlier was attached to the end of the tubing (where the needle was removed). Two pieces of tubing were inoculated for each bacterial strain, one tubing was used with TSB to serve as negative control. All tubings were incubated on an absorbant pad at 37°C for the 2-week incubation period, except *Y. enterocolitica* and *S. marcescens* which were incubated at 30°C. On day 4, the two tubings for each strain either received 3 ml of TSB or 3 ml of treatment (at concentrations of 100 mg/ml, 10 mg/ml, or 1 mg/ml of PEA or EAA supplemented in TSB). New sterile syringes were used, attached, and left on the tubings. No new media was pushed through the control tubing. The subsequent treatments were administered on days 8 and 11. After 2 weeks had elapsed, the tubings were processed.

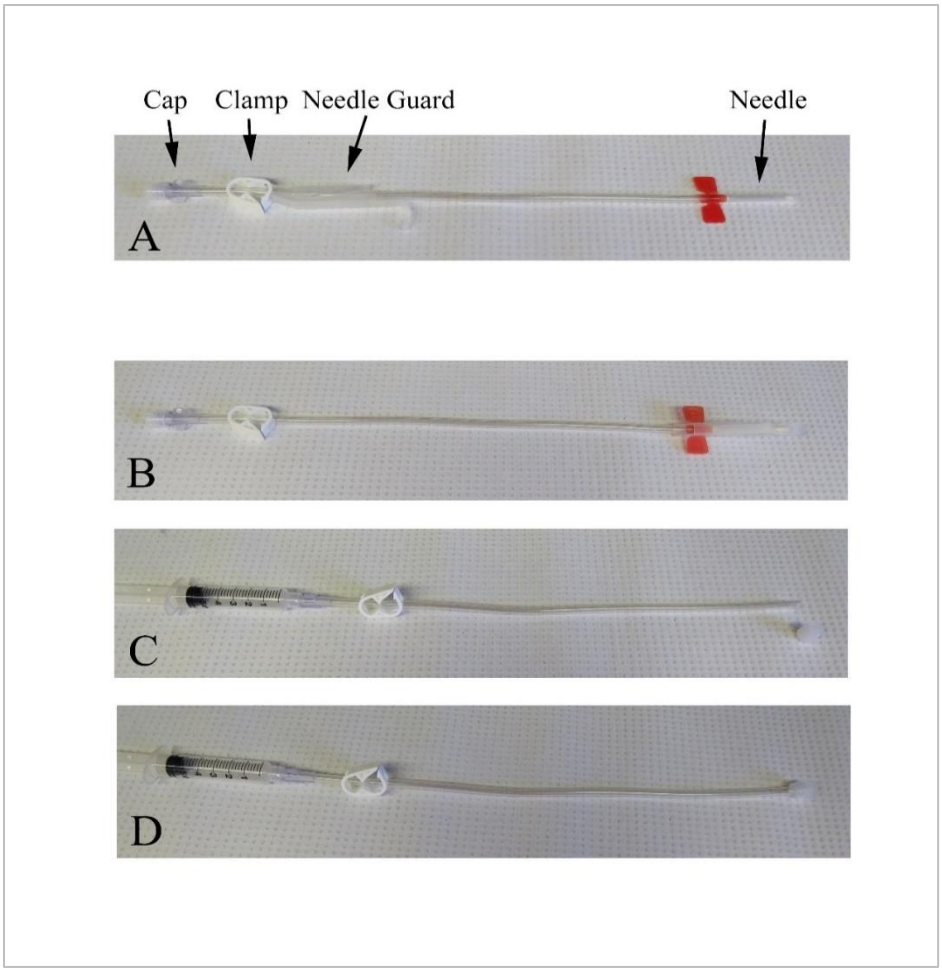

Figure 24. Preparation of the hemodialysis fistula set to grow biofilm in silicone tubing. Panel A, fistula set as it was removed from the plastic protector. Panel B, the needle guard was pushed over the needle. Panel C, the needle was cut off, the cap was removed, and a syringe was inserted. Panel D, the cap was attached to the opposite end for incubation.

Performance of the biofilm assays in silicone tubing

At the end of the incubation, biofilms were characterized with the CV and ATP assays. The tubings were flushed with 3 ml of PBS using a new sterile syringe. The syringe was detached, and 3 ml of air was pushed through the tubing to flush out any liquid. Starting at the end that originally had the syringe attached, a 4-inch section of tubing was cut off with a sterile razor blade. From the 4-inch piece of tubing, six sections of about 1 mm were cut for the biofilm assays. Three of these smaller pieces of tubing were transferred to one microcentrifuge tube to be

used for the CV assay. The other three sections of tubing were transferred into three separate microcentrifuge tubes, each filled with 1 ml of PBS to be used for the ATP assay and the determination of the viable cell counts. A new razor blade was used to cut each catheter.

For the CV assay, 1.0 ml of 0.1 % CV in ddH_2O was added to the microcentrifuge tube. The tube was incubated for 15 min. The silicone tubings were rinsed 3 times by using sterile forceps and dipping for 10 s into 3 separate and consecutive microcentrifuge tubes filled with 1 ml of ddH_2O. The silicone tubings were then transferred into new individual microcentrifuge tubes, where they were allowed to dry at room temperature for at least 1 h. The CV was extracted by adding 500 µl of 20 % acetone 80 % ethanol to each microcentrifuge tube. The tubes were vortexed for 30 s and then incubated for 15 min. 0.15 ml of each extract was then transferred into one well of a 96 well polystyrene plate and the OD_{600} values were determined with the Synergy H1 Hybrid Reader.

For the ATP assay, the three microcentrifuge tubes that contained one section of silicone tubing and 1 ml of PBS were vortexed thoroughly for a minute. Each individual tube was then vortexed for 10 seconds prior to transferring 100 µl to 1 well of a 96-well white plate. Then, 100 µl of BacTiter-Glo™ reagent was added to each well. We covered the plates with tinfoil and thoroughly mixed solutions by placing the plate on a rotating shaker for 5 min. After this incubation, we read luminescence using the Synergy H1 Hybrid Reader.

For the viable cell counts from the biofilms, 100 µl were obtained from the microcentrifuge tube that was used for the ATP assay, serially diluted in 1:10 steps and plated onto TSA plates. Plates were incubated for 24 h at 37°C. In a second experiment, the viable cell counts were determined for the planktonic bacteria in the liquid in the tubing. Before each treatment was administered, the fluid in the tubing was collected in a microcentrifuge tube and

serial diluted in 1:10 steps. Fluid was collected before treatments on day 4 (1st treatment), day 8 (2nd treatment), day 11 (3rd treatment), and on day 14 at the end of the incubation period (4th treatment). The serial dilutions were plated onto TSA plates and the plates were incubated for 24 h at 37°C. Viable cell counts were expressed as CFU per ml. It is important to note, that the lower limit of CFU/ml is 999 for the tubing that received PEA and EAA treatments.

Data analysis

All assays were performed in six replicates, across which the averages and standard errors were calculated. Two inoculated tubings per strain constituted the biological replicates (from independent overnight cultures) and the three slices per tubing the technical ones. Percent reduction was calculated as ((a-b)/a) x 100, where a is the data (*e.g.* OD_{600}, RLU) at the control condition (*e.g.* 0 mg/ml PEA) and b is the data at the experimental condition (*e.g.* specific concentration of PEA). Student's *t*-test determined the statistical significance of the difference between the mean data for each experimental condition and the mean data at the control condition. Differences were considered significant when the *p*-value was below 0.05.

Results

Flushes of PEA reduced the ATP content and total biofilm biomass in silicone tubing of bacterial pathogens

To test one application of PEA, we used the silicone-based model, resembling an intravenous catheter with ALT. In the first experiment, the bacterial strains *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, and *E. coli* K-12 AJW678 were tested with a treatment flush of 100 (Fig. 25) mg/ml of PEA. The bacterial strains *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enetercolitica,* and *L. monocytogenes* were tested with the treatment flushes of 10 (Fig. 26) and 1 mg/ml (Fig. 27) of

PEA. Bacterial strains were grown in the tubings, which were flushed with PEA three times on day 4, 8, and 11 during the 2-week incubation period.

When the PEA flush was used at a concentration of 100 mg/ml, the ATP content of all bacteria was reduced drastically (Fig. 25A). Percent reductions ranged from 98.5 % for *E. coli* CFT073 to 99.9 % for *S. aureus* (Supplemental Table 4). When the PEA flush was used at a concentration of 100 mg/ml, the reduction of CV content of all bacteria was variable across strains (Fig. 25B). Percent reductions ranged from about 49.12 % for *P. aeruginosa* to 80 % for *E. coli* UMN026 and AJW678 *S. aureus* had about 70 % reduction of total biofilm biomass. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for all pathogens, while there was a significant difference between the biofilm biomass for *S. aureus* and *E. coli* UMN026. Only *P. aeruginosa* was found not to be statistically significant with a p-value of 0.0596.

In the next experiment using a treatment of 10 mg/ml of PEA, we expanded our list of pathogens to include *Y. enterocolitica* and *L. monocytogenes*, which have been associated with forming biofilms on processing equipment resulting in outbreaks of foodborne illness.

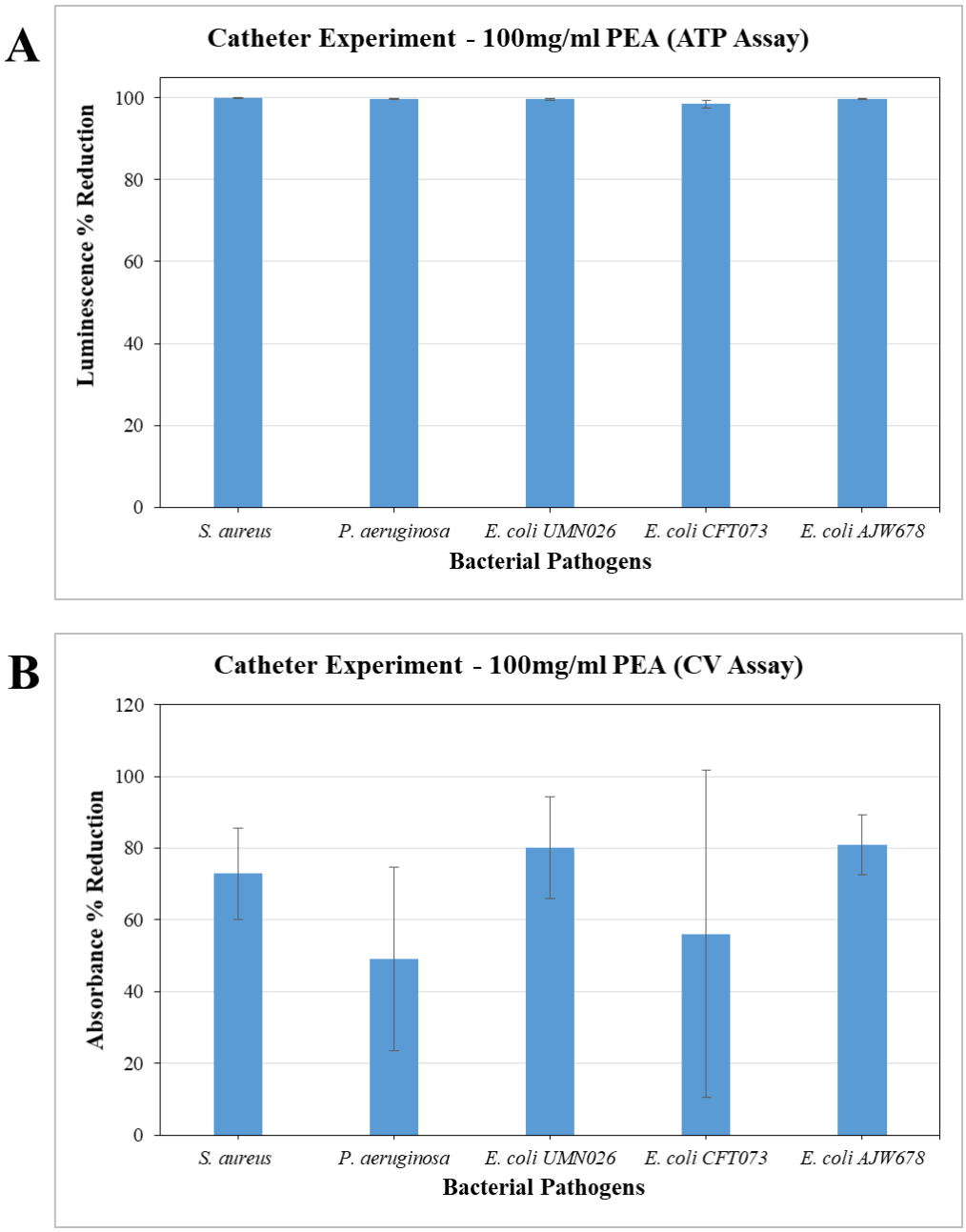

Figure 25. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 100 mg/ml PEA flush compared to biofilms treated with 0 mg/ml of PEA. Panel A shows the ATP content of biofilm grown in the presence of 100 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. PEA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

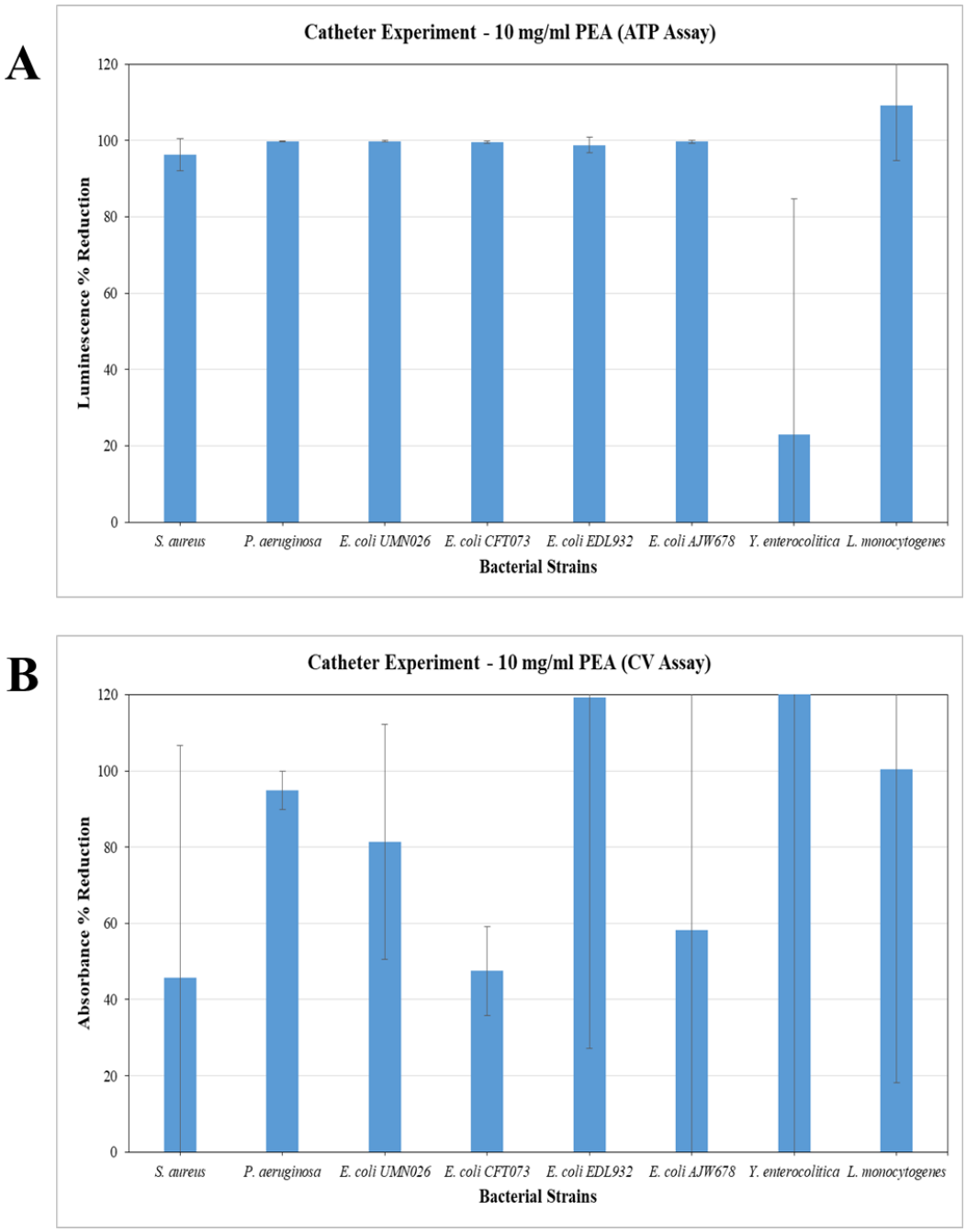

Figure 26. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 10 mg/ml PEA flush compared to biofilms treated with 0 mg/ml of PEA. Panel A shows the ATP content of biofilm grown in the presence of 100 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. PEA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

When the PEA flush was used at a concentration of 10 mg/ml (Fig. 26), reductions of ATP were very similar to the 100 mg/ml flush with all the pathogens having close to 100 % reduction in ATP energy content, except for *Y. enterocolitica*. *Y. enterocolitica* only had an average 20 % reduction in ATP content. *S. aureus, E. coli* AJW678, and *Y. enterocolitica* exhibited the lowest percent reduction with a lot of variation in the amount of biofilm biomass present. All three bacterial strains (*S. aureus, E. coli* AJW678, and *Y. enterocolitica*) had error bars that indicated some treatment of PEA encouraged biofilm formation. The rest of the strains percent reduction of biofilm biomass ranged from 50 % to near 100 % reduction of biofilm biomass. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for all pathogens, except *Y. enterocolitica,* while there was a significant difference between the biofilm biomass for *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* AJW678, *and L. monocytogenes.*

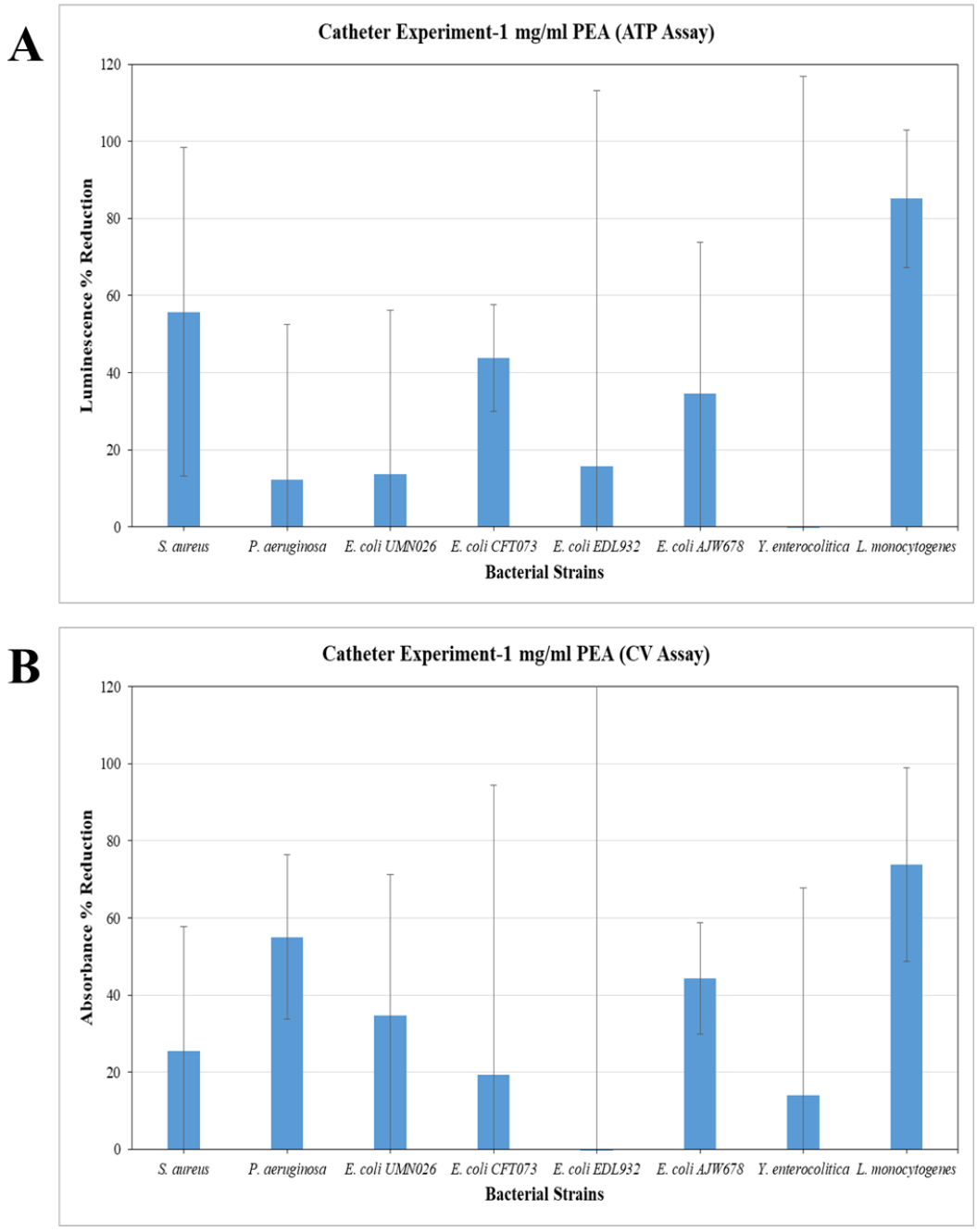

Figure 27. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 1 mg/ml PEA flush compared to biofilms treated with 0 mg/ml of PEA. Panel A shows the ATP content of biofilm grown in the presence of 100 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. PEA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

A lower concentration of PEA at 1 mg/ml was then tested as a flush to see if it had similar effects on ATP as the 100 and 10 mg/ml treatment of PEA did. When the PEA flush was used at a concentration of 1 mg/ml (Fig. 27), the outcome changed for the ATP content. All strains still showed a reduction in the ATP content of their biofilm, but all the strains except *L. monocytogenes* showed a reduction that was insignificant. *L. monocytogenes* had about an 80 % reduction in ATP content when treated with 1 mg/ml of PEA. In general, we observed a decrease in the amount of biofilm biomass being reduced with a lower concentration of PEA. Again, we observed a lot of variability in the amount of biofilm biomass that is reduced depending on the strain. Most of strains still showed a reduction in the ATP content of their biofilm, but the percent reduction of most of the strains was below 50 %.

E. coli EDL932 had almost no reduction in biofilm biomass, while *L. monocytogenes* had about a 75 % reduction. Interestingly, *P. aeruginosa* had a very little reduction in ATP content of cells only reducing ATP by about 15 % at 1 mg/ml of PEA, while the biofilm biomass percent reduction of *P. aeruginosa* was around 55 %. In summary, the PEA flush was very effective at reducing the ATP content of biofilms grown in silicone tubing when used at a concentration of at least 10 mg/ml. However, reduction in total biofilm biomass was dependent on the strain, as well as the treatment concentration being administered. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for *E. coli* CFT073, *E. coli* EDL932, *E. coli* AJW678, and *L. monocytogenes*, while there was a significant difference between the biofilm biomass for *P. aeruginosa, E. coli* AJW678, *and L. monocytogenes.*

Flushes of EAA reduced the ATP content and total biofilm biomass in silicone tubing of some bacterial pathogens

To test one application of EAA, we used the silicone-based model, resembling an intravenous catheter with ALT. The bacterial strains *S. aureus, P. aeruginosa, E. coli* UMN026, *E. coli* CFT073, *E. coli* EDL932, *E. coli* K-12 AJW678, *Y. enetercolitica, S. marcescens* and *C. sakazakii* were tested with the treatment flushes of 100 (Fig. 28), 10 (Fig. 29), 5 (Fig. 30), and 1 mg/ml (Fig. 31) of EAA. Bacterial strains were grown in the tubings, which were flushed with EAA three times on day 4, 8, and 11 during the 2-week incubation period.

When the EAA flush was used at a concentration of 100 mg/ml (Fig. 28), the ATP content of most of the bacterial strains was reduced drastically. Interestingly, *P. aeruginosa* was the pathogen that was the least effected by the EAA treatment and had the lowest amount of reduction of ATP content. However, there was a large amount of variation in the data, which resulted in larger error bars. *S. aureus* had the second lowest reduction of ATP content around 75 % reduction. Percent reductions ranged from 98.5 % for *E. coli* CFT073 to 105 % for *Y. enterocolitica.* When the EAA flush was used at a concentration of 100 mg/ml, the CV content of all bacteria was variable across strains. Percent reductions ranged from 11 % for *E. coli* CFT073 to 100 % for *E. coli* EDL932. There was no reduction in total biofilm biomass for *P. aeruginosa.* However, there was more variation in the data at 100 mg/ml of EAA for biofilm biomass in comparison to the results of the PEA flush at the same concentration. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for all the pathogens except *P. aeruginosa,* while there was a significant difference between the biofilm biomass for *E. coli* UMN026, *E. coli* EDL932, *Y. enterocolitica*, and *S. marcescens.*

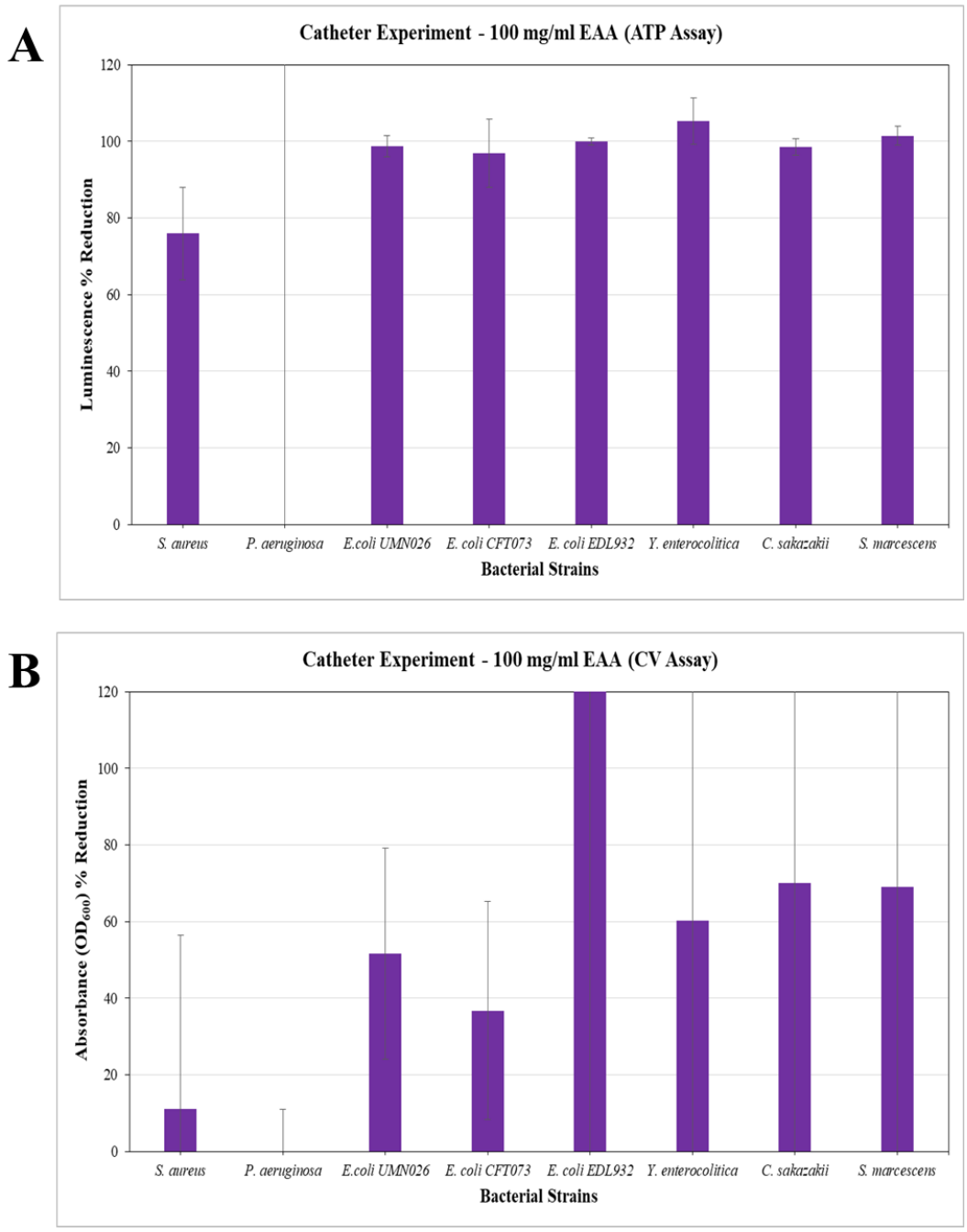

Figure 28. Percent Reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 100 mg/ml EAA flush compared to biofilms treated with 0 mg/ml of EAA. Panel A shows the ATP content of biofilm grown in the presence of 100 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. EAA was flushed through the tubing three times during the 2 weeks of incubation at 37^{o}C, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

When the PEA flush was used at a concentration of 10 mg/ml (Fig. 29), reductions of ATP were similar to the 100 mg/ml flush for some pathogens having close to 100 % reduction in ATP energy content, except for *S. marcescens*. *S. marcescens* biofilms when treated with 10 mg/ml of EAA had almost no percent reduction of ATP content. There was a slight decrease in the percent reduction of *E. coli* O157:H7 and *C. sakazakki* in comparison to the percent reduction of ATP content observed when the EAA treatment was at a concentration of 100 mg/ml. *P. aeruginosa* had 0 % reduction at a treatment of 100 mg/ml of EAA, but it was observed to achieve a 76 % reduction of ATP content when treated with 10 mg/ml of EAA. When we look at the CV data for total biomass, we see the effectiveness of EAA to reduce biofilm biomass depends on the strain. We observed no reduction in biofilm biomass for *E. coli* CFT073 and *Y. enterocolitica.* Percent reductions of biofilm biomass treated with 1 mg/ml of EAA flushes ranged from 56 % for *E. coli* BAA to 71 % for *E. coli* O157:H7 (Supplemental Table 4). Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for all the pathogens except *S. marcescens,* while there was a significant difference between the biofilm biomass for all the pathogens except *E. coli CFT073* and *Y. enterocolitica.*

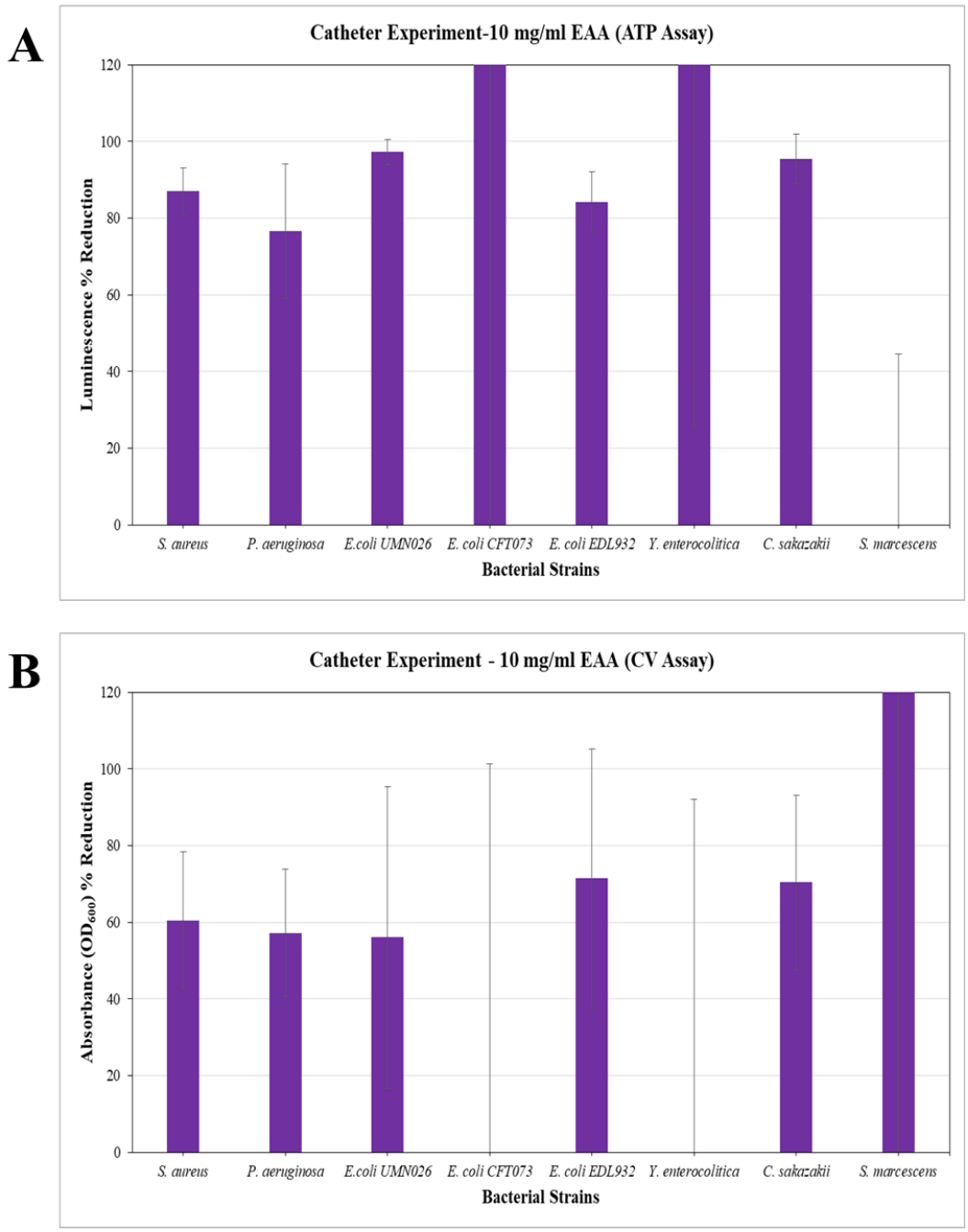

Figure 29. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 10 mg/ml EAA flush compared to biofilms treated with 0 mg/ml of EAA. Panel A shows the ATP content of biofilm grown in the presence of 10 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. EAA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

A lower concentration of EAA at 5 mg/ml was then tested as a flush to see if it had similar effects on ATP as the 100 and 10 mg/ml treatment of PEA did (Fig. 30). When the PEA flush was used at a concentration of 5 mg/ml, the outcome changed for the ATP content. Most strains still showed a reduction in the ATP content of their biofilm, but all the strains showed a reduction that was not significant except *E. coli* CFT073 and *S. marcescens. E. coli* CFT073 and *S. marcescens* showed near 100 % reduction in ATP content of the biofilm, while the rest of the strains either showed no reduction in ATP content or had large error bars due to variation within the data. At 5 mg/ml of EAA overall, we observed very little reduction in total biofilm biomass for most of the strains, except *S. marcescens* had about 98 % reduction and *P. aeruginosa* had about a 67 % reduction in biofilm biomass. Interestingly, *E. coli* CFT073 had close to 100 % reduction of ATP content, but almost no reduction in total biofilm biomass. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA for all the pathogens except *E. coli* UMN026, *E. coli* EDL932, and *C. sakazakii*, while there was a significant difference between the biofilm biomass for *P. aeruginosa* and *E. coli* UMN026.

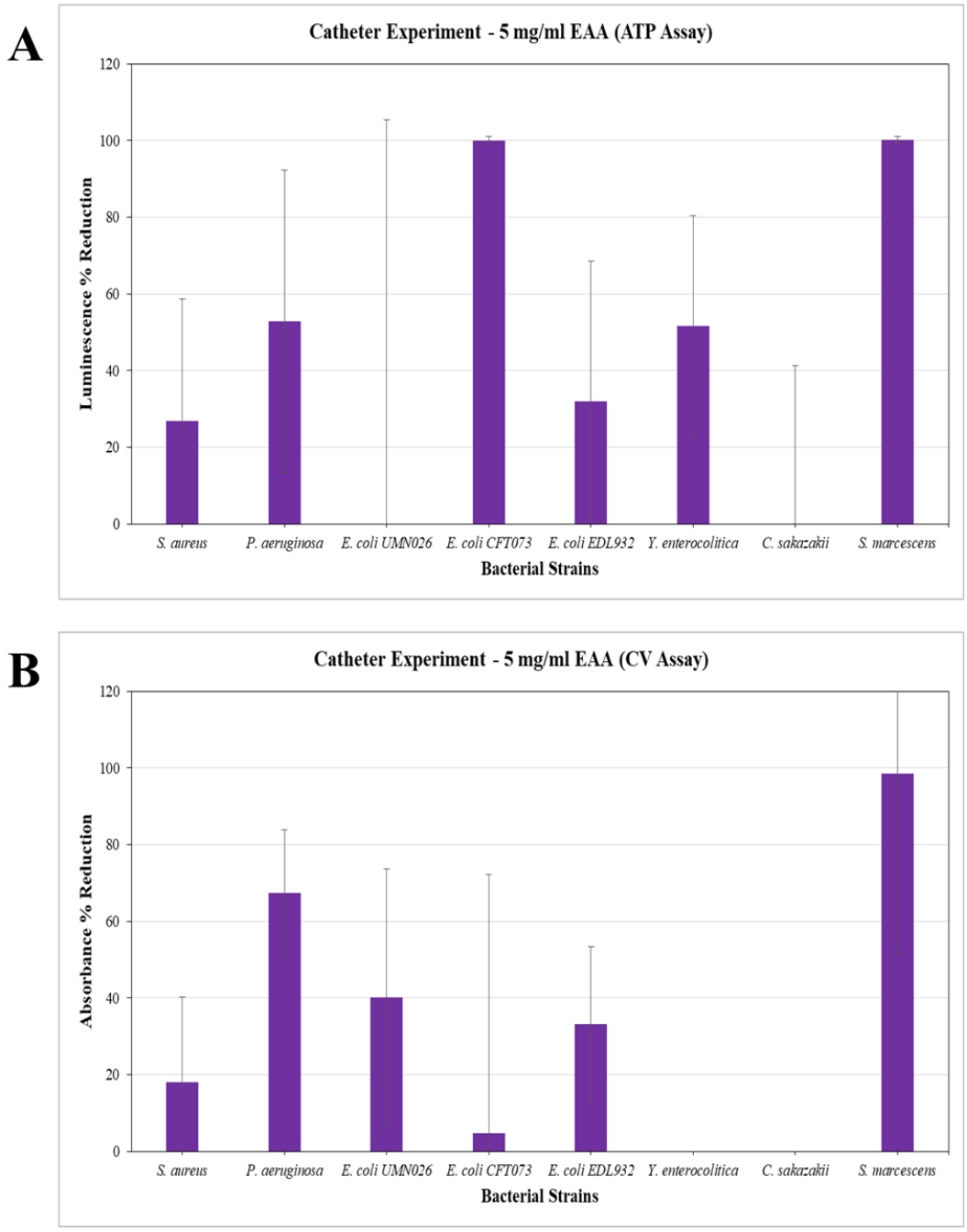

Figure 30. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 5 mg/ml EAA flush compared to biofilms treated with 0 mg/ml of EAA. Panel A shows the ATP content of biofilm grown in the presence of 5 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. EAA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

An even lower concentration of EAA at 1 mg/ml was then tested as a flush to see if it had any antimicrobial effects at this concentration for any of our bacterial pathogens tested (Fig. 31). When the EAA flush was used at a concentration of 1 mg/ml, there was very little reduction in ATP content. None of the reduction in ATP content were significant, except for *Y. enterocolitica* which had a 71 % reduction in ATP content in the biofilm in comparison to the non-treated biofilm. All of the other strains had below 40 % reduction of ATP content and all the other strains error bars were large indicating large variation among the data. The absorbance data for the CV assay, when 1 mg/ml of EAA flush was used showed little reduction in total biofilm biomass. All the strains' biofilm biomass was not significantly reduced in comparison to the non-treated biofilms. The highest reduction of biofilm biomass content was seen for *P. aeruginosa* at 40 %, while *S. aureus* and the pathogenic *E. coli* strains had a reduction of biofilm biomass between 20 % and 37 %. However, all of the strains tested had calculated error bars greater than the average percent reduction of biofilm biomass. Student's *t*-test was able to calculate a statistically significant difference between the ATP content of the biofilms in the absence and presence of PEA only for *P. aeruginosa,* while there was only a significant difference between the biofilm biomass for *Y. enterocolitica.* At 1 mg/ml of EAA, overall, we observed very little reduction in total biofilm biomass for most of the strains, except *S. marcescens* had about 98 % reduction and *P. aeruginosa* had about a 67 % reduction in biofilm biomass. Interestingly, *E. coli* CFT073 had close to 100 % reduction of ATP content, but almost no reduction in total biofilm biomass.

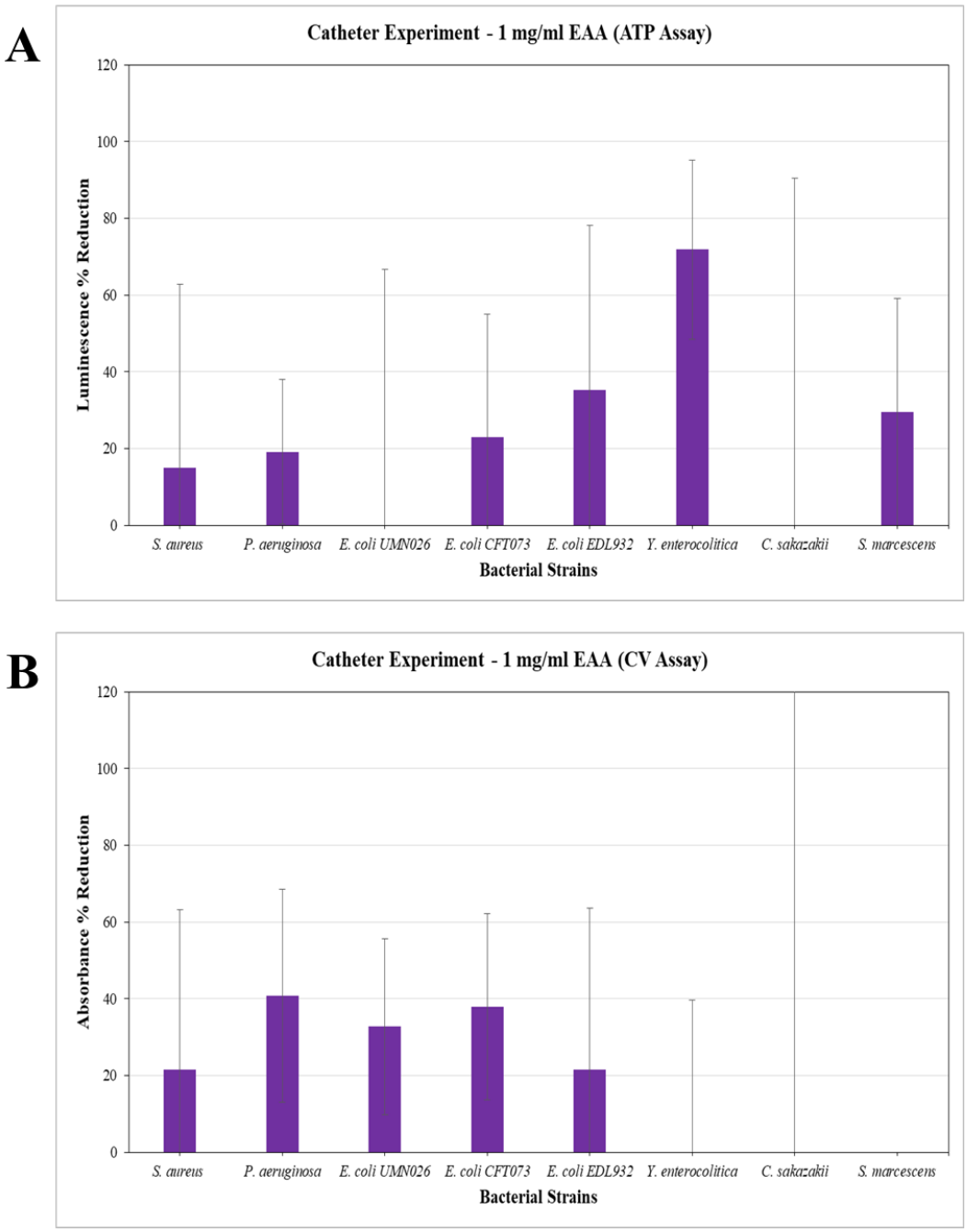

Figure 31. Percent reduction of luminescence from the ATP and absorbance from the CV assay when biofilms were treated with a 1 mg/ml EAA flush compared to biofilms treated with 0 mg/ml of PEA. Panel A shows the ATP content of biofilm grown in the presence of 1 mg/ml, Panel B shows the absorbance data of the total biofilm biomass from the CV assay. EAA was flushed through the tubing three times during the 2 weeks of incubation at 37ºC, biofilm was characterized with the ATP and CV assay at the end of the incubation. Averages and standard errors were determined across 6 replicates.

PEA and EAA reduce ATP content and biofilm biomass of S. aureus and P. aeruginosa in part due to reduction in viable bacterial cell numbers

The previous experiments were repeated with *S. aureus* and *P. aeruginosa* to determine the viable cells in the liquid dwelling in the catheter (Fig. 32 & 33) and the viable cells within the biofilm at the end of incubation (Fig. 34). Fig. 32A shows the cell counts of the planktonic cells of *S. aureus* in the liquid collected before being flushed with 100 mg/ml of PEA (blue bars) or media (orange bars). Fig. 32B shows the cell counts of the planktonic cells of *P. aeruginosa* in the liquid collected before being flushed with 100 mg/ml of PEA (blue bars) or media (orange bars).

We observed that the cell counts of *S. aureus* collected before the first treatment was administered (1st treatment) were around 10^8 CFU/ml and were not significantly different from each other. This indicates that roughly the same amount of viable cells of *S. aureus* were present in both the non-treatment and treatment tubing. The liquid was always first collected before each treatment was administered. We observe that there is roughly a 4 to 5-log difference between the viable cells of *S. aureus* in the liquid of the catheter receiving 100 mg/ml treatment of PEA (orange bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (blue bars) at the 2nd, 3rd, and 4th treatment and were found to be statistically significant. We observed that the cell counts of *P. aeruginosa* collected before the first treatment was administered (1st treatment) were around 10^9 CFU/ml and were not significantly different from each other. This indicates that roughly the same amount of numbers of viable cells of *P. aeruginosa* were present in both the non-treatment and treatment tubing. Interestingly though, we observed only about a 3-log difference at the 2nd treatment (2nd treatment) between the viable cells of *P. aeruginosa* in the liquid of the catheter receiving 100 mg/ml treatment of PEA (blue

bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars). A 6-log difference was observed at the 3nd treatment between the viable cells of *P. aeruginosa* in the liquid of the catheter receiving 100 mg/ml treatment of PEA (blue bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars), while we only saw about a 5-log reduction at the 4th treatment. However, the difference between the viable cells in the liquid of the catheter receiving 100 mg/ml treatment of PEA (blue bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars) at each time point was only found to be significant after the 1st and 2nd treatment.

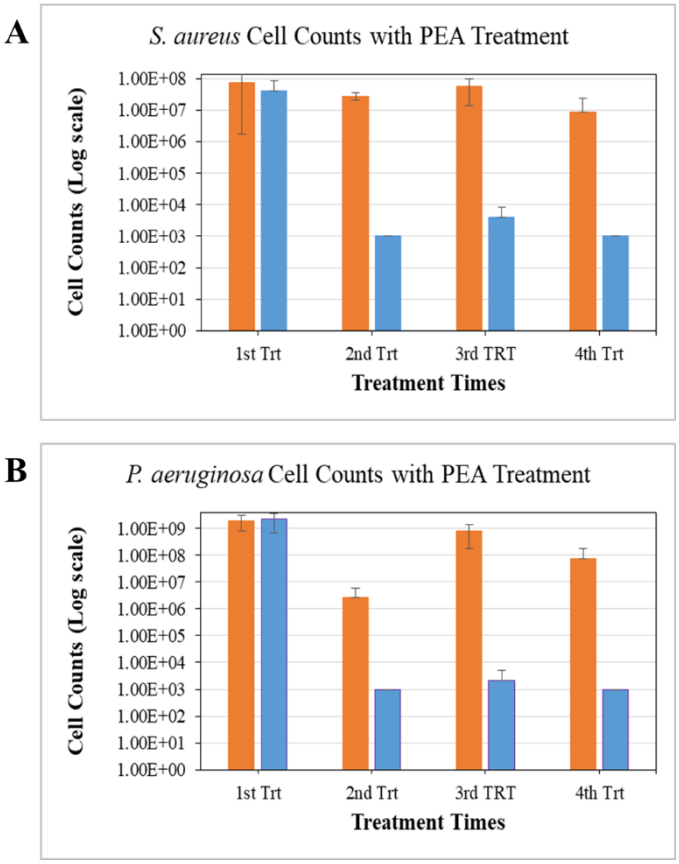

Figure 32. Log reduction of the cell counts of *S. aureus* and *P. aeruginosa* from the collected liquid after treatment 100 mg/ml of PEA. *S. aureus* (Panel A) and *P. aeruginosa* (Panel B) viable cell counts in the liquid collected before being flushed with 100 mg/ml of PEA (blue bars) or media (orange bars). Averages and standard errors were determined across 6 replicates.

Fig. 33A shows the cell counts of the planktonic cells of *S. aureus* in the liquid collected before being flushed with 100 mg/ml of EAA (purple bars) or media (orange bars). Fig. 33B shows the cell counts of the planktonic cells of *P. aeruginosa* in the liquid collected before being flushed with 100 mg/ml of EAA (purple bars) or media (orange bars).

We observed that the cell counts of *S. aureus* collected before the first treatment was administered (1st treatment) were around 10^8 CFU/ml and were not significantly different from each other. Interestingly though, we observed only about a 2-log difference before the 2nd treatment (2nd treatment) between the viable cells of *S. aureus* in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars). There was no difference between the viable cells in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars) before the 3rd and final treatment were administered. Only at the 2nd treatment was there found to be a statistically significant difference in the viable cells of *S. aureus* in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars). We observed that the cell counts of *P. aeruginosa* collected before the first treatment was administered (1st treatment) were around 10^9 CFU/ml and were not significantly different from each other. We observed less than a 2-log difference before the 2nd treatment between the viable cells *P. aeruginosa* of in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars). The viable cells of *P. aeruginosa* in the liquid of the catheter receiving only a media flush (orange bars) was reduced by 2-logs between the 2nd and 3rd treatment. However, the viable cells

of *P. aeruginosa* in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars) maintained about a 2-log difference. Interestingly though, there was about a 1-log increase between the viable cells in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars) after the before the 4th treatment. At the 2nd and 3rd treatment was there found to be a statistically significant difference in the viable cells of *P. aeruginosa* in the liquid of the catheter receiving 100 mg/ml treatment of EAA (purple bars) in comparison to the viable cells in the liquid of the catheter receiving only a media flush (orange bars).

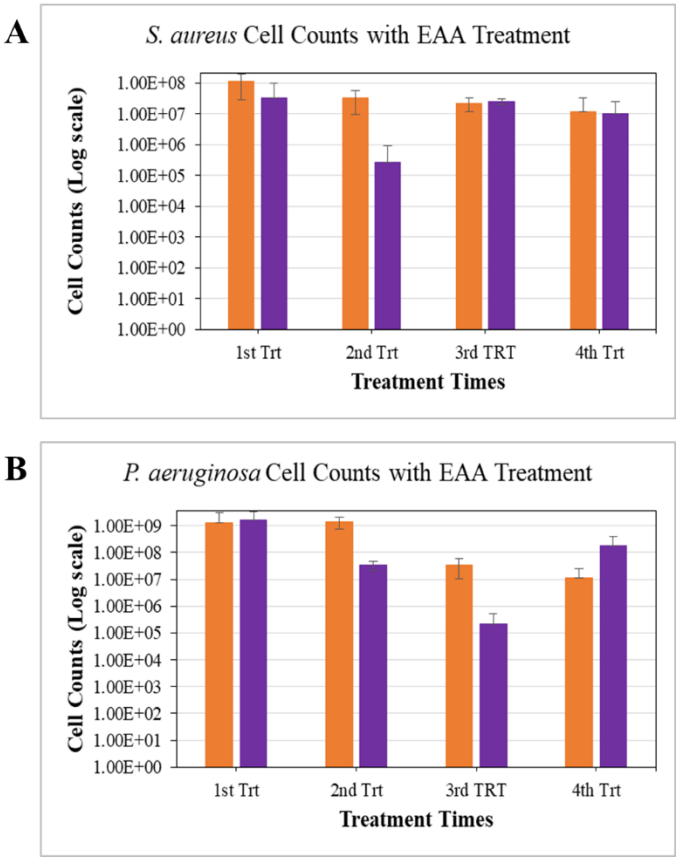

Figure 33. Log reduction of the cell counts of *S. aureus* and *P. aeruginosa* from the collected liquid after treatment 100 mg/ml of EAA. *S. aureus* (Panel A) and *P. aeruginosa* (Panel B) viable cell counts in the liquid collected before being flushed with 100 mg/ml of EAA (purple bars) or media (orange bars). Averages and standard errors were determined across 6 replicates.

Finally, we determined the biofilm biomass, ATP content of the biofilm, and viable cells within the biofilm of *S. aureus* and *P. aeruginosa* to at the end of inoculation (Fig. 34). Biofilm biomass (CV assay) yielded a much smaller difference between the three treatments (100 mg/ml of PEA (blue bars), 100 mg/ml of EAA (purple bars), and 0 mg/ml of PEA and EAA (orange bars)) for *S. aureus*, but still showed a reduction by PEA for *P. aeruginosa* from 0.78 to 0.24 OD_{600} (Fig. 34A). Biofilm biomass of *P. aeruginosa* that were treated with 100 mg/ml treatments of PEA and 100 mg/ml treatments of EAA were found to be statistically significant, while biofilm biomass of *S. aureus* that was treated with 100 mg/ml treatments of EAA were found to be statistically significant. For the ATP assay (Fig. 34B), there was almost no ATP content of the biofilm for *S. aureus* and *P. aeruginosa* treated with 100 mg/ml treatments of PEA and 100 mg/ml treatments of EAA in comparison to the n ATP content of the biofilm for *S. aureus* and *P. aeruginosa* treated with 0 mg/ml of PEA and EAA. Both treatments of 100 mg/ml of PEA and EAA were found to be statistically significant for *S. aureus* and *P. aeruginosa*. With the viable cell counts (Fig. 34C) we observed, *S. aureus* had a reduction of about 1-log in viable cells when the bacteria were treated with 100 mg/ml of PEA or EAA, while *P. aeruginosa* had about a 3-log reduction of viable cells when the bacteria were treated with 100 mg/ml of PEA or EAA. It is possible that the log reduction for *S. aureus* and *P. aeruginosa* in the viable cell counts is an underestimate due to the low cell number in the untreated control and the fact that 999 CFU/ml was the lower limit of detection for our assay. At this point, we do not know whether a reduction in the viable cell counts can serve as the sole explanation for the reduction in the ATP content of the biofilm for this bacterium.

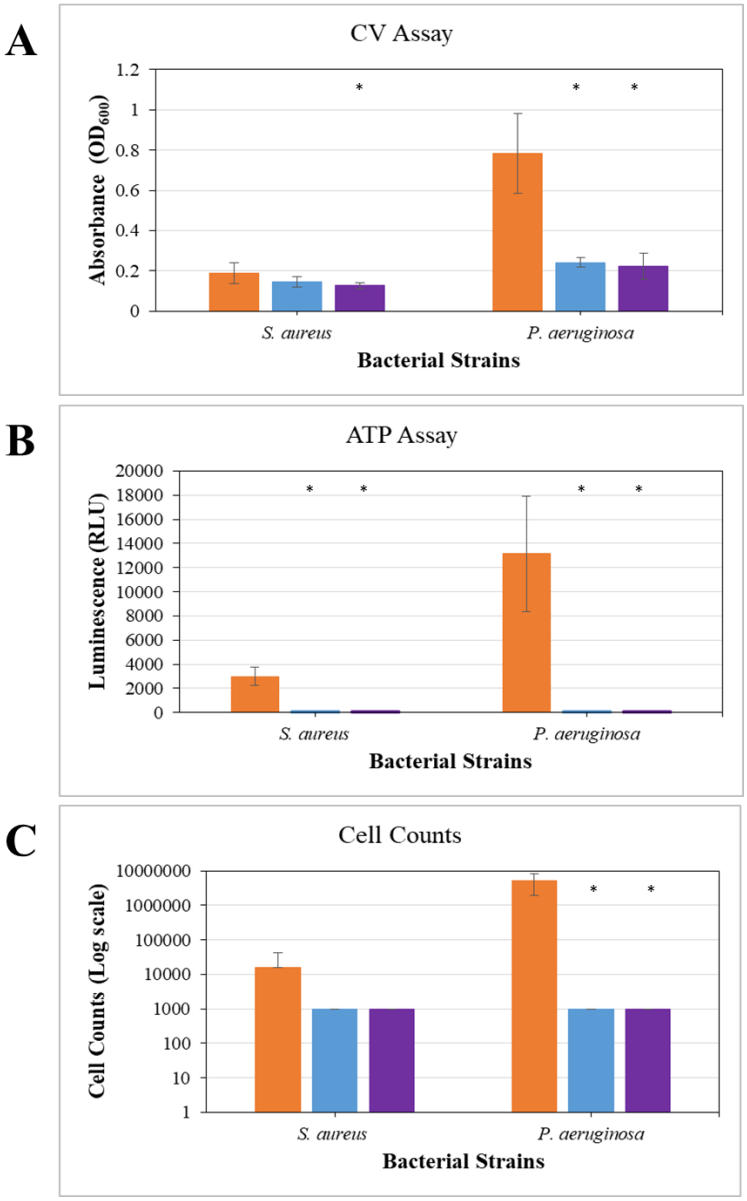

Figure 34. Effectiveness of treatment flushes of 100 mg/ml of PEA and EAA in comparison to 0 mg/ml of EAA and PEA on ATP content, biofilm biomass, and cell counts of biofilms at the end of inoculation in silicone tubing. Biomass (Panel A), ATP content (Panel B) and viable cell counts (Panel C) of biofilm grown in the absence (orange bars) or presence of 100 mg/ml of PEA (blue bars) and 100 mg/ml of EAA (purple bars) in silicone tubing. Averages and standard errors were determined across 6 replicates.

Discussion

A major finding of this study was that PEA was able to reduce ATP content, biofilm biomass, and cell counts of bacterial biofilms that had formed in the inside of small pieces of

silicone tubing, modeled after the antibiotic-lock treatment for intravenous catheters. Our experiment was designed to mimic antibiotic-lock treatments in an intravenous catheter, which are surgically implanted into the heart. The bacteria for this study were selected for their relevance to CLABSI and other medical device infections. In a new report on the pathogen distribution among pediatric healthcare-associated infections to the National Healthcare Safety Network, *S. aureus* ranked first in overall incidences, second in the occurrence of CLABSI, and 12th for catheter related urinary tract infections [225]. *E. coli* ranked first in urinary tract infections, third overall, and 5th in CLABSI. *P. aeruginosa* ranked second for urinary tract infections, and 7th each overall and for CLABSI. The most relevant pathogen for our catheter model is likely *S. aureus* which has been associated with CLABSI for a long time [226, 227] and catheter related urinary tract infections more recently [228]. This pathogen exhibited log reductions of 2.3-logs for the ATP assay and 1.2-logs for the viable cell counts in response to three flushes of 100 mg/ml PEA in our catheter model. Our data for *P. aeruginosa* are even more impressive. The log reductions for this pathogen caused by PEA were 2.9 for the ATP assay and 3.7 for the viable cell counts. For the UPEC strains, reductions in ATP content of the biofilm for both these strains were in the 3-logs range at 10 mg/ml of PEA.

The EAA flush was also found to be effective at reducing the ATP content of biofilms grown in silicone tubing depending on the strain and depending on the concentration of EAA being administered. In general, as we reduced the concentration of EAA, the percent reduction of ATP content and biofilm biomass decreased. However, some strains still had a percent reduction even at 5 mg/ml of EAA. Interestingly we observed *P. aeruginosa* to be the pathogen that was least effected by EAA and had the lowest amount of reduction of ATP content, whereas this bacterial strain was most effected by PEA. EAA was found to reduce growth and biofilm

biomass of both gram positive and gram-negative bacteria. The addition of EAA to a mature biofilm as a treatment could be used as a broad-spectrum treatment against gram positive and gram-negative bacteria. However, EAA did not appear to be as effective at reducing the percent reduction of ATP content and biofilm biomass in comparison to PEA at the same concentrations.

Another important observation from this study is PEA and EAA had a greater effect on ATP content of the bacteria, in comparison to the total biofilm biomass. We observed that increasing concentrations of PEA and EAA (10 and 100 mg/ml) were able to reduce ATP content around 90-99 % depending on the strain. However, biofilm biomass reduction was significantly less than the reduction in ATP content. One explanation for this could be the differences in measurement of the ATP and CV assay. The CV assay stains all negatively charged surface molecules, including live and dead bacteria, as well as the EPS matrix [192]. This assay has been used in many biofilm studies, including some by our own research laboratory [154, 193]. Since the CV assay does not distinguish between live and dead bacteria, we used the ATP bioluminescence assay to quantify the metabolic activity of live cells. This assay relies on the conversion of luciferin and ATP to oxyluciferin and AMP by means of the enzyme luciferase with the concomitant emission of luminescence. One hypothesis is that PEA may be killing all the viable cells but leaving behind the EPS and is stained with the CV assay. This would explain the discrepancies between the ATP and CV assay.

To determine whether the discrepancies with the results of the ATP and CV assay was due to dead cells left behind, we took cell counts of the planktonic bacteria after treatment with PEA and EAA. We observed about a 3-log reduction in *S. aureus* cell counts of planktonic bacteria of the tubing that was treated with 100 mg/ml of PEA in comparison to the tubing that received only media. While we saw about a 6-log difference of *P. aeruginosa* cell counts of the

planktonic bacteria in the tubing that was treated with 100 mg/ml of PEA in comparison to the tubing that received only media. Unfortunately, EAA was less effective at reducing *S. aureus* and *P. aeruginosa* cell counts of the planktonic bacteria when the bacteria were treated with 100 mg/ml of EAA. We observed a reduction in the ATP content, biofilm biomass, and cell counts of *S. aureus* and *P. aeruginosa* biofilms formed on the silicone tubing. Overall, these results support the hypothesis that the differences observed between the ATP and CV assay are due to dead cells and left-over EPS of the biofilm, which are stained by the CV assay. Therefore, using the ATP assay, the CV assay, and cell counts to assess biofilm should be used in the future in order to accurately determine the antimicrobial properties of other nutrients in the future.

Overall, flushes of PEA and EAA at high concentrations were able to effectively reduce ATP content and biofilm biomass of a variety of clinically relevant pathogens. Our studies indicate that PEA or EAA could be used as an antibiotic lock therapy for catheters in the future. This could help reduce the use of antibiotics and decrease the emergence of antibiotic resistant bacteria. PEA and EAA are nutrients which may delay the occurrence of resistance to PEA by other bacterial strains. In addition, when assessing the antimicrobial effects of nutrients, cell counts should also be used to assess the growth and biofilm formation of bacterial pathogens.

Development of a microfluidic system to deliver vancomycin and PEA to a mature *Staphylococcus aureus* biofilm

Introduction

Worldwide each year, millions of people receive essential joint replacement surgery providing pain relief, restoring function, and improving patient quality of life [52]. Over the years there has been a steady increase in TJR, with more than a million people in the United States receiving a joint replacement annually [52]. Almost 10 % of TJRs fail early (< 10 years) with 10-

15 % of early failures attributed to infection. In spite of advances in orthopedic procedures and biomaterials, infection remains a major complication in TJR surgery, with rates ranging from as low as 1-2 % to as high as 4-12 % [53]. Infections can be caused by bacterial biofilms, that can form on the interface of the bone or implant, which are 100-1000 times harder to treat with antibiotics. If all bacterial cells are not completely eradicated, persister cells can remain and develop resistance to the antibiotics [11, 164]. Last-resort antibiotics, such as methicillin and vancomycin, which were once very effective at treating biofilms are becoming insufficient due to overuse. Increasingly antibiotic resistant strains including *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *A. calcoaceticus-baumannii* have been associated with TJR [229].

New drug discovery and formulation development is a tedious and lengthy process, particularly for extended duration and sustained release formulations. This can take decades (~12 years) and billions of dollars (~2.5 bn USD), that must be absorbed by the already over-burdened healthcare system [230]. One approach to solving the problem of antibiotic resistance and avoiding the development of new antibiotics has been to focus on extending the longevity of the current antibiotic arsenal by developing local, controlled release strategies, particularly for hard to penetrate tissues such as bone [231]. Although promising, the development of new delivery formulations is further hindered by the lack of technology available that can monitor the drug release kinetics and quickly assess extended, controlled-delivery formulations; while simultaneously monitoring the effect on bioactivity of a biofilm in real-time activity. Biofilms are a group of bacteria that firmly attach to surfaces and one of the hallmarks of a mature biofilm is the formation of EPS. Many different techniques are used to assess biofilm formation, but most of these assessments occur at the end the incubation period. One option that can be used to measure biofilm in real-time is fluorescence. Green fluorescent proteins conjugated to a

carbohydrate-binding module 3 (GFP-CBM3) has been used to measure EPS production and biofilm amounts of an *E. coli* strain [232].

Our research focuses on the development of a real-time spectroscopy system (Fig. 35), that will allow us to determine antibiotic concentrations while simultaneously monitoring biofilm growth. This will exponentially expedite the drug development process and lower the associated costs. This technology could provide more accurate *in vitro*/*in vivo* translatability through real-time monitoring and automated feedback, saving man-hours and allowing for necessary modifications of the release system. Thus, a multifunctional, modular device is needed. System components include (1) absorbance measurements to detect antibiotic drug concentration from an extended duration release device, (2) fluorescence measurements for real-time biofilm biomass detection, and (3) a pump to automatically draw the released drug and transfer it onto a bacterial biofilm to observe the real-time effect (Fig. 35B). Previously, the absorbance detection device for vancomycin (also used in the current study) was developed (Fig. 35A) [233]. In the current work, a proof of concept for the real-time biofilm monitoring and fluid flow pumping are presented (Fig. 35C). Integration of the two designs (shown in Fig. 35A and 35B) will require both fluorescence and absorbance detection capabilities. A bacterial strain that contains a green fluorescent protein was purchased from BEI Resources (American Type Culture Collection, Manassas, VA). To decouple the fluorescence and UV sensors to reduce interference, the pump component will be used. This real-time system will, for the first time, allow for the determination of drug release kinetics and bioactivity simultaneously. In this proof of concept, we assessed the efficacy of vancomycin and PEA on *S. aureus* biofilm under flow conditions. This was done using a syringe pump to flow vancomycin and PEA onto a mature *S. aureus* biofilm. The effect of drug on biofilm was observed over time via a fluorescent microscope and bacterial viability

was validated using cell counts and an ATP assay. Vancomycin was chosen to be assessed because it is commonly used to treat *S. aureus* infections associated with TJR surgeries. In addition, it builds upon previous work in our lab that has developed a novel vancomycin releasing bone void filler that could be used in the future to help combat osteomyelitis associated with TJR surgeries [234]. PEA is a carbon source bacterium utilize to grow, but has been identified to have antimicrobial properties in high concentration. Previous research identified PEA to be have the greatest inhibitory effect on growth, cell counts, and biofilm amounts of *E. coli* 0157:H7 on treated pieces of beef [204]. PEA could be used in the future as an alternative treatment option to traditional antibiotics to treat biofilm associated infections, such as osteomyelitis, but further investigation is needed.

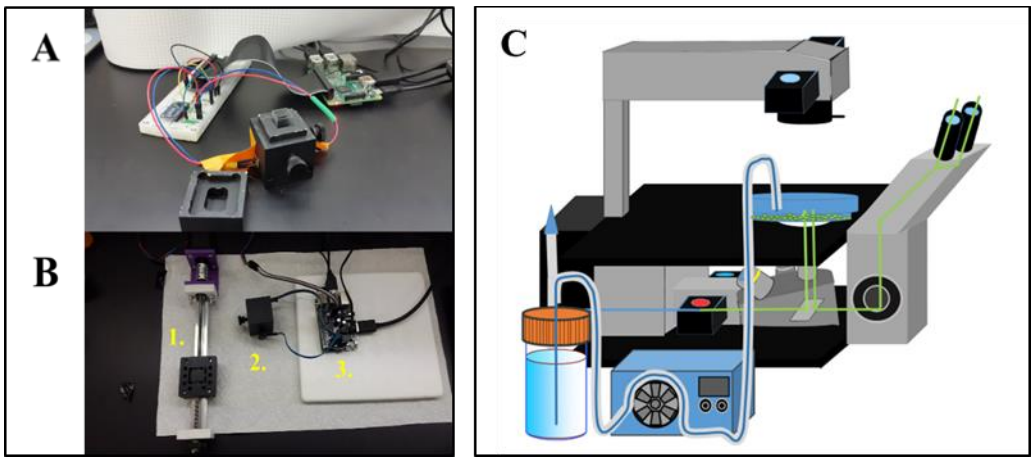

Figure 35. Real time dissolution and bioactivity monitoring device. Panel A shows the previously developed detection device modified to read absorbance and fluorescence simultaneously. Panel B shows the microfluidic device and the flow system that will be used in future experiments. Panel B-1 represents the syringe pump that will flow in media with drug, B-2 is the microfluidic device, and B-3 is the breadboard designed to connect the device to the computer to relay data readings. Panel C shows the current experimental setting of the proof of concept. Antibiotic was pumped onto bacterial biofilm and the effect was observed under fluorescent microscope.

Materials and methods

Bacterial strains

Bacterial strains used in this was *S. aureus* RN4220 with pFP650-R plasmid (BEI Resources, Manassas, VA) that contains a green fluorescent protein integrated into the chromosome. Bacterial strains were maintained as freezer stocks at -80ºC in 8 % DMSO. Prior to each experiment, *S. aureus* were plated onto TSA (15 g/l tryptone, 5 g/l soytone, 5 g/l NaCl, 15 g/l agar) plates. Plates were incubated over night at 37°C.

Formation and assessment of the biofilms in a black 24-well polystyrene plate

Liquid overnight cultures in TSB (17 g/l tryptone, 3 g/l soytone, 2.5 g/l glucose, 5 g/l NaCl, 2.5 g/l K_2HPO_4) were centrifuged at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of 2 x TSB and adjusted until their optical density values at 600 nm (OD_{600}) were 1 ± 0.05. The final inocula were prepared by diluting the 2 x TSB cultures 1:10 in 2 x TSB. Dilutions of vancomycin hydrochloride (0 – 16 mg/ml) were prepared in PBS; vancomycin concentrations ranged from 0 mg/ml to 16 mg/ml. A 1:1 dilution of final inocula and vancomycin solutions were used to inoculate individual wells of a 24-well black polystyrene plate with 1 ml/well. This yields an inoculation OD_{600} of approximately 0.05 in 1 x TSB and a vancomycin concentration range from 0 to 8 mg/ml. The vancomycin solutions were added to the biofilm at the time of the initial inoculation or at 24 h. The plates were then incubated statically at 37ºC for 48 h. At the end of the incubation period, planktonic growth was determined as end point growth by using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), which recorded the OD_{600} at the end of the incubation period. Fluorescence was determined as end point fluorescence using Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), with an excitation of 488 nm and an emission of 525 nm. The CV was used to characterize the total biomass of the biofilm

on the 24-well styrene plates, which were performed as described in Section A. Averages and standard deviations were determined across two replicates.

In a second experiment, the correlation between fluorescence and viable cell counts was assessed to determine if fluorescence could be used to measure increases and decreases in viable cell counts in a biofilm. Bacterial cultures were prepared as described previously above, but the cultures were inoculated in a black 24-well polystyrene plate. At different time points (0 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h) fluorescence was measured using the Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), with an excitation of 488 nm and an emission of 525 nm. The media was then carefully removed from the appropriate wells and rinsed three times with 1 ml of PBS. Bacteria were recovered with nylon flocked swabs (Copan Diagnostics Inc., Murrieta, CA) and immediately transferred into a 15 ml test tube containing 10 ml of PBS. The tubes with the flocked swab were vortexed vigorously for 1 min to thoroughly mix the recovered cells into solution. Serial dilutions were plated in three replicates onto TSA agar plates and reported as CFU/ml. Averages and standard deviations were calculated for fluorescence and viable cell counts across six replicates (3 biological replicates from independent overnight cultures and two technical replicates per plate). Student's *t*-tests were performed to determine the statistical significance of the differences between the flow conditions.

Biofilm formation and assessment in a microfluidic system (Fig. 36)

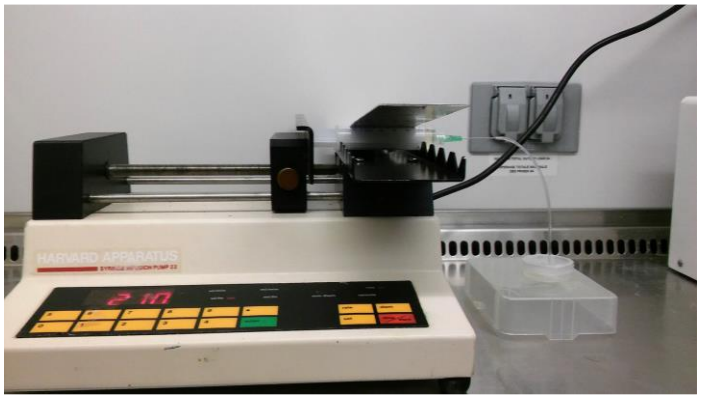

Figure 36. Experimental setup, where liquid media was flowed over a mature *S. aureus* biofilm grown in a small petri dish via a syringe pump.

Prior to the beginning of the experiment, a small hole was drilled in the top of a 35mm mini petri dish (ThermoFisher Scientific, Waltham, MA), which was then sterilized in a biological safety hood with UV radiation for at least 15 min. Bacterial cultures were prepared as described previously, except the inocula was prepared in 1X TSB. This yielded a final inoculation OD_{600} of 0.05. In a biological safety hood, 3 ml of the final inocula was added to a sterile 35mm mini petri dish (ThermoFisher Scientific, Waltham, MA). The hole and the plate were covered with parafilm and allowed to incubate for 24 h at 25°C. After 24 h, the growth media was carefully removed and discarded before fluorescent images were taken of the biofilm with a Leica Microsystems' fluorescence stereo microscope (Leica Microsystems, Buffalo Grove, IL). After imaging, 3 ml of growth media was carefully added to the plate without disrupting the biofilm. A 1.6 mm piece of plastic tubing was inserted into the hole in the lid of the plate and connected to a sterile syringe hooked up to the Syringe Infusion Pump 22 (Harvard Apparatus, Holliston, Massachusetts). The syringe pump then administered 3 ml total of 1X TSB as a control and 3 ml of treatment solution (100 mg/ml of vancomycin or PEA supplemented to

PBS) for 24 h at the rate of 210 µl per hour. After an additional 24 h, the media was removed, discarded, and final fluorescent images of the biofilm were taken with the microscope.

After images were taken, bacteria were recovered from the bottom of the mini petri plates with nylon flocked swabs (Copan Diagnostics Inc., Murrieta, CA), which were immediately transferred into a 15 ml test tube containing 10 ml of PBS. The tubes with the flocked swab were vortexed vigorously for 1 min to thoroughly mix the recovered cells into solution to be used to assess ATP content and viable cell counts of the biofilm. For the ATP assay, the BacTiter Glo™ kit (Promega, Madison, WI) was used. Reagents were prepared per manufacturer instructions. The flocked swab in the conical tube was vortexed again prior to 100 µl being transferred to an opaque white 96-well plate, this was repeated two more times. Then 100 µl of BacTiter-Glo reagent was added to the 100 µl of bacterial cell. We covered the plates with tinfoil and thoroughly mixed solutions by placing the plate on a rotating shaker for 5 min. After this incubation, luminescence was read using the Synergy H1 Hybrid Reader. Averages and standard deviations of luminescence values were determined across 9 replicates.

For the viable cell counts from the biofilms, 100 µl were obtained from the 15 ml conical tube with the flocked swabs. The flocked swab in the conical tube was vortexed again, serial diluted in 1:10 steps, and plated onto TSA plates. Plates were incubated for 24 h at 37^{o}C. Viable cell counts were expressed as CFU per ml. It is important to note, that the lower limit of detection for this assay was 999 CFU/ml. Averages and standard deviations were determined across 9 replicates. Student's *t*-test was run to determine the statistical significance of the difference between the mean of the control plates that received no flow in comparison to the mean of the plates that received treatment but no flow. Student's *t*-tests were performed to determine the statistical significance of the difference between the mean of the control plates that

received flow in comparison to the mean of the plates that received treatment under flow conditions.

Results

Vancomycin reduces planktonic growth and biofilm biomass of S. aureus when added at the time of inoculation (0 h), but not when it is added to a mature S. aureus biofilm (24 h)

To determine our experimental parameters, we investigated the effect of varying concentrations of vancomycin on the early stages of biofilm formation and on a mature biofilm. Concentrations of vancomycin (ranging from 0 – 16 mg/ml) were added to *S. aureus* at the time of inoculation (0h) and after the bacteria had formed a biofilm (24h). End point planktonic growth, total biofilm biomass, and fluorescence were measured after 48 h (Fig. 37). In a first experiment, *S. aureus* was grown on 24-well polystyrene plates in TSB supplemented with a range of concentrations of vancomycin (0-8 mg/ml), that were supplied to the liquid growth medium at the beginning of biofilm development (0 h) or a mature biofilm (24 h). Fig. 37A shows the planktonic growth measured after 48 h of incubation at 37°C. When vancomycin was added at the time of inoculation, it greatly reduced growth at 0.25 mg/ml of vancomycin. Whereas, when vancomycin was added to a mature biofilm at 24 h, we observed only about a 50 % reduction in planktonic growth at 8 mg/ml. Fig. 37B shows the absorbance data from the CV assay that was performed on the biofilm. When vancomycin was added at the time of inoculation the biofilm biomass was almost completely gone at 0.25 mg/ml. Interestingly, when vancomycin was added to a mature biofilm (24 h), we observed an increase in biofilm biomass as the concentration of vancomycin increased. Fig. 37C shows the fluorescence data that was performed to further characterize the biofilm. Very little fluorescence was observed at all the concentrations of vancomycin when it was added at the time of inoculation. When vancomycin

was added to a mature biofilm we see an increase in fluorescence between 0 and 4 mg/ml, with a slight decline in fluorescence seen at 8 mg/ml of vancomycin.

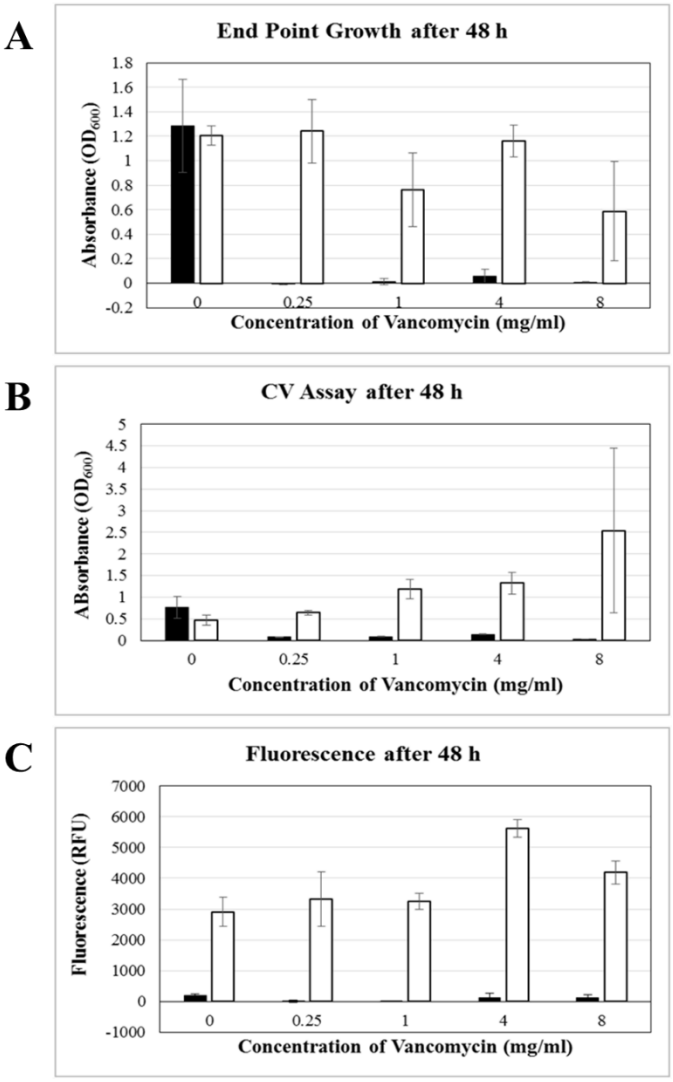

Figure 37. Vancomycin's effect on growth, biofilm amounts, and fluorescence when it was added at the time point 0 h and 24 h. Panel A shows the end point growth in OD_{600} of *S. aureus* in the presence of varying concentrations of vancomycin at 0 h (black bars) and at 24 h (white bars). Panel B shows the absorbance data from the CV assay that was done on the biofilm in OD_{600}. Panel C shows the fluorescence in relative fluorescence units (RFU). Averages and standard deviations are calculated.

Fluorescence of S. aureus correlates with increases and decreases in bacterial cells

In a second experiment, we measured planktonic growth and fluorescence at different time points to compare them to the corresponding cell counts (Fig. 38). Fig. 38A shows the

planktonic growth (orange line) and cell counts (black line) measured at 2, 4, 6, 8, 12, and 24h of incubation at 37°C. We observed a steady increase in planktonic growth between 0 and 12 h, but then a slight decline between 12 – 24 h. We measured a small increase in cell counts between 0 - 4 mg/ml, a slight decline in cell counts between 4 – 8 mg/ml, and then a steep increase in cell counts from 8 – 24 mg/ml. Fig. 38B shows the fluorescence (blue line) and cell counts (black line) measured at 2, 4, 6, 8, 12, and 24h of incubation at 37°C. We observed a small decrease in fluorescence between 0 – 2 mg/ml, an increase between 2 – 6 mg/ml, a decrease between 6 – 12 mg/ml and finally a sharp increase in fluorescence between 12 – 25 mg/ml. The same cell counts data is shown in both Fig. 38A and B. Overall, our data shows very little correlation between planktonic growth and cell counts, which even appears to be inversely correlated. Whereas, fluorescence and cell counts had similar shaped curves, which appear to have increases and decreases occurring at the same time. This is an indication that there is a moderate correlation between fluorescence and cell counts.

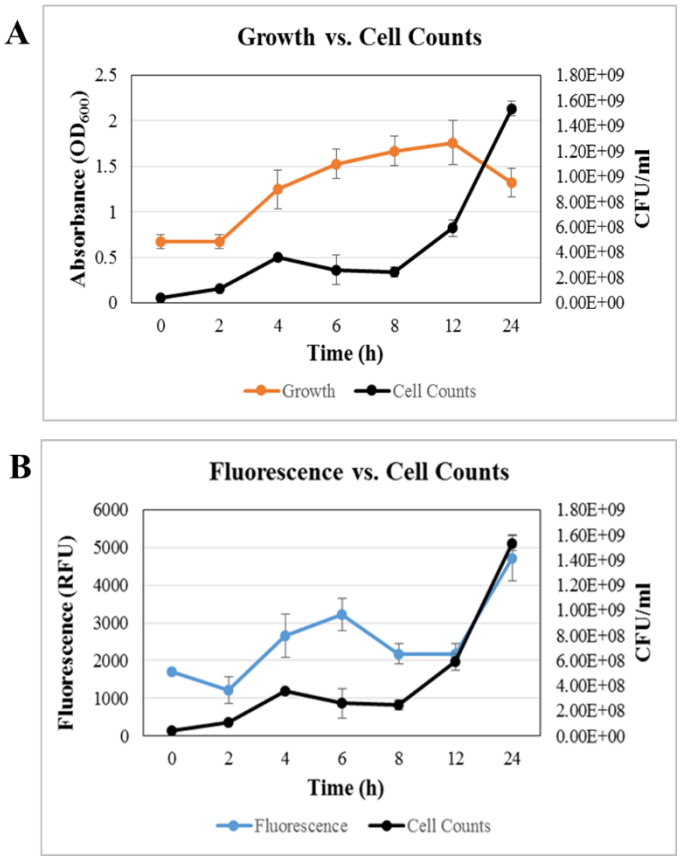

Figure 38. Correlation between fluorescence and growth or cell counts of AJW678 over 24 h. Panel A shows the growth (orange line) of *S. aureus* versus the associated cell counts (black line) at different time points over 24 h. Panel B shows the fluorescence (blue line) of *S. aureus* versus the associated cell counts (black line) at different time points over 24 h. Averages and standard deviations were calculated over 3 replicates.

Effect of vancomycin and PEA on S. aureus in a microfluidic system

To determine the effect of vancomycin and PEA on *S. aureus* biofilm, we flowed media or our treatment solutions (100 mg/ml of vancomycin or PEA) over a mature *S. aureus* biofilm grown in a 35mm mini petri dish at a rate of 210 µl per hour. The biofilms were subjected to different flow conditions: no flow (NF), no flow and treated with vancomycin (VO), media flowed over the biofilm (MO), vancomycin flowed over the biofilm (VF), PEA flowed over the biofilm (PF), and vancomycin/PEA flowed over the biofilm (V+PF). Fig 39A. shows the luminescence data from the biofilm after 24 h of incubation at 25°C. In comparison to the control

plate that received no flow, we observe an increase in ATP energy content of the biofilm that was treated with vancomycin and no flow. We observed about a 50 % reduction in ATP content of the biofilm that was treated with media flow only in comparison to the control plate that was that received no treatment or flow. The reduction in ATP content of the biofilm that was treated with media flow only was found to be statistically significantly different in comparison to the control plate that was that received no treatment or flow with a p-value of 4.5×10^{-6}. The biofilm that was treated with vancomycin and flow had about the same amount of ATP content as the control plate that received no treatment or flow. There was about a 99% reduction in the ATP content of the biofilm that was treated with PEA and flow in comparison to the plates that were treated with media flow only and the control plate that received no treatment or flow, which was found to be statistically significant with a p-value of 8.9×10^{-6} and 3.5×10^{-8} respectively. The combination treatment of PEA and vancomycin under flow caused a substantial increase in ATP energy content in comparison to the control plate. Fig. 38B shows the cell counts measured after 24 h of incubation at 37°C. All of the treatment including the control plate showed similar cell counts around 10^8 CFU/ml, except the biofilms treated with PEA and flow. We observed about a 4-log reduction in ATP content of the biofilms treated with PEA and flow in comparison to to the plates that were treated with media flow only and the control plate that received no treatment or flow, which was found to be statistically significant with a p-value of 5.7×10^{-10} and 0.03 respectively.

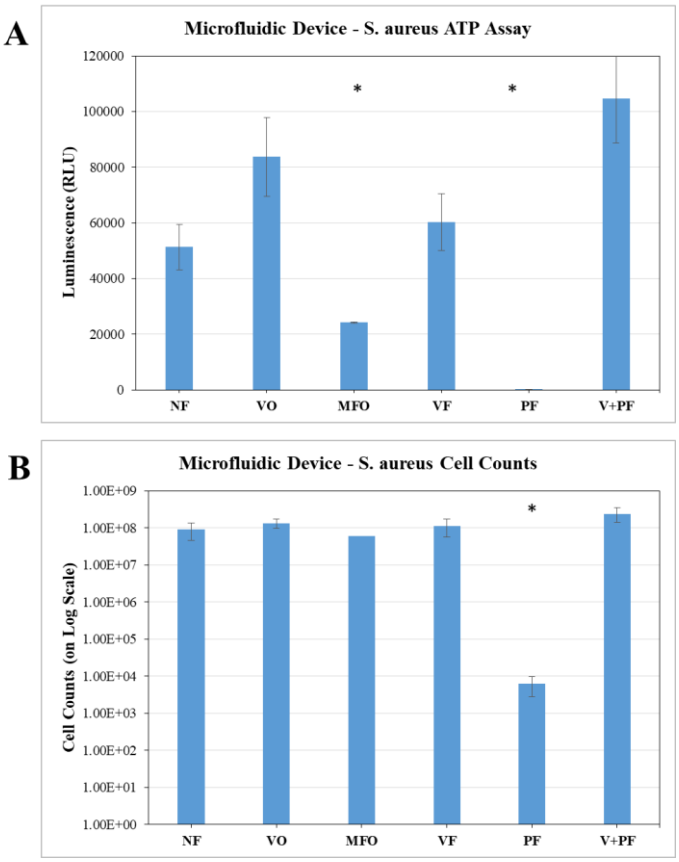

Figure 39. Effectiveness of PEA and flow on the ATP content of the biofilm and cell counts of *S. aureus.* Panel A shows the luminescence of *S. aureus* biofilms under different flow conditions and treatments, while Panel B shows the associated cell counts after 24 h. Averages and standard deviations were calculated over 3 replicates. Asterisks indicate a statistically significant difference between the mean energy content (ATP) of mean viable cell counts of the biofilm at the respective treatment in comparison to the mean of the NF treatment and the MFO treatment.

Discussion

In this preliminary study, we first determined the experimental parameters we would use for future experiments in our microfluidic device system. We investigated the effect vancomycin had on growth, biofilm amounts, and fluorescence of *S. aureus* grown in nutrient media. We observed that when vancomycin (concentrations ranging from 0.25 - 8 mg/ml) was added at the time of inoculation we saw complete wipe out of growth, biofilm amounts, and fluorescence. Whereas, when the vancomycin (concentrations ranging from 0-8 mg/ml) was added 24 h after

inoculation we see great variability in the growth, biofilm amounts, and fluorescence. We see a general decrease in absorbance as the concentration of vancomycin increases. However, the amount of biofilm and fluorescence varied based on the concentration of vancomycin.

One explanation could be that the vancomycin wasn't at a high enough concentration to clear the biofilm biomass completely from the surface (Fig. 38B). This could be attributed to biofilms being 100-1000 times harder to treat with antibiotics, chemical disinfectants, and physical cleaning. As a biofilm matures, the bacteria form a slime layer (or EPS) around themselves, which creates a physical barrier between the bacteria and its environment but can allow the bacteria to sequester itself from antibiotics and chemical treatments. If all bacterial cells are not completely eradicated, persister cells can remain and develop resistance to the antibiotics or antimicrobials used as treatment [11, 164]. Biofilms being much more resistant to antibiotics could be another possible reason for variability in both the CV assay and fluorescence assays. Many strains of *S. aureus* have become resistant to vancomycin due to the overuse of antibiotics [55].

Another possible explanation could be the type of assays we are using to characterize the biofilm (Fig. 38). The CV assay stains live and dead biomass, as well as EPS and the ATP assay measures the energy content of live bacteria [186, 187]. This could mean that because we added vancomycin to an already mature biofilm, the biofilm that is being stained at the end of the experiment is composed of dead cells and EPS. This would mean that the even though the absorbance values of the CV assay are high they aren't very indicative of what is actually going on within the biofilm. This seems to be supported by the data from the microfluidic system. We observed that when a 100 mg/ml of PEA was flowed over a mature *S. aureus* biofilm there was

about a 99 % reduction in both the ATP content of the biofilm and the viable cell counts in comparison to when only media was flowed over the biofilm.

Our microfluidic system was designed to assess drug concentration with absorbance, while simultaneously assessing biofilm formation using fluorescence. In order to confirm that our green fluorescent protein, integrated into the *S. aureus* chromosome could be used as a way to measure biofilm amounts, we compared the absorbance data (growth) and fluorescence data versus the cell counts taken at varying intervals over 24h. We observed that as growth increases the cell counts of *S. aureus* also increases, however this doesn't occur proportionally. However, as the fluorescence increases we see the cell counts of *S. aureus* also increases proportionality. This is shown by the similar shapes of the two lines in the graph. Previously, green fluorescent proteins have been shown to increase and decrease with viable cells of *Aureobasidium pullulans* when treated with biocides. However, environmental pH has been shown to impact the loss of green fluorescent protein and viability, maximum losses of fluorescence and viability were observed to occur at a pH 4 in this study [235].

In Fig. 38 we observed that the growth and cell counts of *S. aureus* is much less correlated than the relationship between fluorescence and cell counts. In general, as growth increases, so do the cell counts. However, scientists have yet to agree upon how we should calculate the correlation between growth and cell counts. Growth is measured in absorbance (OD_{600}) and doesn't distinguish between the live and dead cells. Thus, as cells start to proliferate the absorbance will go up. When we add vancomycin to kill the cells, those dead cells can impact the absorbance values recorded making them higher values that can mislead someone to assume the cells are still proliferating. To account for this some scientists use calibration curves, such as the direct linear relationship model based on Lambert–Beer law [236], while others use a

logarithmic transformation to get a linear relationship between the log OD and log CFU/ml by normalizing the variance [237]. Therefore, cell counts were performed as verification of how many cells are present that are actually still alive. We found that cell counts, and fluorescence increased and decreased nearly proportionately. This indicates that as fluorescence increases and decreases, so does the number of cells; allowing us to use fluorescence as a way to measure increasing biofilm amount. Then when we flow vancomycin over a mature biofilm, we will be able to see a decrease in fluorescence that correlates to a decrease in biofilm amounts.

Since biofilms are capable of growth under both flow and stagnate fluid conditions, it is important to evaluate antibiotic administration and biofilm robustness under flow *in vitro*. In Fig. 39, we see the luminescence data from our simulated microfluidic flow system and that there was a decrease in ATP content when the media only (MFO) was flowed over the biofilm in comparison to the ATP content when there was no flow at all. This could be attributed to the shear force exerted on the biofilm when the liquid is flowed over the biofilm. Previous research with algae, observed that increased jet pressure was correlated to increase biofilm removal when a custom, automated water jetting system was used. However, duration of the jetting was not found to be correlated with increased biofilm removal [238]. No significant difference in the viable cells of the MFO and NF was observed.

Interestingly, the ATP content of the plates that had been treated with vancomycin whether with or without flow showed increased ATP content in comparison to the control plates (MFO and NF). One explanation for this is vancomycin has a high binding affinity to protein, with one study finding that on average about 41 % of vancomycin was found to be bound to protein when tested with 50 patients. However, estimates of vancomycin's binding affinity to protein have been as high as 50 %. Unfortunately, this explains why there wasn't any decrease in

ATP content or cell counts of any of the biofilms treated with vancomycin. The media we used is a nutrient rich media, which is high in protein. Vancomycin wasn't able to be active in killing the bacterial cells because it was bound to the protein in the media. In addition, the treatment with both vancomycin and PEA has a similar explanation where vancomycin could have been bound to not only the protein in the media, but PEA itself making both antimicrobials inactive. This would explain why the biofilm that had been treated with vancomycin and PEA had the highest ATP content of all the treatment.

The only treatment that reduced both ATP content and the viable cell counts of *S. aureus* was PEA. The ATP content of the *S. aureus* biofilm treated with PEA was almost completely abolished. In addition, all of the plates that had received flow or no flow with treatment or no treatment after incubation had viable cells at 10^8 CFU/ml, except the biofilm that was treated with PEA and flow. We observed about a 6-log reduction in viable cells in comparison to the controls (MFO and NF), the biofilms treated with vancomycin, and the biofilms treated with vancomycin+PEA. From previous experiments in the lab, we expected to see a reduction in biofilm amounts of *S. aureus* regardless of when it was added. This confirms that flow doesn't impact the antimicrobial ability of PEA to reduce ATP content and viable cells of bacterial pathogens.

CHAPTER 5. DISCUSSION AND FUTURE OUTLOOK

Our results confirm that the small molecules PEA and EAA reduce the growth, ATP content, and biofilm biomass of a variety of bacterial pathogens. We then demonstrated that these small molecules could be used to reduce bacterial biofilms in three different applications: 1) as an antimicrobial lock flush in a catheter, 2) as an antimicrobial material, and 3) in a microfluidic system. These findings support the idea that small molecules which bacteria utilize to grow could be used as an alternative treatment to traditional antibiotics for bacterial biofilms, which are becoming ineffective due to overuse and the increasing emergence of antibiotic resistant bacteria. This work could help develop new treatments with PEA or EAA of biofilm associated infections in the medical and food processing industry.

One of the major findings of this study was we determined PEA could be used as both a prevention and treatment method of bacterial biofilms of a non-pathogenic *E. coli*, when supplemented to liquid growth media. PEA was observed to reduce ATP content and biofilm biomass of AJW678 between 10 mg/ml and 100 mg/ml of PEA, regardless of when it was added. This builds upon previous research in our lab that identified PEA to be have the greatest inhibitory effect on growth, cell counts, and biofilm amounts of *E. coli* 0157:H7 on treated pieces of beef [204]. Other researchers have had success identifying small molecules with antimicrobial effects. One study found that citrus limonoids were able to reduce biofilm formation of *E. coli* through cell surface organelle genes shown to play a role in biofilm formation, including type 1 pili, flagella, and EPS [239-242]. Current research is focused on using small molecules like these to reduce biofilm formation and reduce the emergence of antibiotic resistance by modulating signal transduction pathways to regulate bacterial phenotypes [87]. Signal transduction pathways are an integral part in the formation and development of bacterial

biofilm, so we hypothesize that PEA may be reducing biofilm formation through some aspect of signal transduction.

PEA could be used as treatment method of a broad spectrum of bacterial pathogens, including gram positive and gram-negative bacteria. We observed that PEA was able to reduce planktonic growth and ATP content of four gram-negative (*E. coli* K-12, *P. aeruginosa,* and two UPEC strains of *E. coli*) and one gram-positive bacterial strain (*S. aureus*) under a variety of experimental conditions. However, the extent of the reduction in growth and ATP content was dependent upon the bacterial strain and the conditions under which these biofilms had been cultured. Higher concentrations of PEA were required to get a complete reduction in ATP content of *S. aureus* and *P. aeruginosa*. This could be due to the fact that clinical strains of *S. aureus* and *P. aeruginosa* have been found to have more resistance to antibiotics and antimicrobials in recent years [55, 197]. Currently, the approach to combat antibiotic resistance include the development of new antibiotics or extending the longevity of currently used antibiotics by developing localized, controlled drug release strategies [243]. However, these strategies come with their own challenges [244]. Our hypothesis is that using nutrients like PEA could be a better option to treat bacterial biofilms than antibiotics because bacteria utilize these nutrients to grow and it may take longer for resistance to develop.

Another major finding of this study was that another small molecule, EAA, was able to reduce the ATP content and biofilm biomass of some of the bacterial pathogens tested. EAA was found to inhibit biofilm formation of four gram-negative bacterial strains (*E. coli* K-12, *P. aeruginosa, C. sakazakii,* and *S. marcescens*) and one gram-positive bacterial strain (*L. monocytogenes*). In comparison to PEA, EAA appeared to be more effective at reducing ATP content and biofilm biomass of some of the bacterial pathogens (*Y. enterocolitica, C. sakazakii,*

and *S. marcescens*) tested. However, the efficacy of EAA on biofilm formation is strain dependent and was unable to reduce biofilm formation of all of the bacterial pathogens tested. The similar chemical structure of acetic acid and EAA may be attributed to the antimicrobial effects seen on some pathogens. Acetic acid has been used as an antiseptic in medicine for over 6000 years and was found in a recent study to have bactericidal effects, particularly for *P. vulgaris, A. baumannii* or *P. aeruginosa*. However, like our study, it found that acetic acid was better in comparison to traditional antiseptics for some bacterial strains, but not for all [205]. Interestingly, it has also been shown that derivatives of 2-aryl EAAs have antifungal, antibacterial, and antimalarial effects [206-208]. The other nutrients investigated showed very little effect on bacteria biofilms and there was no increased antimicrobial effect when EAA, GP, or lyxose were used in combination with PEA when supplemented to the liquid media. In the future, investigating other small molecules may be beneficial to developing new treatment for bacterial biofilms.

PEA was found to prevent and treat bacterial biofilms in 3 different applications: 1) as an antimicrobial material physically integrated with PEA, 2) as a liquid flush in a catheter, and 3) under flow conditions in a microfluidic system. We were able to physically blend PEA into polyurethane using a hot-melt extrusion process and were able to get at least a 20 % reduction in ATP content of all the bacterial pathogens tested on our 5 % PEA-PU material in comparison to our 0 % PEA-PU material. There was even up to an 80 % reduction of *S. aureus* biofilms. Liquid flushes of PEA and EAA were able to reduce ATP content, biofilm biomass, and cell counts of bacterial biofilms that had formed in the inside of small pieces of silicone tubing, modeled after the antibiotic-lock treatment for intravenous catheters. We observed that 3 treatment flushes of 10 and 100 mg/ml of PEA and EAA over 2 weeks resulted in almost 100 % reduction in ATP

content of most of the bacterial pathogens tested. In a microfluidic system, we observed that a treatment of 100 mg/ml of PEA completely abolished the ATP content of *S. aureus* biofilms and reduced the cell counts about 4-logs. We also determined that fluorescence could be used in the future as a way to assess biofilm formation in a microfluidic device designed to simultaneously assess drug concentrations. This data supports our previous observations that high concentrations of PEA and EAA reduce ATP content and total biofilm biomass of multiple bacterial pathogens, but at lower concentrations PEA and EAA effectiveness is strain dependent. PEA or EAA could be used in multiple applications to reduce bacterial biofilm of pathogens in the medical and food processing industry.

Throughout the manuscript, we observed that it took higher concentrations to reduce the biofilm biomass in comparison to the ATP content of the biofilm of multiple bacterial strains under different environmental conditions when treated with PEA. One explanation for this difference between biofilm biomass and biofilm ATP content could be that there are no energetically active cells (measured by the ATP assay) within the biofilm at lower concentrations of PEA, but the EPS of the biofilm is still left behind and gets stained with the CV assay [191]. The CV assay will stain any negatively charged surface molecules, including live bacteria, dead bacteria, and the EPS [192]. Whereas, the ATP bioluminescence assay is used to quantify the metabolic activity or energy content of live bacterial cells. Luminescence occurs through the enzymatic reaction where luciferin and ATP is converted to oxyluciferin and AMP when the luciferase enzyme cleaves off ADP causing an emission of light. The ATP content of bacteria does not vary with the growth rate, which is essential for using ATP bioluminescence to assess cell viability [194]. Previous research in our lab using the BacTiter Glo™ kit from Promega, demonstrated a linear correlation between the luminescence signal and the number of

viable bacteria in planktonic culture [195]. However, the correlation between the ATP signal and viable bacteria is less clear in biofilm bound bacteria than in planktonic bacteria. We have observed a decrease in viable bacteria as the ATP signal decreases with increasing concentrations of PEA, which has been seen in multiple experimental settings (for data see Chapter 3). This supports the hypothesis that there is a correlation between ATP signal and viable bacteria.

Overall, PEA and EAA were found to be the best options for treatments of bacterial biofilms, but further research needs to be done. In the future, PEA or EAA could be used to develop custom materials as coatings for catheters or food processing equipment, as an antimicrobial lock solution for catheters, or as a liquid treatment to clean surfaces, tubing, or even as a spray for foods. Treatments like these could be used in conjunction with current prevention methods to reduce bacterial biofilm associated infections and outbreaks. Advantages to using nutrients PEA or EAA is they have GRAS status from the FDA and are considered safe substances. Other researchers have used biofilm preventing technologies that were based on antibiotics or silver coatings [212, 245]. However, these strategies encourage microbial resistance and have high production costs. Other small molecules could also be investigated from our previous study to be used in conjunction with PEA or EAA to enhance their inhibitory effect on bacterial biofilms or could be used independently as another treatment alternative [204]. This work is significant because we desperately need alternative treatment options for bacterial biofilms in the medical and food processing industry to try to combat antibiotic resistance and reduce bacterial biofilm associated infections.

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