WPI

Design of a Topical Antimicrobial Peptide Delivery Vehicle for the Treatment of Acne Vulgaris

A Major Qualifying Project

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Degree of Bachelor of Science

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Authorship

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Abstract

Acne is the eighth most prevalent disease worldwide. Current treatments include over-the-counter and prescription options, both of which are inadequate due to adverse effects such as redness and inflammation of the skin, and growing antibiotic resistance, respectively. Antimicrobial peptides (AMPs) have been investigated as an alternative, but have suffered due to their high cost and cytotoxic risk. This project aimed to develop a topical antimicrobial peptide delivery vehicle to deliver modified antimicrobial peptide *f*CBD-LL37 to the sebaceous glands in the skin for the safe and effective treatment of acne vulgaris. The final delivery vehicle must not inhibit the antimicrobial activity of the peptide, must allow for peptide activity within a safe therapeutic window, must facilitate penetration of *f*CBD-LL37 to the sebaceous glands, and must be non-cytotoxic to human skin cells. The team utilized Minimum Inhibitory Concentration (MIC) assays to assess the antimicrobial activity of *f*CBD-LL37 with and without the delivery vehicle candidate against *Propionibacterium acnes* (*P. acnes*). In addition, the team used histological, and immunohistochemical (IHC) staining techniques to ensure that the *f*CBD-LL37 peptide in the delivery vehicle is able to effectively diffuse into the sebaceous glands of a porcine ear skin model. Through experimental validation, the team was able to demonstrate that *f*CBD-LL37 in the final delivery vehicle successfully killed *P. acnes* bacteria within a safe therapeutic window and was able to penetrate into the sebaceous glands for the safe and effective treatment of acne vulgaris.

1. Introduction

Acne is the eighth most prevalent disease worldwide, with an estimated 9.4% of the global population affected [1]. In the United States alone, the American Academy of Dermatology reports that 85% of people between the ages of 12 and 24 experience at least minor acne [2].

Acne vulgaris is a skin disease of the pilosebaceous unit. The sebaceous glands produce and excrete sebum, the oil used to lubricate and waterproof the skin [3]. Acne occurs when dead skin cells clog the hair follicles and prevent sebum drainage [4]. This problem is exacerbated by androgen overproduction, which occurs during adolescence. Androgens are hormones that stimulate sebum excretion [3]. This provides an ideal microenvironment for *Propionibacterium acnes* (*P. acnes*) colonization. During periods of sebum overproduction, their growth catalyzes an inflammatory response from the body, which causes pimples to form [3].

Recurrent acne has been shown to be a significant cause of depression and anxiety, especially among teenagers and young adults [5]. Additionally, even mild acne cases have the potential to cause resultant facial scarring, further exacerbating the self-esteem issues that result from acne [2]. Due to these two common problems, acne can cause potentially severe, long-term morbidity despite being a non-life-threatening condition on its own [5]. The prevalence paired with comorbidities of acne vulgaris necessitates better treatment options [5].

Acne treatment is currently dominated by three companies that are collectively responsible for 64% of the Over-The-Counter (OTC) acne treatment market [6]. These companies manufacture four acne treatment product lines: Proactiv, Clearasil, Neutrogena, and

Clean & Clear. The aforementioned over-the-counter acne treatments use either benzoyl peroxide or salicylic acid as their active ingredient, which primarily function by removing dead skin cells and unclogging pores [7-11]. Additionally, benzoyl peroxide has bactericidal properties. These chemicals are harsh on the skin when used too frequently, creating redness and inflammation [12]. Prescription acne treatments commonly utilize antibiotics as their active ingredients. However, *P. acnes* are rapidly developing resistance to the antibiotics that are typically used in these products. A 2015 study conducted in the UK found that 80% of *P. acnes* infections in acne patients were resistant to erythromycin, clindamycin, or both [13]. This rise in antibiotic resistance is causing both practitioners and the United States Food and Drug Administration (FDA) to be reluctant to endorse antibiotic use for non-life-threatening conditions. Therefore, a novel acne treatment that effectively targets *P. acnes* is needed before these bacteria become completely resistant to current antibiotic treatments.

A promising alternative to traditional antibiotics and OTC treatments is antimicrobial peptides (AMPs). AMPs serve as the first line of defense in the innate immune system, and thus play an essential role is protecting the body and skin from invading pathogens [14]. They are generally cationic and amphipathic, meaning they have both hydrophobic and hydrophilic regions [14]. This structure allows AMPs to effectively attack and destroy bacterial membranes, ultimately leading to bacterial cell death. This mechanism allows for broad-spectrum antimicrobial activity, meaning they are able to target both Gram-positive and Gram-negative bacteria [15]. Additionally, due to the nonspecific nature of their attack mechanism, AMPs are less likely to induce bacterial resistance.

While AMPs offer a promising, effective alternative to traditional antibiotics, limitations that exist for nearly all AMPs are the expensive cost of production, and the threat of cytotoxicity to mammalian cells, due to the nonspecific mechanism [14]. Additionally, a significant challenge is delivery of a large protein such as an AMP across the skin barrier. For this project, the AMP of interest is LL37, the only human-derived AMP of the cathelicidin class.

Cathelicidins are typically produced by neutrophils, macrophages, and keratinocytes, and demonstrate potent antimicrobial activity and healing properties [15]. LL37 is inactive when synthesized intracellularly until it is cleaved extracellularly by serine protease proteinase-3 and upregulated by keratinocytes during infection or injury to the skin [14]. When activated, this peptide exhibits antimicrobial activity and is transported by white blood cells to the wound site [15]. LL37 promotes proliferation of endothelial cells while also assisting in the modulation of inflammatory responses [15]. In recent years, LL37 has been investigated in clinical trials for therapeutic use, however its success has been limited due to its cytotoxic effects against mammalian cells. To combat the problems of cytotoxicity, a chimeric, modified version of LL37 that contains a fibronectin collagen binding domain (*f*CBD) has been developed at Worcester Polytechnic Institute, which has exhibited reduced cytotoxicity *in vitro* [15]. For this reason, this peptide, known as *f*CBD-LL37, became the focus of this project.

The goal of this project was to design a topical antimicrobial delivery vehicle to deliver *f*CBD-LL37 into the sebaceous glands for the safe and effective treatment of acne vulgaris. Four primary design objectives were identified to design and assess this topical biologic.

1. The delivery vehicle must not inhibit the antimicrobial efficacy of *f*CBD-LL37 against *P. acnes.*
2. The delivery vehicle must allow for peptide activity within a safe therapeutic window.
3. The delivery vehicle must facilitate penetration of the peptide to the sebaceous glands.
4. The delivery vehicle must be non-cytotoxic to human skin cells.

Because *f*CBD-LL37 had not been tested against *P. acnes* before, the team had to first ensure that the modified peptide was effective against the pathogen. To do this, the minimum inhibitory concentration (MIC) of the peptide was determined using an MIC assay. The MIC is defined as the lowest concentration of peptide where 100% visible growth of the bacteria in culture is inhibited. The results of this initial MIC assay confirmed that *f*CBD-LL37 would be effective against *P. acnes* on its own.

Natural, antimicrobial, essential oils were then identified as potential delivery vehicle components. Various essential oils including tea tree oil, manuka oil, eucalyptus oil, orange citrus oil, sunflower oil, and lemongrass oil are commonly used in skincare products. Each of the aforementioned oils have beneficial properties for the skin, and also exhibit their own antimicrobial properties [5,16]. Because of their innate antimicrobial properties, the team believed that their inclusion would ensure successful accomplishment of objective one. In order verify that they would not inhibit peptide activity, their MICs were also tested against *P. acnes*, and then the two best-performing oils were chosen to be combined with *f*CBD-LL37. The team

believed that a combination of peptide plus essential oil would enhance peptide activity so that it would be active well below its cytotoxic concentration, accomplishing the second objective.

Once the team was able to determine a delivery vehicle for *f*CBD-LL37, the challenge of delivering a large molecule protein into the pilosebaceous unit was addressed, posing the question: Will our delivery vehicle facilitate delivery of the peptide into the hair follicles and sebaceous glands?

A skin diffusion experiment was conducted to answer this question, requiring an accurate and cost-effective *ex vivo* skin model. Through extensive research of the literature, porcine ear skin was identified as the most suitable model, as it is most comparable to human forehead skin in both hair follicle density and stratum corneum thickness [17]. Oil-peptide formulations of the delivery vehicle were created and applied to harvested porcine ear skin samples. The skin samples were then subject to standard histological procedures (Eosin staining) and anti-LL37 immunohistochemistry to visualize peptide diffusion across the stratum corneum and into the hair follicles.

In order to verify non-cytotoxic effects of the delivery vehicle, peer-reviewed literature was consulted in the interest of time and budget constraints. The *f*CBD-LL37 peptide, as discussed previously, does not have the same risk of cytotoxicity, as it has been shown to be non-cytotoxic at concentrations up to 24 times greater than the cytotoxic threshold for the unmodified LL37 [15]. Additionally, the essential oils that were chosen for the final delivery vehicle formulation, eucalyptus oil and manuka oil, have been found to be non-cytotoxic and non-irritating at concentrations up to 10% v/v, well below the concentrations that are present in the delivery vehicle [18,19]. This evidence, paired with a suggested future cell-based

cytotoxicity assay, indicates that the delivery vehicle along with *f*CBD-LL37 will not be cytotoxic to human skin cells.

Effective delivery of an AMP in a topical acne treatment using low concentrations of peptide and essential oils, paired with the mitigation of cytotoxic effects that have plagued AMP products, has significant implications for the future of AMP-based therapeutics and acne treatment. Developing this delivery vehicle into a viable commercial therapeutic would both revolutionize the innovation-starved acne treatment market and offer a solution to the antibiotic resistance crisis threatening current prescription treatments.

2. Literature Review

2.1 Acne as a Disease

2.1.1 What is Acne?

Acne vulgaris is an inflammatory disease that occurs in the pilosebaceous unit, which includes hair follicles and associated sebaceous glands. This skin disease most often affects the face, upper chest, and upper back, as these areas have the highest number of sebaceous glands and hair follicles [20]. Acne is characterized by the presence of comedones, papules, pustules, and cysts, or nodules.

Comedones are non-inflammatory acne such as blackheads and whiteheads that are either open or closed [20]. Papules are small, inflammatory raised elevations on the skin. Pustules are similar to papules, but have a central pocket of pus [20]. Finally, cysts and nodules are large, painful, and swollen pimples that are typically more than five millimeters in size [20]. When nodules occur in acne, the wall of the hair follicle unit ruptures, which results in intense inflammation and pain, with a risk of later scarring [20].

2.1.1.1 Mild to Moderate Acne Vulgaris

The characterization of acne depends on the amount of acne as well as the type of raised elevations present. The American Academy of Dermatology developed an acne classification grading scale in 1990 that categorizes acne into three levels: mild, moderate, and severe. The classification of acne vulgaris allows appropriate treatment to be identified. Mild acne is generally classified as a mixture of a few to several papules and pustules, with no nodules

present. Moderate acne is classified as having several to many papules and pustules, with a small amount of nodules present.

2.1.1.2 Causes of Acne

Acne vulgaris is a skin disease of the pilosebaceous unit (*Fig. 1*), which is comprised of hair follicles and sebaceous glands that span from the stratum corneum, the top layer of skin, into the dermis, but not reaching the arteries.

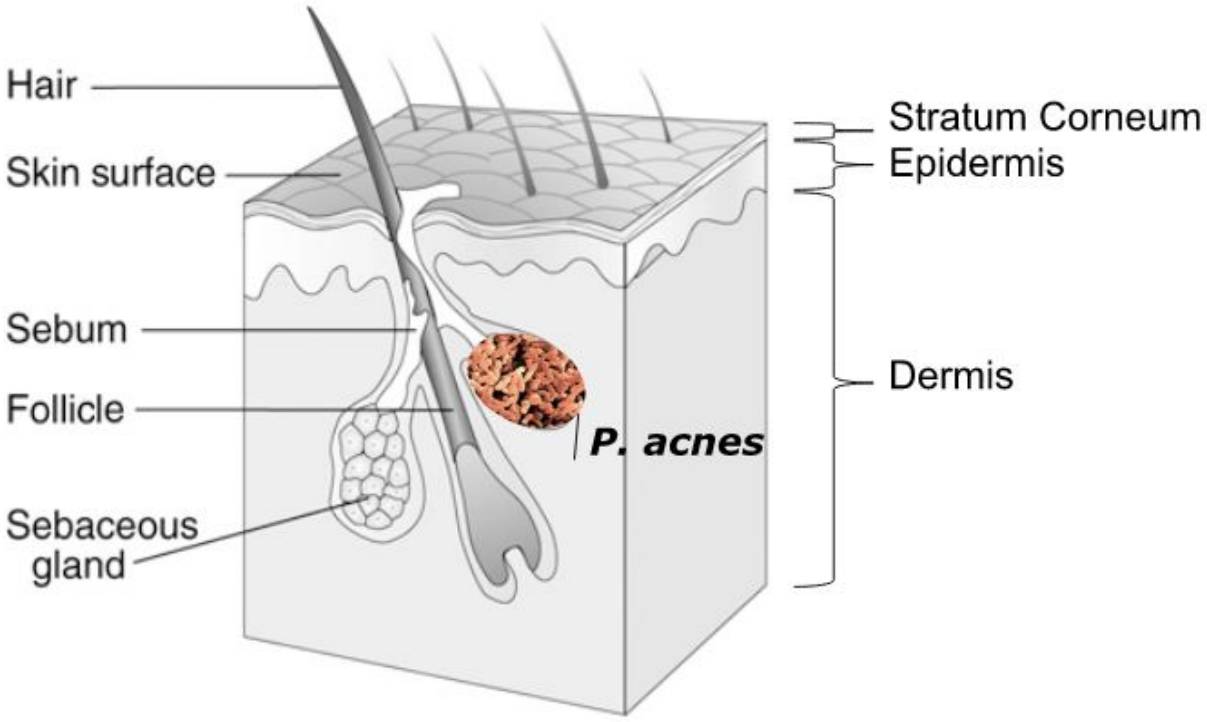

Fig. 1: **Anatomy of the pilosebaceous unit in relation to skin [21]**

The sebaceous glands produce and excrete an oil known as sebum that is used to both lubricate and make the skin waterproof [3]. Within the sebaceous glands, dead skin cells may block the gland, a process known as follicular plugging. This blockage prevents the sebum from draining and provides a microenvironment ideal for the primary acne-causing bacteria, *P. acnes*, colonization [6,22].

The other root cause of acne revolves around androgens, or hormones expressed most often during pubescent years [3]. The presence of androgens creates excess sebum production and excretion, which further promotes *P. acnes* proliferation in the sebaceous gland [3]. This creates a cycle of increased bacterial colonization and therefore, the persistent recurrence of acne vulgaris.

2.1.1.2.1 *Propionibacterium acnes*

Propionibacterium acnes, known as *P. acnes*, is the primary bacteria associated with acne vulgaris [20]. It is a Gram-positive, rod-shaped bacterium that can cause inflammation and cellular damage deep within the pores and hair follicles [20]. Additionally, *P. acnes* is an aerotolerant anaerobe, with an oxygen-deficient environment offering ideal growing conditions.

2.1.3 Populations Affected

Acne is estimated to affect 9.4% of the global population, making it the eighth most prevalent disease worldwide [1]. It is the most common skin disease among adolescents, with approximately 60% suffering from mild acne, accompanied by the use of non-prescription acne treatments. The remaining 40% suffer from severe acne, requiring consultation with a physician for prescription treatment [23].

It is important to note that acne affects populations of all ages, and is not exclusive to adolescents. An international observational study of adults older than age 25 found that more than 88% of women had acne in multiple facial zones, including cheeks, forehead, mandibular area, and temples, with a spectrum of facial acne severity similar to adolescents [24]. Similarly, a large-scale survey distributed to over a thousand participants ages twenty and older explored the

prevalence of acne reported by women and men in the U.S. [25]. Over 50% of women and 42.5% of men 20-29 years old reported having acne, and these numbers steadily decreased as the age increased.

Although acne vulgaris has effectively been treated through the use of traditional antibiotics and topical products, there has been a dramatic increase in antibiotic resistance in recent years, which has threatened their effectiveness.

2.1.4 Antibiotic Resistance

In 2015, the United States Centers for Disease Control and Prevention reported that from 2010-2011, doctors wrote over 154 million prescriptions for antibiotics [26]. Of those, 30%(~47 million) were ultimately deemed as unnecessary [26]. When antibiotics are prescribed unnecessarily, sub-inhibitory concentrations of the antibiotic can lead to bacteria adapting to the drug's mechanism of action [26].

Spanning over five decades, antibiotics, both oral and topical, have been widely used for their antimicrobial properties against *P. acnes* [27]. However, in just two decades after being used for the first time, 62% of *P. acnes* strains were reported to be resistant to common antibiotic treatments [27]. As of 2016, many countries such as France, Greece, Spain, and the UK have reported *P. acnes* antibiotic resistance levels of up to 90% against common antibiotic treatments, especially to macrolide-class antibiotics like clindamycin [27]. These high levels of international antibiotic resistance require that novel treatments be developed in order to combat the growing crisis. Additionally, with antibiotic resistance on the rise, the FDA has discouraged the use of antibiotic treatments for non-life-threatening conditions, including acne [28].

2.1.5 Psychological Problems Associated with Acne

While acne vulgaris on its own is not a life-threatening disease, it has been shown to cause significant morbidity due to its side effects, especially in adolescents [29]. Adolescents with acne have lower self-esteem and self-worth [29]. A lack of self-esteem can cause development of psychological conditions like depression and anxiety [29]. A study performed in Norway published in 2011 found that 9.5% of adolescents with mild acne reported instances of suicidal ideation, which rose to 18.6% of adolescents with moderate acne and 24.1% of adolescents with severe acne [29]. Additionally, the increasing severity in acne has been found to result in different types of social deficiencies including losing attachment with close friends, a lack of romantic relationships, and struggling with academics [29]. Mild to severe acne cases all have the potential to cause resultant facial scarring, further exacerbating the self-esteem issues that result from acne [2]. The prevalence paired with comorbidities of acne vulgaris necessitates better treatment options.

2.2 Current Acne Treatment & Their Limitations

2.2.1 Current State of the Art

There are many acne treatment products that are currently available for patients, both over-the-counter (OTC) and prescription-based. Over-the-counter products often come in the form of creams or solutions that utilize small molecules with varying effects to treat and prevent acne. Prescription acne treatments, however, are almost always antibiotic-based.

2.2.1.1 OTC Solutions

The most common OTC brands for acne treatment are Clean & Clear, Proactiv, and Clearasil. The three companies which make and market each of the respective products have control over 64% of the OTC acne treatment market on their own [30]. The three brands mentioned above utilize similar formulations and the same active ingredients in the majority of their products. The two most commonly used small molecules are salicylic acid and benzoyl peroxide. Salicylic acid works by softening the keratin in the top layer of the skin, loosening old, dry skin and making it easier to remove [31]. Because old skin cells shedding into pores is one of the main factors that cause acne, the salicylic acid can help unclog the pores and treat acne [31]. On the other hand, benzoyl peroxide kills the bacteria that cause acne. Additionally, it is comedolytic, which means it reduces the number of comedones on the skin [32].

2.2.1.2 Prescription Treatments

Antibiotics for the treatment of acne are administered either orally or topically. Because of the increased tendency of bacteria to become resistant to topical antibiotics, oral antibiotics are prescribed more often [33]. Three of the most commonly prescribed oral antibiotics are doxycycline, minocycline, and clindamycin. Doxycycline and minocycline are part of the tetracycline class of antibiotics, which work by preventing protein synthesis by inhibiting the binding of tRNAs to the A-binding site of ribosomes [34]. This mechanism allows them to be effective against both Gram-negative and Gram-positive bacteria, including *P. acnes* [34]. Clindamycin differs from both doxycycline and minocycline in that it belongs to the macrolide-lincosamide-streptogramin B (MLS) class of antibiotics [35]. While tetracyclines

block the binding of tRNAs to the 30s ribosomal subunit, MLS drugs block the exit of newly synthesized peptides from the 50s ribosomal subunit [34,35].

In addition to prescription antibiotics, synthetic retinoids derived from vitamin A are prescribed for the treatment of acne, usually of the moderate-to-severe variety [20]. Retinoids may be in oral and topical forms, and block the formation of new comedones [20].The most well-known type of retinoid is oral isotretinoin. Typically, isotretinoin is only prescribed for the most severe types of nodular acne, but it can also be prescribed when a patient's acne will not respond to any oral antibiotic treatments [33].

2.2.2 Limitations of Current Acne Treatments

Although there are a variety of options to treat acne that are available both over-the-counter (OTC) and by prescription, no treatment is perfect. Each carries its own variety of limitations and side-effects.

2.2.2.1 OTC Treatments

OTC solutions like salicylic acid and benzoyl peroxide, while common and popular, are still not ideal for the treatment of acne. Salicylic acid in particular belongs to the salicylate family of chemicals, and is very similar to the active ingredient in Aspirin, acetylsalicylic acid [36]. Salicylate sensitivity is a condition that affects around 2% of Americans, and has a multitude of potentially severe side effects [37]. People with asthma and other chronic sinus conditions are especially prone to salicylate sensitivity [37]. Some side effects include itchy and watery eyes, atopic dermatitis, and urticaria, or hives [38]. In the most extreme cases, it can induce potentially fatal anaphylaxis [38]. Because of this, many people who need acne treatment options cannot use salicylic acid at all, limiting what they can purchase for themselves without a

prescription. Additionally, while salicylic acid has proven to be an effective acne treatment, it does not address the root problem behind acne—*P. acnes* bacteria.

Benzoyl peroxide is an effective, fast-acting treatment for acne, but comes with its own side-effects. Most notably, benzoyl peroxide can be a skin irritant and a drying agent, especially when it is used in concentrations of 5% to 10% [32]. Allergies to benzoyl peroxide are also a factor, although they are not nearly as common as salicylate sensitivities [32]. Symptoms of benzoyl peroxide allergy include contact dermatitis, and most severely, anaphylaxis [32]. In addition, benzoyl peroxide is a bleaching agent, and can cause discoloration of fabrics if the fabric is exposed to the molecule before it has fully dried on the skin [32].

2.2.2.2 Prescription Treatments

Aside from the threat of antibiotic resistant *P. acnes*, prescription antibiotics (e.g. clindamycin, minocycline and doxycycline) have a number of problems that are linked to their usage. Clindamycin has the most severe side-effects. Research has shown that the oral consumption of clindamycin can lead to dramatic decreases in the diversity of intestinal bacteria [39]. This decrease in gut microbiota diversity allows pathogenic bacteria like *Clostridium difficile* to infect the small intestine, leading to severe colitis and diarrhea in patients [39].

Although it is rare, minocycline has the potential to cause liver damage and lupus-like symptoms. Females are slightly more prone to experiencing these drug side effects, and have been found to contract them much more rapidly than males that experience the same side effects [40].

Of the three antibiotics, doxycycline has the least severe side effects. It can cause gastrointestinal distress and photosensitivity, or sensitivity to light [40]. This makes patients

more likely to become sunburned after long periods of time spent outside [40]. Additionally, it can cause both reversible and irreversible levels of tooth discoloration [40]. Members of the tetracycline class of antibiotics can also impair the growth of adolescents, and should not be used by patients who are aged twelve years or younger [41].

Isotretinoin has the most severe side effects, which cause it to be reserved for only the most extreme circumstances before it is prescribed [33]. It typically causes photosensitivity and excess drying of the skin, which can cause atopic dermatitis [33]. The most important side effects of isotretinoin, and what makes dermatologists so hesitant to prescribe it, are caused by its teratogenic properties [33]. This means that it can inhibit or disturb embryonic development, causing severe birth defects [33].

Overall, the multitude of potentially severe side effects of currently prescribed acne drugs means that a novel prescription acne treatment is absolutely necessary, in order to ensure patient comfort and safety.

2.3 Antimicrobial Peptides as an Alternative Solution

With antibiotic resistance on the rise, opportunities for alternative treatments to conventional antibiotics are being investigated. Antimicrobial peptides offer a promising, effective alternative to antibiotics for the treatment of acne.

2.3.1 What is an AMP?

Antimicrobial peptides (AMPs) serve as the first line of defense in the innate immune system, secreted by keratinocytes and other resident cells in the skin such as eccrine gland cells, mast, cells, and sebocytes [14]. AMPs play an essential role in protecting the skin and body

against invading pathogens, demonstrating broad-spectrum antimicrobial activity against a range of bacteria (both Gram-negative and Gram-positive), fungi, and viruses [14].

Antimicrobial peptides are generally cationic, amphipathic peptides with less than 100 amino acids [14]. AMPs are soluble in water, and their amphipathic structure allows them to bind both hydrophilic phospholipid head groups and hydrophobic tail groups that comprise the lipid bilayers of microbial cells (*Fig. 2*) [14]. The positive charge allows AMPs to adsorb onto and effectively disrupt the anionic phospholipids of the bacterial cell membranes, causing leakage of cellular contents and ultimately microbial cell death [14].

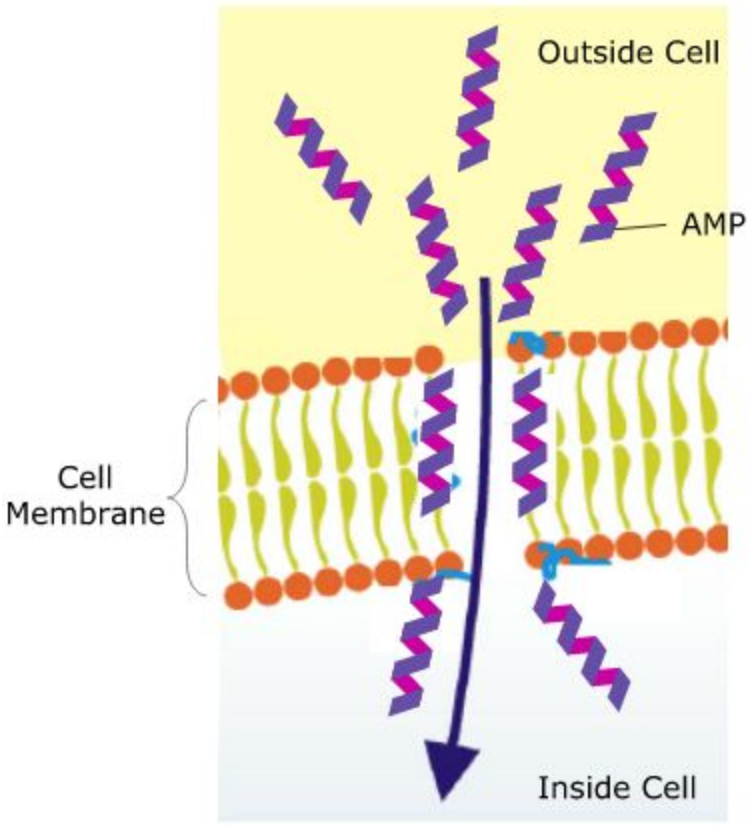

Fig. 2: **The membrane-destruction mechanisms of cationic AMP alpha-helices [42]**

Compared to conventional antibiotics, which are typically directed toward single enzymatic targets on bacteria, this mechanism of action of AMPs makes them less likely to

induce resistance within bacteria [43]. In order to acquire resistance to AMPs, bacteria would need to redesign their lipid membrane composition, which would take a greater amount of time [43].

2.3.2 Human-Derived AMP, LL37

LL37 is a human-derived 37-amino acid AMP belonging to the cathelicidin family. Cathelicidins are a class of mammalian AMPs produced typically by neutrophils, macrophages, and keratinocytes. In instances of infection or wounded skin, cathelicidins are considerably upregulated [14]. Cathelicidins are generally stored in an inactive state, mostly within granules of circulating immune cells such as neutrophil secretory granules [44]. They become activated when their characteristic N-terminal segment, known as the cathelin domain, is cleaved by proteases to generate a mature, active peptide within the C terminus [44]. Once cleaved, the mature peptide may take on a variety of tertiary structures, such as the alpha-helix of LL37 [44]. The active peptide is then transported via white blood cells to the wound site [14]. In addition to antimicrobial activity, LL37 has been shown to have several wound healing activities and assists in the modulation of the inflammatory response. This includes stimulation of wound vascularization and re-epithelialization of healing skin [45].

It has been shown that the cathelicidin LL37 has *in vitro* activity against *P. acnes* at a concentration of 15 μM [45]. Expression of LL37 in the sebaceous glands and its induction in HaCaT keratinocytes and sebocytes by culture supernatants of *P. acnes* suggests a beneficial role of LL37 in preventing acne by limiting the growth of *P. acnes* [46]. Furthermore, studies have provided evidence of the anti-inflammatory properties of LL37, further supporting that this peptide has potential for use as a therapeutic acne treatment [46].

2.3.3 Pitfalls of Past AMP Treatments

While AMP-based therapeutics offer a promising alternative to traditional antibiotic treatments, there are challenges that need to be considered, as they have limited success in clinical trials. AMPs have exhibited cytotoxic activity towards human tissue above a certain concentration. This property limits the concentration of peptide that can be included in topical AMP biologics, and thus hinders their antimicrobial effectiveness. This narrows their therapeutic window, or the difference between their cytotoxic concentration and their effective concentration. Additionally, AMPs can be subject to rapid clearance by bacterial proteases when delivered to infection sites [47]. This causes a problem if the peptide is degraded by proteases before it can effectively attack bacteria.

Topical AMP therapeutics have entered the clinical trial phase of development, but few have made it through clinical trials to become approved therapeutics. A promising topical AMP therapeutic that failed in stage III clinical trials, Pexiganan, is an illustrative example of the failures of AMP therapeutics as a whole. Pexiganan is an analog of the AMP magainin, derived from African clawed frogs. In the stage III trials, 0.8% pexiganan cream was tested against a placebo cream and evaluated for its antimicrobial efficacy in the treatment of infected diabetic foot ulcers [48]. The Pexiganan cream failed against the placebo cream in several metrics: fewer patients had a clinical response to it, fewer patients experienced microbiological success with it, and more patients experienced adverse side effects [48].

2.3.3.1 Chimeric LL37 with Collagen-Binding Domains (CBD-LL37)

In an effort to overcome the aforementioned challenges, two modified versions of the LL37 peptide with collagen binding domains (CBDs) were synthesized at WPI—*c*CBD-LL37

(6459 Da) and *f*CBD-LL37 (6645 Da) [15]. Their collagen binding domains are derived from collagenase (*c*CBD) and fibronectin (*f*CBD), respectively [15]. In a study evaluating the modified peptides, *c*CBD-LL37 and *f*CBD-LL37, as well as normal LL37, were adsorbed onto collagen-based scaffolds. The goal was to generate and evaluate whether this new delivery strategy would improve the stability of the peptide while also lowering the cytotoxicity of the AMP for potential therapeutic use. The authors showed that CBDs may increase the stability and retention of AMPs on collagen by limiting the peptide exposure to proteases and providing high affinity, specific binding onto collagen [15].

While *c*CBD-LL37 did not display a marked decrease in cytotoxicity, *f*CBD-LL37-loaded collagen scaffolds were not cytotoxic to fibroblasts at concentrations 24 times higher than the reported concentrations in solution for unmodified LL37 but still maintained antimicrobial activity at concentrations lower than 10 μM [15]. Based on these encouraging results, incorporation of *f*CBD-LL37 into a delivery vehicle provides an opportunity to develop an alternative AMP treatment that is both non-cytotoxic and effective for the treatment of acne.

2.4 Delivery Vehicle Versus Formulation

2.4.1 What is a delivery vehicle?

A delivery vehicle is an engineered material or technology that is designed to carry and protect a drug, as well as facilitate its transport to a specific site within the body [49]. The term "delivery vehicle" can be used to describe a wide range of substances including injectables, nanoparticles, implants, and topicals. Topical delivery vehicles are designed to carry and deliver

drugs onto or into the skin, and they can have varying physical, mechanical, and chemical properties depending on their specific application [49].

2.4.2 What is a formulation?

The term “formulation” is used to describe the specific ingredients used in a topical delivery vehicle, including the specific ratio of ingredients. Within the same type of topical delivery vehicle, the formulation can be altered to tailor the specific properties of the delivery vehicle to suit its intended application and the properties of the drug. Alterations of oil and water ratios, as well as the inclusion of and variations in quantities of ingredients, fall under the umbrella of formulation design. Some categories of ingredients commonly included in topical delivery vehicles in various concentrations include antioxidants, emulsifiers, gelling agents, preservatives, permeation enhancers, acidifying agents, and alkalizing agents [50].

3. Project Strategy

In order to design a successful delivery vehicle for the modified CBD-LL37 antimicrobial peptide for the treatment of mild to moderate acne vulgaris, a project strategy was created that satisfies advisor needs while maintaining a measurable and attainable scope, given project time and budget constraints. Through both team meetings as well as advisor meetings, the goal of the project within the client statement was revised based on literature research to better understand the opportunities for such a product. The developed project outline serves as a basis for what the team believes is critical to accomplish in order for the project goal to be attained successfully. This strategy evolved over time.

3.1 Initial and Revised Client Statement

The initial client statement was provided to the team directly from the project advisor and is as follows:

> "The goal of this project is to develop a topical antimicrobial delivery system that can be applied to skin abrasions, or anti-acne products. The final product should be comprised of FDA-approved ("generally regarded as safe") materials, be non-toxic, effectively deliver antimicrobial peptides (retain their activity and deliver AMPs) to the skin, and kill bacteria known to cause acne or infect abrasions."

As expected, the initial provided statement is open-ended with a broad scope, and therefore required revisions to be able to create a measurable and approachable project strategy. The initial client statement was consolidated and revised based on literature reviews of both the acne market

and the abrasions market. While the abrasions pipeline offers market opportunity for alternative treatments to traditional oral and over the counter antibiotic treatments, the team found that there is an extreme lack of innovation in the current acne market, leaving the market in desperate need of new, effective treatments. With these points in mind, the revised client statement is as follows:

> " The goal of this project is to design a topical antimicrobial delivery vehicle to deliver *f*CBD-LL37 to the sebaceous glands for the safe and effective treatment of acne vulgaris."

In order to accomplish this goal, a number of design objectives and requirements were determined to assess the safety and efficacy of the delivery vehicle.

3.2 Design Requirements: Technical

3.2.1 Objectives

Four objectives were developed for this project based on the overall project goal and client statements. These objectives can be seen in Table 1, with the importance of each objective described in more detail below.

Table 1. **Key Project Objectives**

1. Antimicrobial Activity	The delivery vehicle must not inhibit the antimicrobial activity of *f*CBD-LL37 against *P. acnes.*
2. Combined Delivery Vehicle + Peptide Activity	The delivery vehicle must allow for peptide activity within a safe therapeutic window.
3. Skin Penetration	The delivery vehicle must facilitate penetration of *f*CBD-LL37 to the sebaceous glands.
4. Biocompatibility	The delivery vehicle must be biocompatible with human skin cells.

1. **The delivery vehicle must not inhibit antimicrobial activity of the peptide**. If the *f*CBD-LL37 in the final chosen delivery vehicle design is unable to kill the *P. acnes*

bacteria, it will not be able to fulfill the goal of being an acne-treatment product. Antimicrobial activity against *P. acnes* in each designed delivery vehicle must be evaluated to ensure the peptide retains its efficacy in the vehicle.

2. **The delivery vehicle must allow for peptide activity within a safe therapeutic window.** The therapeutic window describes the difference between the cytotoxic concentration and the minimum effective concentration of the peptide. Therefore, in order to be safe and effective, *f*CBD-LL37 must be able to kill *P. acnes* below its reported cytotoxic concentration, ~20 μM [15].
3. **The delivery vehicle must facilitate penetration of the peptide to the sebaceous glands.** Effective topical delivery of biologics is a constant challenge due to the barrier function of the stratum corneum preventing transdermal diffusion of molecules greater than 500 Daltons in molecular weight [51, 52]. This is a challenge faced in clinical development that necessitates a delivery mechanism that is able to permeate through the skin.
4. ***f*CBD-LL37 in the final delivery vehicle must be non-cytotoxic to human skin cells**. The delivery vehicle should not exacerbate adverse cytotoxic effects of *f*CBD-LL37, nor should it contain other components that are harmful to mammalian cells or tissues. Due to a nonspecific attack mechanism, AMPs, including unmodified LL37, have posed a cytotoxic limitation on mammalian cells when investigated for clinical use. To succeed where other peptide therapeutics have failed, *f*CBD-LL37 must be verified to be non-cytotoxic to human cells.

The project objectives were ranked by importance, and the numbered list above correlates to the

pairwise chart results (*Table 2*).

***Table 2*. Pairwise Comparison Chart of Project Objectives**

Objectives	Antimicrobial Activity	Therapeutic window	Skin penetration	Non-cytotoxic	Total Score
Antimicrobial Activity	X	0	0.5	0	0.5
Therapeutic window	0	X	1	0	1
Skin penetration	0.5	0	X	0	0.5
Non-cytotoxic	1	1	1	X	3.0

Safety is always the primary concern, and therefore the non-cytotoxic objective was evaluated to be most important. Creating a design that works within a safe therapeutic window further increases the safety of the final delivery vehicle. Finally, skin penetration and antimicrobial activity are essential components for the successful treatment of acne, however, because they pertain to efficacy rather than safety, they were ranked below the safety objectives.

3.2.2 Design Functions

Based on the objectives, primary design functions were identified that the delivery vehicle must satisfy in order to successfully deliver *f*CBD-LL37 to the sebaceous glands for acne treatment (*Table 3*).

***Table 3*. Primary Functions and Means**

Design Function	**Possible means of accomplishing function**	
Has antimicrobial activity towards *P. acnes*	*f*CBD-LL37 AMP	Natural antimicrobial ingredients
Facilitates penetration of biologic	Nanoparticles, Microneedle Array	Liquid carriers: Cream, Ointment, Serum
Be effective at a non-cytotoxic concentration	*f*CBD-LL37 AMP	Ingredients to lower minimum required dose of AMP

In order to be effective, *f*CBD-LL37 in the delivery vehicle must exhibit complete antimicrobial activity towards the target bacteria, *P. acnes*, as it has never before been tested against this bacteria. Natural antimicrobial ingredients, such as essential oils and honey, are cited in the literature to be naturally toxic to *P. acnes* [53]. By incorporating these ingredients as potential carriers, a combination effect with *f*CBD-LL37 may help the peptide retain its antimicrobial activity.

As a large protein biologic (6645 Da), it is a significant challenge in delivery *f*CBD-LL37 through the skin barrier after topical application, and therefore the delivery vehicle must help facilitate this diffusion into the pilosebaceous unit. Liquid carriers, mechanical disruption with microneedles, or incorporation into nanoparticles are potential means to accomplishing this significant function.

The concentration of *f*CBD-LL37 in the delivery vehicle must be non-cytotoxic to human skin cells. LL37 is reported in literature to be cytotoxic at concentrations more than 13 μM [15]. *f*CBD-LL37 has been evaluated to have reduced cytotoxicity compared to unmodified LL37,

allowing for treatment concentrations of up to 20 μMbefore toxic effects are observed [15]. However, this will need to be tested and confirmed with cell viability assays or skin irritation models.

3.2.3 Design Constraints

While the previously identified objectives and functions serve as a measurable guide for accomplishing the goal of the project, constraints that limit the scope of this project must be considered when designing delivery vehicles. These constraints are divided into the six categories, and explored below.

Safety Compliance: To satisfy FDA regulations, the delivery vehicle must contain formulation ingredients that are considered GRASE (Generally Recognized As Safe and Effective) by the FDA. Additionally, the delivery vehicle must adhere to other standards found in the FDA Guidance for Industry publication "Acne Vulgaris: Developing Drugs for Treatment", and discussed in further detail in Section 3.3.

Topical Environment: In order to be effective, the peptide in the delivery vehicle must be stable at skin pH conditions (4.8 - 5.3). In this context, stability refers to maintaining antimicrobial effects against *P. acnes* while preventing conformational changes that threaten peptide activity.

Pilosebaceous Unit: While topical biologics hold potential advantages as an alternative treatment to antibiotics and over the counter treatments, delivery of large protein molecules presents a significant challenge that must be defined. In a scientific release published in 2015 by Tergus

Pharma, a leader in topical biologics, large biologics are identified as having a molecular weight over 500 Daltons, which includes most peptides and proteins [51]. Because skin acts as a protective barrier for the human body, the delivery of the biologic must disrupt the skin layer, usually by penetration [51].

Testing Models: Due to the project budget and timeframe, only *in vitro* and *ex vivo* testing models are available. Additionally, many *in vitro* tissue engineered human skin constructs are too expensive to purchase for the project. Other desirable testing models, like a pilosebaceous unit model, are unavailable because they have not been developed yet or are too complicated to fabricate for this project.

Project Budget: The budget for this project is $1000. All materials purchased for delivery vehicle formation, as well as testing equipment and other resources, must fit within this budget.

Project Timeframe: The project timeframe demands that all testing procedures be completed by April of 2018.

3.2.4 Specifications

Following the determination of the design functions and constraints, the team identified several specifications that the delivery vehicle must satisfy in order to be deemed successful.

First, the peptide in the final delivery vehicle should exhibit a minimum inhibitory concentration (MIC) equal to or lower than 10 μM, which is the reported MIC of the *f*CBD-LL37

peptide against Gram-positive bacteria [15]. The MIC of the modified peptide against the Gram-positive *P. acnes* will be determined through testing, defined as the lowest concentration of the antibacterial agent that prevents visible growth of a microorganism after incubation overnight.

In order to penetrate into the pilosebaceous unit, the modified peptide in the delivery vehicle has to be smaller than 7000 Daltons [51]. The *f*CBD-LL37 peptide is 6645 Daltons, falling within the specification range.

In order to prevent cytotoxicity to the surrounding skin cells and tissues, the concentration of the unmodified LL37 peptide has to be less than 13μM, with the optimal concentration for wound healing and other positive effects of LL37 at or below 1μM [15]. The *f*CBD-LL37 has been shown to be non-cytotoxic at concentrations up to 20μM, so the non-cytotoxic specification for the modified peptide will be determined upon testing.

3.3 Design Requirements: Standards

In order to design a viable product that has a chance of approval and getting to market, it is essential to consider international standards and national regulations beginning in the early stages of the design process. A number of ISO (International Organization for Standardization) standards are relevant in designing and testing each delivery vehicle and formulation. Some of these are ISO 20776-1:2006, ISO 20776-2:2007, ISO/TS 17518:2015, and ISO 21148:2017. The first standard contains the proper methods for testing the *in vitro* activity of antimicrobial agents, and the second standard contains performance criteria of devices used to determine MIC as well as criteria for whether or not bacteria are susceptible, intermediate, or resistant to the treatment method [54,55]. The first standard was incorporated into the antimicrobial activity testing for

*f*CBD-LL37 against *P. acnes*. Additionally, in antimicrobial activity testing, the provided criteria from the second standard will allow *P. acnes* to be classified against each *f*CBD-LL37 designed delivery vehicle treatment. ISO/TS 17518, the third standard, lists different reagents for staining biological material, which will be important when designing mammalian cell viability assays to test drug cytotoxicity [56]. Finally, ISO 21148 concerns general instructions for microbiological examination of cosmetics, which describes how to ensure product quality and safety [57]. This will assist in ensuring that the product is free of any potentially harmful pathogens, especially of the type that are not susceptible to *f*CBD-LL37.

Because the product is being designed in the United States, it is especially important to abide by FDA regulations in the design. At the moment, the list of FDA GRASE materials is being considered in order to decide on inactive ingredients in the formulation that should ideally cause no problems for approval. Additionally, in early 2017, the FDA released a Guidance for Industry describing the current good manufacturing practice (CGMP) requirements for combination products [58]. Because they define combination products as a drug/biologic, a drug/drug, or a biologic/biologic, the chosen delivery vehicle design will have to abide by these guidances as a topical biologic.

Finally, and most importantly, in 2005 the FDA released a Guidance for Industry on developing drugs for the treatment of acne vulgaris [59]. It describes both the clinical background of acne as well as instructions for drug development, including clinical considerations like baseline lesion counts [59]. Keeping any FDA and ISO regulations/standards at the forefront of the product design ensures that there will be little to no regulatory problems as

the product eventually moves forward into preclinical and clinical trials after the team confirms *in vitro* safety and efficacy.

3.4 Management Approach

In an effort to help manage time and ensure progress throughout the year in an efficient manner, the team first identified the major milestones towards project completion each term (*Fig. 3*). A term was primarily focused on conducting literature research about the pathology of acne and its treatment methods, along with developing our own experimental design plan that would reflect the project design objectives. This was necessary to create design constraints, functions, and specifications that again, governed the methodology planned for B term. In B term, the experimental design was implemented through preliminary experiments that served as a chance to both learn and master protocols, gather materials, and reevaluate the experimental design for C term in order to accomplish the team's objectives. In C term, the experimental design was repeated and further revised in order to obtain reproducible results with ease and efficiency, which will lead the team to a final formulation design decision. The final formulation design focuses on determining the most synergistic oil and modified peptide combination, as well as the most promising concentrations of various active and inactive ingredients. The project was concluded during D term, with final experiments conducted to further validate the design.

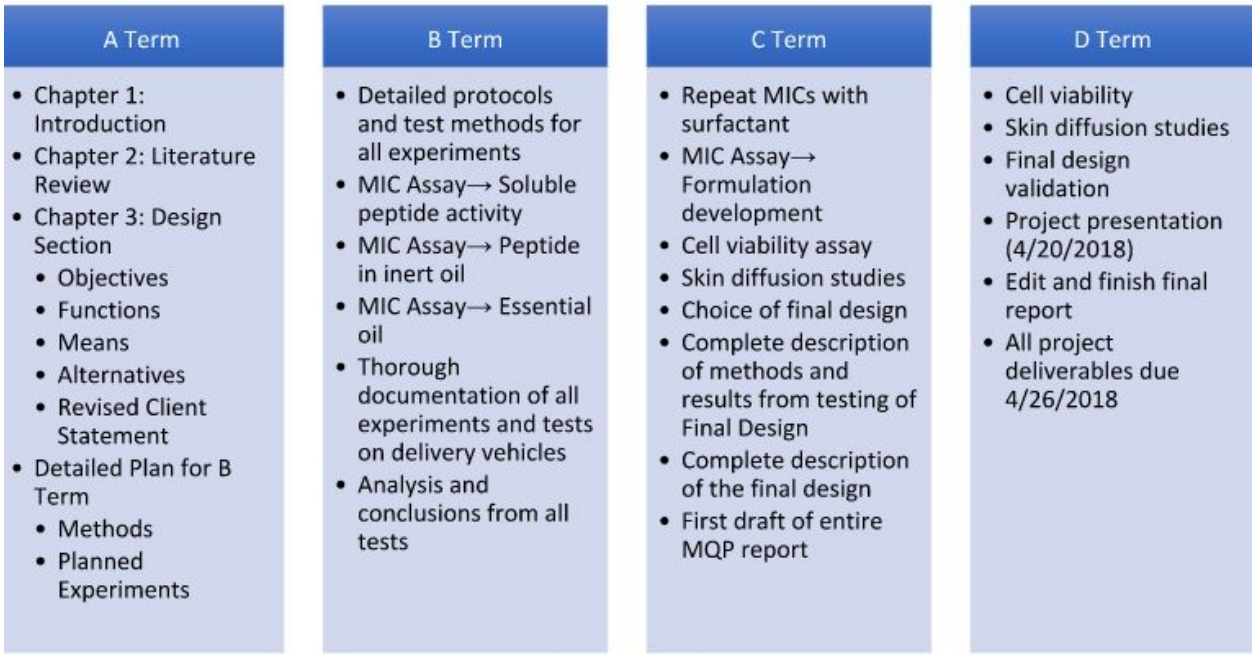

***Fig. 3*: Major milestones for project completion each term**

The Gantt chart (*Fig. 4*) provided a target timeline for when the group would accomplish each of the tasks associated with the project, such as conducting background research, developing formulations, testing alternative designs, final design selection, etc. Following the schedule laid out in these tools assisted the team in tracking the completion of the project tasks and achieving the overall goals and objectives.

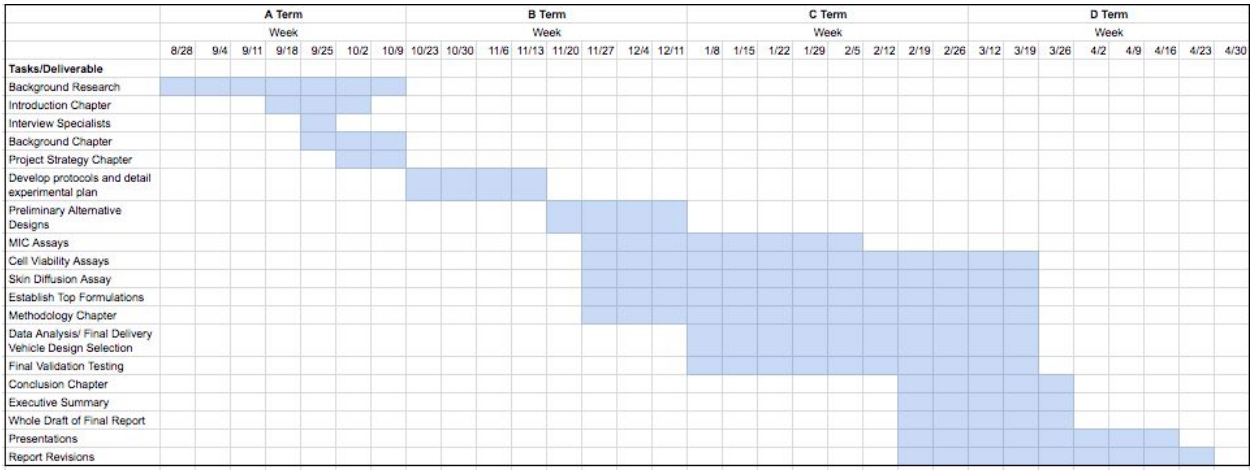

***Fig. 4*: Gantt chart for the 2017-2018 academic year, by week**

4. Design Process

4.1 Needs Analysis

Based on the design objectives, the team identified several "needs" and "wants" for the final delivery vehicle in order to be considered successful. A need is a characteristic or ability of the final product that is absolutely essential to the success of the product, while a want is something that the team aims to obtain, but is not essential. Upon identification, the team used a pairwise-comparison chart to evaluate and quantify each of these criteria against one another (*Table 4*). A "1" was used to signify that the need in the left column was deemed more essential than the need it is being compared to in the top row; a "0" is used to describe a need in the left column that is less of a priority than the need on the top row, and a "0.5" is used to signify that the needs are of equal importance. Based on the total score tallied for each need, the needs were prioritized in the following manner, with the justification for the need subsequently included:

1. **Peptide in delivery vehicle kills 100% of *P. acnes* bacteria.** In order to be effective as an acne product, the peptide in the delivery vehicle has to kill all of the bacteria that are causing the acne. The concentration of peptide in the delivery vehicles must be at or above its demonstrated minimum inhibitory concentration to achieve this.
2. **Peptide in delivery vehicle must enter hair follicle pores into the sebaceous gland.** *f*CBD-LL37 must be able to target the *P. acnes* bacteria within the sebaceous glands to be effective in targeting acne at the root cause.
3. **Delivery vehicle stays applied to skin.** The delivery vehicle has to remain on the skin for a long enough period so that the peptide can be effective. The team evaluated skin

penetration 30 minutes after application onto an *ex vivo* skin model to ensure that the formulation did reach the pilosebaceous unit within that time.

Table 4. **Pairwise Comparison Chart of "Needs" for Final Design.**

	Kills 100% of *P. acnes* bacteria	Must enter hair follicle pores	Not cytotoxic	Stays applied to skin	**Total Score**
Kills 100% of *P. acnes* bacteria	X	0	0	1	**1**
Must enter hair follicle pores	1	X	0	0	**1**
Not cytotoxic	1	1	X	1	**3.0**
Stays applied to skin	0	0	0	X	**0**

The "wants" for the design were quantified in the same manner (*Table 5*). The wants were prioritized in the following manner:

1. **More effective than current acne treatments**

   In order to be a competitive acne treatment, the final design must have at least the same or increased efficacy compared to current acne treatments.

2. **Minimal and natural ingredients**

   Minimal and natural ingredients are not only appealing to consumers, but are also more beneficial to the skin than harsh, skin-irritating chemicals.

3. **Long shelf-life**

   A long shelf-life of the product is desirable, referring to the duration of time that the peptide in the delivery vehicle remains usable (active and effective) or saleable.

Table 5. **Pairwise Comparison Chart of "Wants" for Final Design**

	Minimal and natural ingredients	Long shelf-life	More effective than current acne treatments	**Total Score**
Minimal and natural ingredients	X	1	0	**1**
Long shelf-life	0	X	0	**0**
More effective than current acne treatments	1	1	X	**2**

Finally, the team identified physical limitations and technical constraints that may limit the ability of the team to satisfy these needs and wants. As stated, the peptide must be able to enter the hair follicle pores in order to act within the pilosebaceous unit. While the size of the peptide can be estimated in an effort to ensure this targeted delivery, the team cannot directly measure the absorption and diffusion of the peptide into a pilosebaceous unit without an *ex vivo* human skin construct that serves as an acne model, which is not feasible to produce for this project. Porcine ear skin, a proven animal skin model most similar to human forehead skin, was used to address this limitation [60]. Further, the team cannot ensure it will not penetrate through the stratum corneum and become systemically bioavailable, which may affect the safety of the drug. Recognition of the limitations of the studies conducted through this project are important, and further investigation should be conducted following proof-of-concept development and testing.

4.2 Concept Map, Conceptual Designs, and Feasibility Studies

The team developed a concept map as a way to visualize design ideas and potential design alternatives (*Fig. 5*).

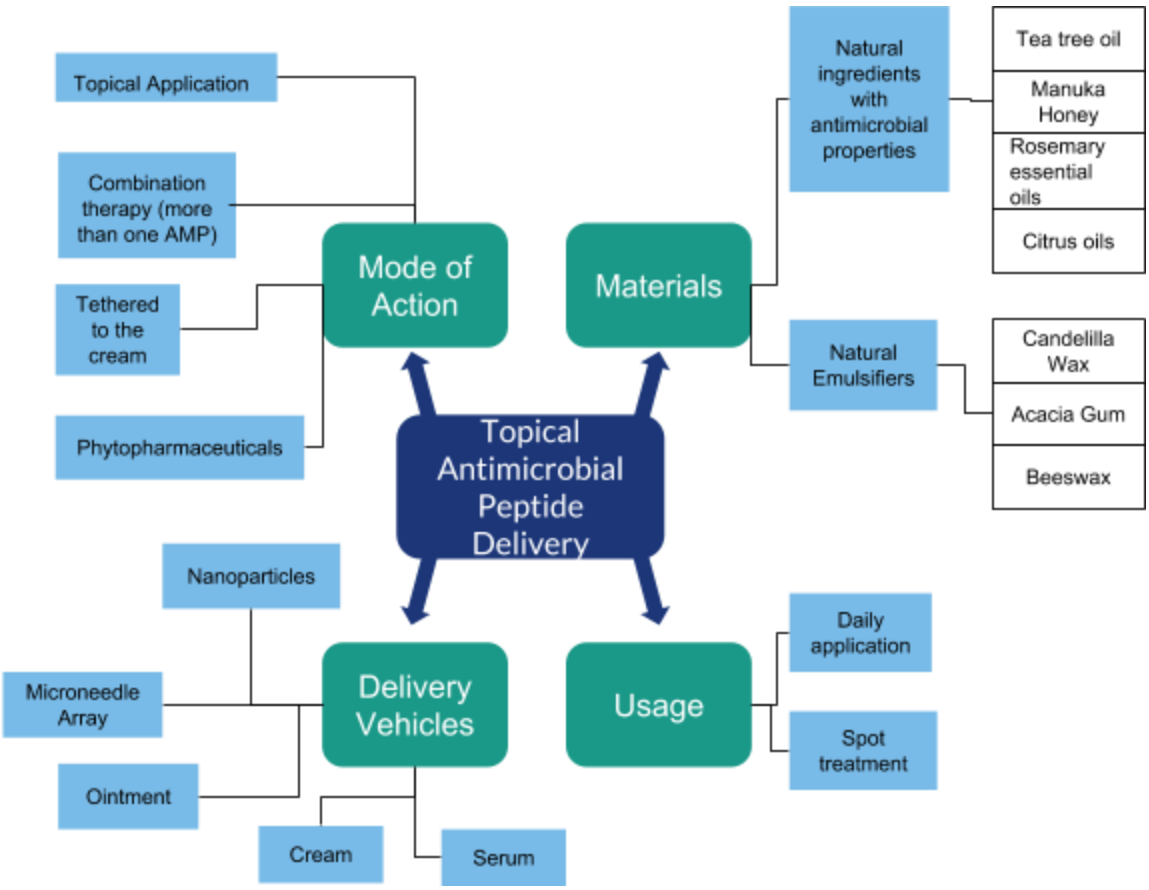

***Fig. 5*: Design concept map**

In brainstorming potential concepts to achieve an effective topical acne treatment product with *f*CBD-LL37, concepts were divided into four overarching categories that the team felt were most critical to a successful delivery system including delivery vehicle, mode of action, material, and usage. Potential vehicles for topical delivery were conceptualized, as well as the mode of actions that would ultimately enhance peptide delivery and functionality as a product. Finally,

the usage of the delivery vehicle was brainstormed, as the usage will depend on what delivery vehicle was chosen as the final design.

4.2.1 Conceptual Designs

4.2.1.1 Why Topical versus other delivery methods?

Peptides used for therapeutic applications require a delivery vehicle for transport due to the widespread evidence that when delivered orally, degradation interrupts the peptides ability to perform its intended therapeutic function [51]. Current peptide-based treatments are delivered instead via injection series, in order to deliver enough peptide for effective treatment [51]. This treatment method is both inefficient and also reduces the patient's comfort and satisfaction, creating a need that is not being met.

Various topical carriers including nanoparticles or oil-based solutions such as creams, ointments, and serums present an opportunity to facilitate diffusion and absorption of large molecules such as biologics.

4.2.1.2 Challenges with Topical Biologics

While topical biologics hold potential advantages as an alternative treatment to antibiotics and over the counter treatments, delivery of larger biological molecules present challenges that must be defined. In a scientific release published in 2015 by Tergus Pharma, a leader in topical biologics, large biologics are identified as having a molecular weight over 500 Daltons, which includes most peptides and proteins [51]. Because skin acts as a protective barrier for the human body, the delivery of the biologic must disrupt the skin layer, usually by

penetration [51]. In addition to bypassing the skin, topical biologics require unique processing considerations, as structural damage and conformation can alter expected activity and properties of the peptide [51]. In contrast, *f*CBD-LL37 costs $21/residue/mg. When buying peptide products, a residue represents an amino acid, and *f*CBD-LL37 contains 62 amino acids, thus costing a total of $1302/mg.

Literature suggests than incorporation into an oil-based carrier may alter the conformation of the peptide and thereby improve its activity. In aqueous solutions, many cationic AMPs exist in a disordered structure, and are converted to the α-helical structure in the presence of hydrophobic oil components, suggesting that the inclusion of oil-based formulations in the designed delivery vehicle would be beneficial [61].

4.2.1.3 Nanoparticle Delivery

Polymeric nanoparticles for topical drug delivery are capable of delivering both hydrophobic and hydrophilic drugs while allowing for controlled drug release, making them potentially advantageous for delivering topical AMPs [62]. Drug-loaded nanoparticles also tend to accumulate in hair follicles, making targeted acne treatment an ideal application [62]. Incorporation into nanoparticles may also increase the efficiency of the peptide by improving stability *in vivo*, and minimize the side effects associated with various therapeutic ingredients [63].

4.2.1.4 Microneedle Arrays

Microneedle arrays consist of hundreds of micrometer-scale needles that are loaded with drug solution and placed on the skin to deliver drugs. Their primary advantage as a delivery

vehicle is their ability to bypass the stratum corneum and create a direct pathway for dermal drug diffusion [64]. Microinjection delivery has been shown to increase drug permeability compared to non-injection topical delivery methods [65]. This technology combines the easy access and availability of topical delivery with the drug penetration efficacy of injections. Microneedle arrays also have flexibility with regards to the length and density of the needle geometry, which can be customized to treat patients with varying skin thicknesses [65].

For this project, microneedle arrays had the potential to simplify some of the delivery and degradation concerns by bypassing the stratum corneum and releasing the peptide closer to the sebaceous gland. However, at this time, microneedles have only been used for transdermal and intradermal delivery of drugs to the bloodstream. This raises concerns that the technology is not accurate or developed enough to specifically target the sebaceous gland.

4.2.1.5 Topical creams

A cream is defined as an emulsion semisolid comprised of more than 20% water and less than 50% hydrocarbons, oils, or waxes [66]. An emulsion is a two-phase mixture of two or more immiscible liquids, typically oils and water, where one immiscible liquid is dispersed as globules in the other [66]. The immiscible liquids are prevented from separating with the inclusion of an emulsifier. The globular suspension property of emulsions can be used to further categorize them into oil-in-water emulsions (oil or hydrocarbon component suspended in an aqueous liquid), or water-in-oil emulsions (aqueous component suspended in an oil/hydrocarbon liquid). A semisolid is a substance that exhibits plastic flow behavior which does not flow to completely conform to the shape of its container at room temperature [66]. Creams are viscous and mildly

greasy-feeling to the touch. Additionally, they will eventually evaporate and/or absorb into the skin after application [66].

4.2.1.6 Topical ointments

An ointment is described as an emulsion semisolid comprised of less than 20% water and more than 50% hydrocarbons, oils or waxes [66]. Ointments are viscous and greasy. Unlike creams, they will not evaporate or absorb into the skin after application, due to their smaller aqueous component compared to creams [66].

4.2.1.7 Topical serums

Serums do not have a concrete definition among topical biologics. For this project, serums are considered thin liquids with relatively low viscosity compared to creams and ointments. Serums also allow for better drug penetration into the skin, mainly due to more lightweight and minimal ingredients [67]. Additionally, they readily absorb into the skin, so manual removal after application is not required [67].

4.2.2 *Ex vivo* Model

Diffusion of the peptide is a model that will be critical to understand. This concept is important because it directly relates to the antimicrobial efficacy and the application of the delivery vehicle to the skin.

The team utilized an *ex vivo* model, specifically porcine ear skin, for absorption and diffusion studies of *f*CBD-LL37 within the designed delivery vehicle [60]. Porcine ear skin has a similar stratum corneum thickness to human skin, at 21-26 μm, and has similar histological properties [60]. Porcine ear skin in particular is a useful model for acne, because its hair follicle

density, at 20/cm^2, is comparable to the hair follicle density of the human forehead, at 14-32/cm^2 [60]. Due to the similar types of stratum corneum lipids found in pigs and humans, pig skin permeability is close to that of humans [60]. Due to the aforementioned reasons, along with the ease in acquiring it and its affordability, pig ear skin will be both an accurate and cost-effective transdermal drug delivery model [60].

4.2.3 Feasibility Studies

Although normal LL37 has been documented to be effective against *P. acnes*, the modified *f*CBD-LL37 has not yet been tested against this strain of bacteria. Because of this, testing results may show that the modified AMP is completely ineffective in killing *P. acnes*, rendering the goal of the project infeasible. Through the design of delivery vehicles for AMP transport, the results of the project will ultimately show whether or not *f*CBD-LL37 is a good potential treatment for acne, and if it has the potential to be a viable replacement for current antibiotics and topical small molecule acne treatments.

4.3 Design Alternatives

Once possible topical delivery vehicle designs were conceptualized, the first step in considering each design alternative was to evaluate each based on feasibility in attaining the project goal, based on extensive consultation of peer-reviewed literature.

Nanoparticle delivery design would first involve determining a suitable polymer material for the particles, and then once a material was chosen, figuring out how to begin and optimize

nanoparticle fabrication. Additionally, the team would have needed to ensure that the *f*CBD-LL37 was loaded properly into the nanoparticles and conducted controlled release studies before attempting the methods required to accomplish the project objectives. These steps would not have fit within the project timeframe, and therefore were not pursued.

Microneedle arrays were a desirable design alternative due to their ability to bypass the stratum corneum layer, offering facilitated diffusion of *f*CBD-LL37. However, a main concern in the implementation of microneedles is that while they allow relatively easy diffusion of the therapeutic agent by providing a microchannel, they also allow easy diffusion of bacteria, potentially increasing the risk of infection if good clinical practice is not utilized [68]. Like nanoparticles, a microneedle array would require complex design techniques and considerations that were beyond the scope of this project. These include patch design and the way the delivery vehicle would be loaded onto or into the microneedles and released. Additionally, because both nanoparticle delivery and mechanical disruption are complex, developing therapies, these alternatives were not ideal for ease of application or user comfort.

This conclusion led us to further investigate liquid-based topicals including creams, ointments, and serums, which are more familiar to patients, and are simple to apply. Creams, ointments, and serums all have varying ratios of oil and water. As previously discussed, both creams and ointments typically have high viscosity. On the other hand, serums are lower viscosity, and have been shown to facilitate improved penetration and absorption of large molecules across the skin barrier [66]. Therefore, a serum-based design was identified as the design alternative that would accomplish the project goal within the project timeframe, and offer the best approach.

4.3.2 Serum Oil Component

Once a serum was determined as the most optimal alternative design, and before a final design selection could be made, further development of the oil component of the serum was necessary. Due to the hydrophobicity of the sebum produced by the sebaceous glands and which coats the skin, the team made the incorporation of an oil component into the delivery vehicle an important priority. The team anticipated that the hydrophobic oil component would mix well with the sebum in the sebaceous glands, facilitating absorption of the peptide in formulation. In addition, as previously discussed, incorporation of a hydrophobic oil component may alter the helicity of the peptide, suggesting that the peptide would be more active in a hydrophobic environment [61].

After extensive literature research, six different essential oils were chosen as potential candidates for the final delivery vehicle, including tea tree oil, manuka oil, orange citrus oil, lemongrass oil, eucalyptus oil, and sunflower oil. Essential oils are concentrated hydrophobic, plant-based liquids that contain compounds that give plants their aroma. They are commonly used in commercial skin products due to their beneficial properties, including being anti-inflammatory and antimicrobial, which are discussed in further detail below.

4.3.2.1 Selected Essential Oils

Tea tree oil (Eve Hansen) is a common essential oil that has been investigated for its anti-acne potential since at least 1990 [69]. It has been shown to have an efficacy level close to benzoyl peroxide for treating acne when both were used at 5% v/v, while also being associated with less potentially adverse effects [69]. *In vitro*, it has also displayed potent activity against *P.*

acnes, with minimum inhibitory concentration (MIC) values ranging from 0.25-1% v/v, as reported by multiple studies that have chosen to investigate its potential [70,71,72]. Additionally, tea tree oil has anti-inflammatory properties, which assist in reducing pimple redness by decreasing vasodilation, microvascular blood flow, and plasma extravasation at application sites [72]. On a molecular/cellular level, tea tree oil inhibits the production of pro-inflammatory cytokines by monocytes and macrophages [72].

The second essential oil chosen for investigation was manuka essential oil (Earth Shield), a product of *Leptospermum scoparium* trees native to New Zealand. Manuka essential oil has well-documented antimicrobial effects against multiple strains of bacteria and yeast, including *Staphylococcus aureus* [18]. Manuka oil, along with tea tree oil, also has its own anti-inflammatory properties, which give it high potential for acne treatment as well [18].

Orange citrus oil, lemongrass oil, and eucalyptus oil are less common essential oils that have been investigated for their antimicrobial and antifungal potential in several studies. One study conducted in 1996, which compared the aforementioned activity of 10 essential oils against 22 strains of bacteria and 12 strains of fungi, found that these three oils effectively inhibited all tested strains of bacteria and fungi [73]. Other studies have experimentally determined the MIC values of these oils against various strains of bacteria. Orange oil was shown to have an MIC of 1.5% v/v against 6 strains of bacteria [74]. Lemongrass oil and eucalyptus oil were shown to have MICs of 0.125% v/v and 4% v/v against *P. acnes*, respectively [74].

Sunflower oil is a natural, cost-effective oil that offers antimicrobial properties and beneficial skin properties. A 2014 study found that sunflower oil was effective against three strains of bacteria at concentrations as low as 0.07% v/v [75]. Along with its potential for a low

MIC, sunflower oil contains tocopherols, which is a group of compounds that make up the vitamin E complex [76]. Tocopherols are antioxidants and offer prevention of collagen cross linking, a factor associated with skin aging [76]. Additionally. vitamin E is essential in maintaining stratum corneum preservation as well as improving hydration [76]. Containing antioxidants and carotenoids allows sunflower seed oil to hold regenerative skin benefits [76]. This oil also contains fatty acid linoleic acid that has been indicated as a potential inhibitor of *P. acnes* [77]. Literature hypothesizes that a decrease in linoleic acid in the sebum may be responsible for disturbed keratinization of the stratum corneum [78]. In a study conducted in 1998, the effect of topically applied linoleic acid on the size of comedones was conducted, resulting in 25% reduction of comedones observed [78]. Sunflower seed oil offers desirable skin benefits and antimicrobial properties that support its selection as a potential oil used in this design.

4.4 Final Design Selection

Experiments began with a series of MIC assays to determine the antimicrobial activity of the modified *f*CBD-LL37 antimicrobial peptide and six essential oils against *P. acnes* individually. The results of these MIC assays determined the formulation components and concentrations that would become part of the final design. Ultimately, the two oils selected to move forward for testing were manuka essential oil and eucalyptus essential oil, as manuka oil consistently killed *P. acnes* at a concentration of ~1% v/v, and eucalyptus oil consistently killed *P. acnes* at a concentration of 0.0625% v/v, discussed below. While the MIC of lemongrass oil

was lower than that of manuka oil, the team chose to move forward with manuka oil because it has more beneficial properties for the skin, apart from its antimicrobial activity.

The two best performing oils were chosen to move forward in the final design selection process because the team believed that the high performing oils would again, be least likely to inhibit peptide activity within the delivery vehicle. Additionally, limiting the number of oils in the delivery vehicle formulation process ensured that safety and efficacy could be thoroughly evaluated. From here, the two oils were each tested in combination with the *f*CBD-LL37 peptide, and again, the best-performing oil formulation was identified for use in later skin penetration studies. A final delivery vehicle with 0.05% eucalyptus oil diluted in water was selected based not only on its consistent antimicrobial activity, but also its ability to lower the concentration of peptide needed to kill *P.acnes* from 7.5μM to 1μM within the delivery vehicle, therefore significantly widening the therapeutic window of the delivery vehicle. The methods used to reach this conclusion are described below for the MIC assays, as well as the subsequent evaluation of the delivery vehicle's diffusion through porcine ear skin.

5. Experimental Design Verification

5.1 Design Verification Methods

The team identified a three-step process of experiments that allowed for verification of the conceptual delivery vehicle design. This process included a series of three minimum inhibitory concentration (MIC) assays, each of the results informing how to conduct the next, and ultimately funneling down to the specific components that formed the final delivery vehicle design that was validated (Chapter 6). The first step was to characterize the soluble, antimicrobial activity of *f*CBD-LL37 against *P. acnes*. The second step was to investigate the antimicrobial activity of each of a total of six essential oils against *P. acnes*. Finally, the third step was to test the combined *f*CBD-LL37 and oil activity, informed by the second MIC assay, to ensure that within the delivery vehicle, *f*CBD-LL37 would be active within a safe therapeutic window. In order to verify and optimize the aspects of the delivery vehicle design, MIC assays were tested in triplicate, and each step was performed at least twice to confirm reproducible results.

5.1.1 MIC Assay Universal Experiment Conditions

Each MIC assay in the three step design verification process required the use of *P. acnes* liquid bacterial culture. Additionally, steps two and three involve essential oils and bacterial media, which required emulsification using 0.5% polysorbate 80, also known as Tween® 80. These components are universal across each assay, and are described individually below.

The complete protocols for each MIC assay are listed in Appendices A-C of this report.

5.1.1.1 *P. acnes* Bacterial Culture

As *P. acnes* (ATCC® 6919, VPI0389 (strain), 70005466 (lot #)) is an aerotolerant anaerobe, it requires minimal exposure to oxygen. Therefore, bacterial cultures were stored in an anaerobic chamber, a BD GasPak™ EZ chamber (Becton Dickinson, Franklin Lakes, NJ), along with anaerobic gas sachets that remove oxygen from the environment within the chamber. The bacteria were cultured using 15 g/L tryptic soy broth and plated on tryptic soy agar plates. *P. acnes* is slow growing, therefore four liquid cultures of ~5 mL each were inoculated one week prior to use in an experiment. Adequate bacterial growth was verified by assessing liquid culture turbidity.

To prepare the $1x10^6$ CFU/mL concentration of bacteria needed for each MIC assay, the team conducted a 0.5 McFarland standard correction. The optical density at 590 nm of the 0.5 McFarland standard tube represents a bacteria concentration of $1x10^8$ CFU/mL. In order to adjust the *P. acnes* liquid culture to the desired concentration, a cycle of pipetting bacteria out of the culture tube and pipetting media in was repeated until visual comparison with a Wickerham card showed that the optical density was close to the liquid in the 0.5 McFarland standard tube. Then, to lower the standardized *P. acnes* culture to $1x10^6$ CFU/mL, it was diluted 1:100 in broth in a Petri dish, which was used for the MIC assays. To prove that the McFarland standardization procedure was done accurately, the $1x10^6$ CFU/mL culture was further diluted to 10^4 and 10^3, respectively, and spread onto n=2 tryptic soy agar plates for colony counts.

5.1.1.2 Media Preparation

As mentioned previously, *P. acnes* were grown in 15 g/L tryptic soy broth. In order to ensure a uniform solution of bacteria with each of the essential oils, 0.5% Tween® 80 (Sigma-Aldrich) was used as an emulsifier and surfactant. The 0.5% Tween® 80 solution was prepared by diluting the polysorbate 80 in tryptic soy broth and vortexing to mix the solution. Due to its viscosity, the Tween® 80 was heated in a 37 ℃ water bath prior to ensure more efficient pipetting.

5.1.1.3 Peptide Preparation

Stock solutions of the modified *f*CBD-LL37 peptide as well as unmodified LL37 were diluted with Dulbecco's Phosphate-Buffered Saline (DPBS) to reach the desired concentration for the relevant assay being performed. Each peptide stock was stored in water, pH 3.5 supplemented with 5 mM ethylenediaminetetraacetic acid at -20°C at a concentration of 670 μM. Therefore, peptides are not anticipated to lose stability at skin pH conditions.

5.1.2 Delivery Vehicle Activity Assay

In order to verify the choice to use *f*CBD-LL37 as a potential acne treatment within the delivery vehicle, it was important first to verify its ability to kill *P. acnes*. This experiment, therefore, focused on achieving the first defined project objective: **The delivery vehicle must not inhibit the antimicrobial activity of *f*CBD-LL37 against *P. acnes*.** This assay utilized unmodified LL37 as a control due to its well-established activity against *P. acnes*. The MIC

assay, performed in a 96-well plate, followed a protocol described by Lozeau *et al* [15]. Appropriate positive and negative controls were included in each assay, including a growth control containing only *P. acnes*, and a sterility control containing growth medium only. Each peptide was tested in triplicate and serially diluted at a 1:2 ratio with 15 g/L tryptic soy broth. After completing the serial dilutions, liquid *P. acnes* culture was added to each well at a concentration of $1x10^6$ CFU/mL, as determined by the 0.5 McFarland standard protocol described in section 5.1.1.1. In the end, each well contained 100 µl total volume, 50 µL of peptide dilution plus 50 µL of liquid bacterial culture. Inoculated plates were incubated for 72 hours before being read for absorbance at 590 nm. Seventy-two hours was chosen as an appropriate incubation time period due to the slow doubling time of *P. acnes.* Additionally, in accordance to the McFarland standard protocol in section 5.1.1.1, the diluted liquid culture was spread on agar plates to confirm that the initial standardization was accurate.

5.1.3 Essential Oil Antimicrobial Activity Assay

Each of the six tested essential oils were primarily chosen due to their established antimicrobial properties, elaborated in detail in Section 4.3. Identifying the MIC of each oil against *P. acnes* indicated whether or not the oil would be likely to inhibit *f*CBD-LL37 activity within the serum delivery vehicle.

Prior to being pipetted into the 96-well plate, each oil was first diluted in tryptic soy broth supplemented with 0.5% Tween® 80 to a concentration of 8% v/v. The final volume of each well was 100 µL, containing 50 µL oil and 50 µL liquid bacterial culture. This resulted in a starting oil concentration of 4% v/v, which was chosen because it is higher than each of the

published oil MICs against *P. acnes* described in Section 4.3. After being pipetted into the wells, the oils were serially diluted 1:2 with tryptic soy broth supplemented with Tween® 80. From then on, the same procedure outlined in subsection 5.1.2 was followed.

5.1.4 Oil-Peptide Combination

The primary goal of oil-peptide combination experiments was to evaluate the activity of the AMP when combined with the oil component in the delivery vehicle against *P. acnes*, conducted to assess the second objective, which stated that **the delivery vehicle must allow for peptide activity within a safe therapeutic window.** This could be reflected by a lower MIC value of the peptide-oil combination compared with that of peptide alone; using a lower concentration of the peptide would improve the safety profile of the delivery vehicle.

The starting peptide concentration for *f*CBD-LL37 was determined based on the delivery vehicle activity assay MIC result. Despite testing MICs of 6 different oils, the two with the lowest MIC results from the oil MIC assay were chosen for combination testing, which were eucalyptus and manuka oil. Two sub-inhibitory concentrations for both eucalyptus and manuka oil were evaluated in order to ensure that the oils could not create false positive results by killing the bacteria on their own. Each experimental well was set up as follows: 50 µL bacteria, 25 µL diluted essential oil, 25 µL peptide. Since the manuka MIC against *P. acnes* was 1% v/v, the concentrations chosen to test in combination were 0.5% v/v and 0.1% v/v; similarly, since eucalyptus had an MIC of 0.0625% v/v, it was tested at 0.05% v/v and 0.02% v/v. Since the oils made up one quarter of the well volume, oil aliquots were first made at four times the desired final concentration. The peptides were added to the 96-well plate and serially diluted 1:2 as

described in 5.1.2. Following the peptide additions, the oils were added before the bacteria procedure was corrected to $1x10^6$ CFU/mL. In addition to the growth and sterility controls, a peptide-only control was set up to corroborate the results found from the first MIC assay with the same *P. acnes* cultures used to test the combination effects. The results of this assay determined which delivery vehicle formulation to move forward with for the skin penetration studies.

5.1.5 Skin Diffusion Assay

Once a delivery vehicle was determined for *f*CBD-LL37, the team again faced that significant challenge of topically delivering a large protein molecule through the skin barrier. In order to assess peptide diffusion, an accurate yet cost-effective *ex vivo* skin model was needed, and porcine ear skin was identified as most comparable to human forehead skin in both hair follicle density and stratum corneum thickness.

The team obtained a set of pig ears from Adams Farm in Athol, MA to complete skin penetration studies. This allowed for a visualization of the peptide location after application to confirm that **the delivery vehicle facilitates penetration of the peptide into the sebaceous glands of skin**, in accordance with the third objective. The team wanted to ensure that the peptide could be effectively delivered to the pilosebaceous unit so that it could be active against the *P. acnes* bacteria in the sebaceous gland. The three-step process that guided diffusion assessment includes skin retrieval through dissection, Eosin staining, and immunohistochemistry. Detailed protocols can be referenced in Appendix E.

5.1.5.1 Skin Model Dissection

Skin from two porcine ears was harvested from the cartilage using surgical tools into roughly 2 cm x 0.5 cm sections for later histology. To isolate the skin, the perimeter of the ear was removed with surgical scissors to expose the layers of skin and cartilage in the ear. Then, the skin was pulled away from the cartilage with forceps while the connective tissue between the skin and cartilage was severed using a scalpel. This process was carried out until most of the skin-cartilage barrier was severed (*Fig. 6*), at which point the skin flap was removed completely from the ear.

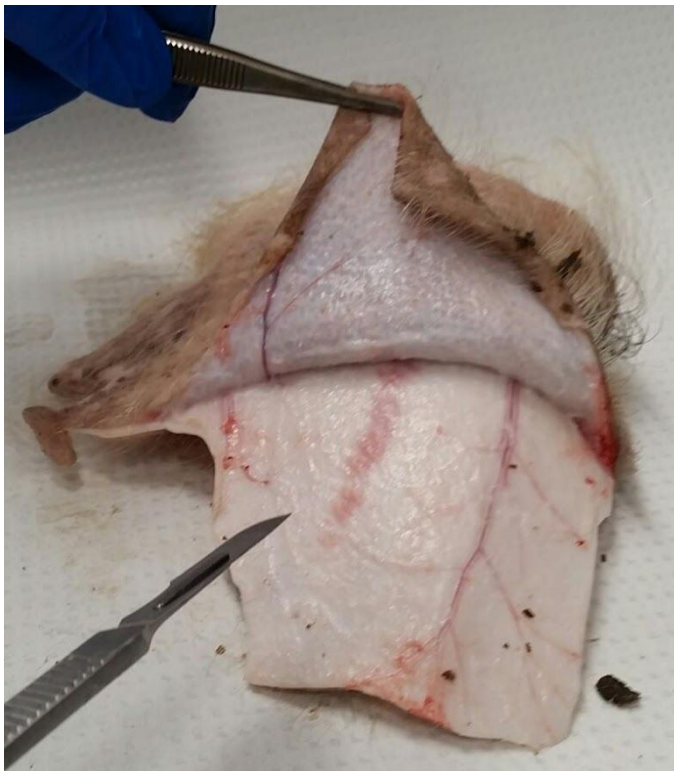

***Fig. 6*: Surgical isolation of skin from porcine ear**

The large skin specimen was cut into approximately 2 cm by 0.5 cm samples. Formulations, mixed with a black tissue marking dye (Ted Pella Inc.) that persists through tissue processing, were applied to these sections for five and thirty minutes, with sets at both room temperature and

37 ℃, and subsequently used for histology experiments (*Fig. 7*). Once formulation incubation time on the skin samples was complete, the samples were placed in histology cassettes and stored in 10% neutral buffered formalin overnight to fix the samples.

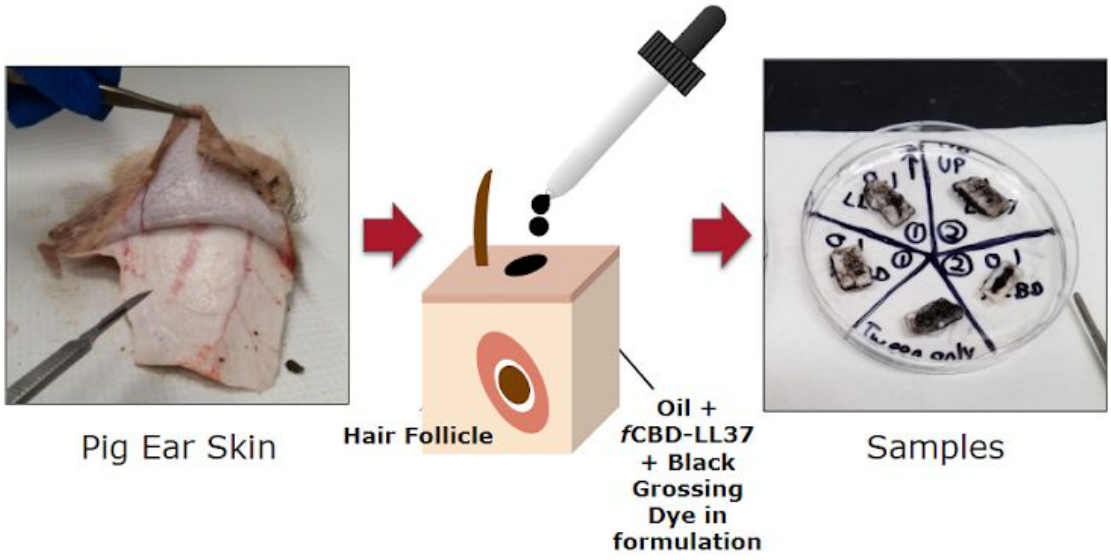

***Fig. 7*: Skin dissection and formulation application summary**

5.1.5.2 Histology

After the skin samples had been fixed in formalin, the cassettes were processed using Sakura's Tissue-Tek VIP 6 AI Vacuum Infiltration Processor. The processing step dehydrates the skin samples by displacing water in the samples with ethanol, and then subsequently displacing the ethanol with xylene. After processing, the tissue samples were embedded in paraffin wax, sectioned into 5µm thick slices, and mounted on glass slides in preparation for staining (*Fig. 8*). Skin samples were stained with Eosin, a colorimetric staining method, to reveal the detailed structure of the tissue. Sister skin sections were used for immunohistochemistry to detect the presence of the modified LL37 peptide.

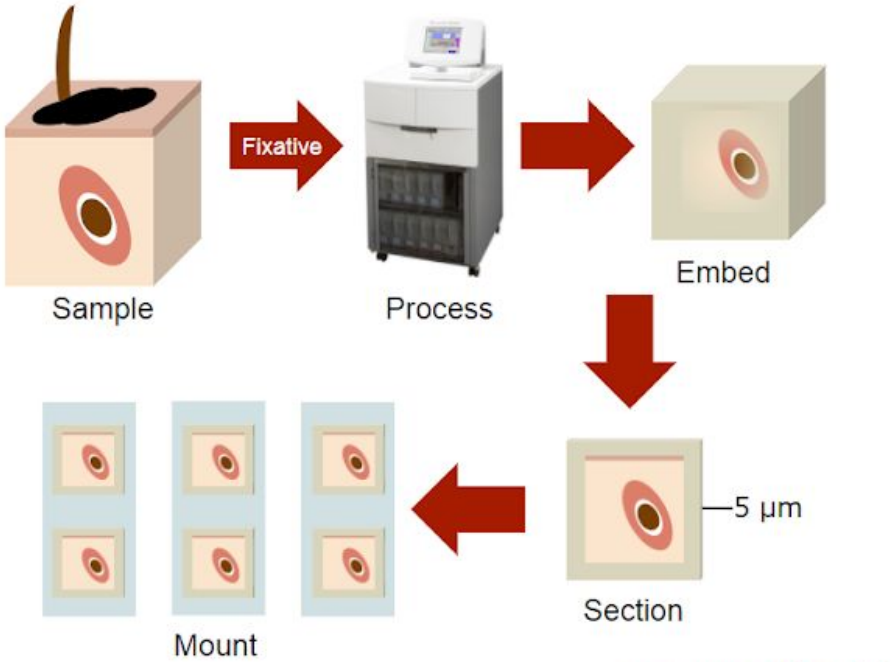

***Fig. 8*: Skin preparation process**

5.1.5.3 Colorimetric Staining

Once mounted, the glass microscope slides were loaded into a slide holder for staining. First, slides were soaked in three rounds of xylene solution for three minutes each to deparaffinize the sections. Slides were then rehydrated via two rounds of 100% ethanol solution for three minutes each, followed by 95% ethanol and 70% ethanol, each for three minutes. Slides were then inserted into the Eosin counterstain for 30 seconds, which colors eosinophilic structures (such as cell cytoplasms and extracellular proteins such as collagen) in shades of pink. Two rinses (3 minutes each) were then completed in 95% ethanol, followed by another three rinses (3 minutes each) in 100% absolute ethanol. Three additional rinses in xylene (2 minutes each) were completed before the slides were coverslipped using xylene-based Permount mounting medium and left to dry before imaging.

5.1.5.4 Hoechst and Anti-LL37 Immunohistochemistry (IHC)

Rabbit anti-LL37 polyclonal primary antibodies and goat anti-rabbit Alexa Fluor 488 secondary antibodies were used to locate the peptides within the skin samples. A Hoechst nuclear counterstain was applied in conjunction with the fluorescent antibodies to visualize the tissue morphology. For this, the team followed the protocol outlined by Lozeau et. al. [15]. Briefly, samples were deparaffinized in xylene and ethanol rinses. Samples were then boiled under pressure with a tris-EDTA buffer antigen retrieval solution (SigmaAldrich) for five minutes. Three PBS rinses (5 minutes each) were completed, followed by blocking with 5% v/v normal goat serum in PBS for 30 minutes at room temperature. After blocking, 10μg/mL primary rabbit polyclonal anti(human)-LL37 (Anaspec) in 3% v/v goat serum was added and samples were incubated overnight in a fridge at 4°C. Three PBS rinses were again completed. Alexa Fluor 488 goat-anti-rabbit antibody (LifeTechnologies), diluted 1:50 in 3% v/v goat serum was then added for 1 hour at room temperature. Samples were kept in the dark from this point forward. A Hoechst nuclear counterstain was then applied, and an addition three PBS rinses were conducted prior to adding coverslips using Prolong Gold mounting medium [15]. All slides were imaged at the same time within three days of staining and stored at 4°C.

5.1.5.5 IHC Pixel Intensity Analysis

In order to quantitatively assess the fluorescence intensity of the IHC samples, the fluorescence intensity of three different regions within each tissue specimen was determined. These regions were the dermal tissue, the epidermis, and the hair follicles (*Fig. 9*). Pixel intensity is defined as the sum of the brightness of each pixel within a given region, divided by the total

number of pixels within the region. This analysis allowed quantitative comparison of the fluorescence within the tissue and follicles between different skin samples and formulations.

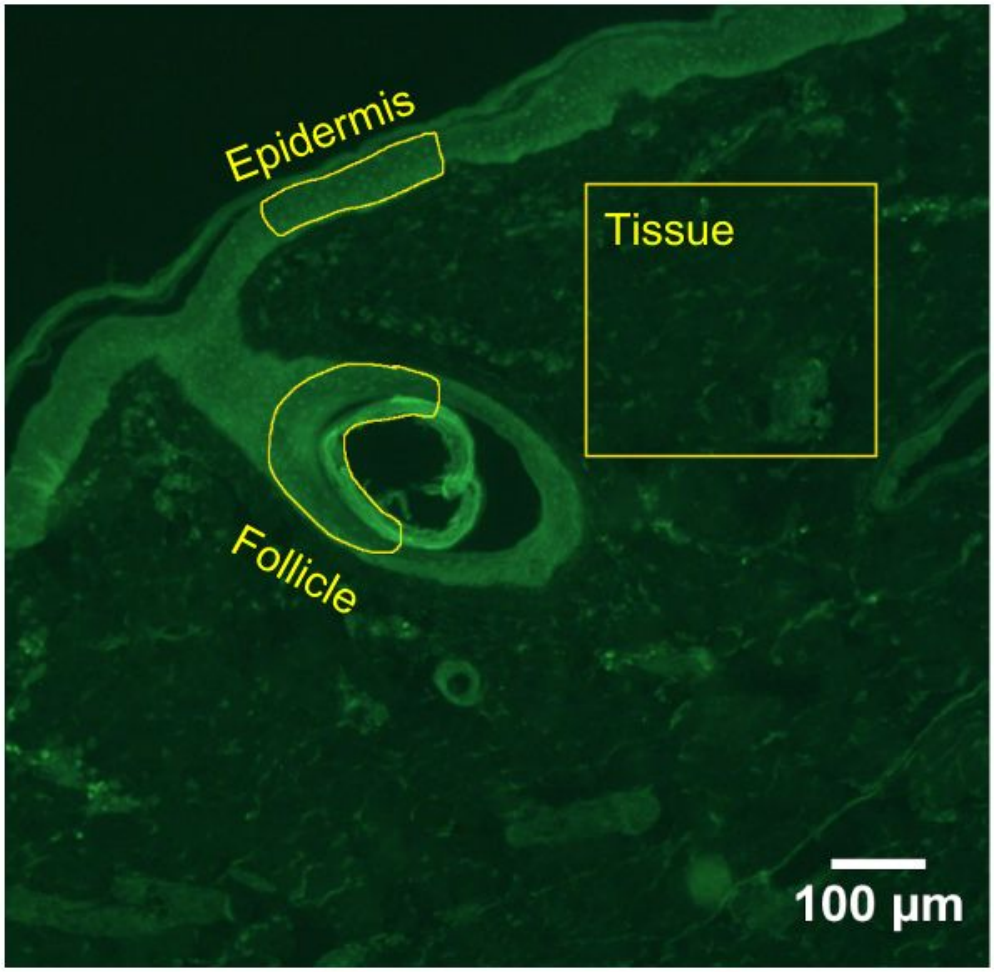

***Fig. 9*: Representative samples taken for IHC pixel intensity analysis.**

Three samples per region per slide were analyzed. Using the average pixel intensity and the variance associated with each group, a two-factor ANOVA was performed in order to determine if there was a statistically significant difference between the mean pixel intensity for each region and each peptide-containing delivery vehicle formulation.

5.2 Design Verification Results

The experiments described above facilitated selection of the final delivery vehicle and formulation components. The results of these experiments, as well as conclusions derived from these experiments that led to the design of the final formulation, are described in detail below.

5.2.1 Delivery Vehicle Activity

The MICs for soluble LL37 and *f*CBD-LL37 against *P. acnes* were found to be approximately 1.88 μM and 7.5 μM, respectively, demonstrated by the concentration at which the OD(590) for each peptide crosses the sterility control line, representing 100% killing. This result verified that the modified *f*CBD-LL37 peptide was active against *P. acnes* bacteria, and indicated the highest concentration of the peptide necessary in the formulation to kill *P. acnes* (*Fig. 10*). Therefore, this concentration of the modified *f*CBD-LL37 peptide was used for the oil-peptide combination assays.

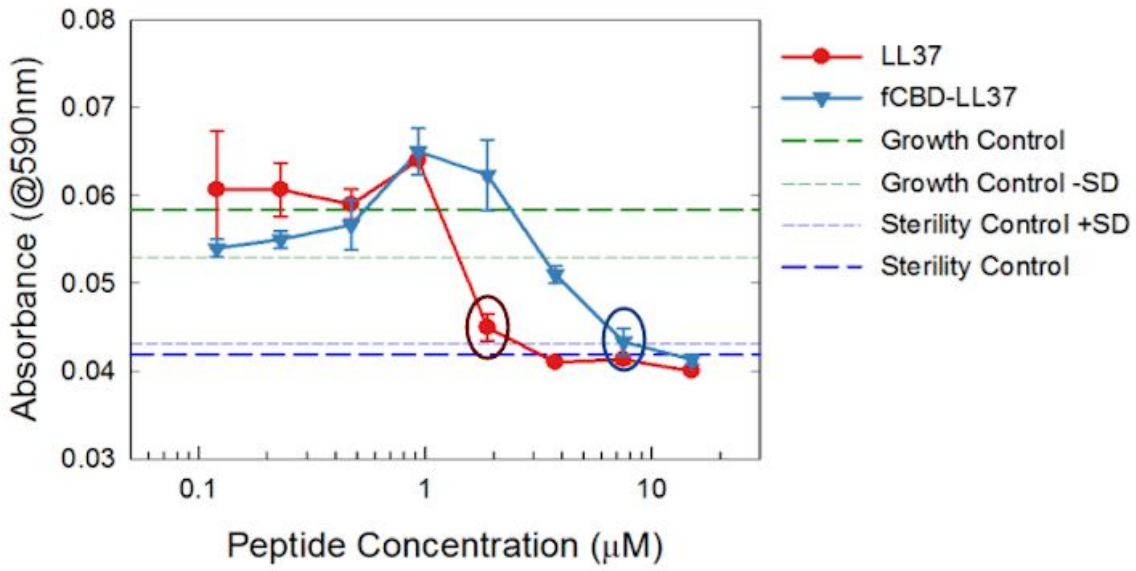

Fig. 10: **Soluble activity of LL37 and *f*CBD-LL37 against *P. acnes*, starting at a concentration of 15 µM. MICs were determined to be 1.88µM and 7.5µM for LL37 and *f*CBD-LL37, respectively.**

5.2.2 Essential Oil Antimicrobial Activity

The MICs for each of the six essential oils against *P. acnes* were determined (n=2) (*Fig. 11, Table 6*). Based on their effectiveness, manuka (MIC of 1%) and eucalyptus (MIC of 0.063%) oils were chosen to investigate in the combined activity assay.

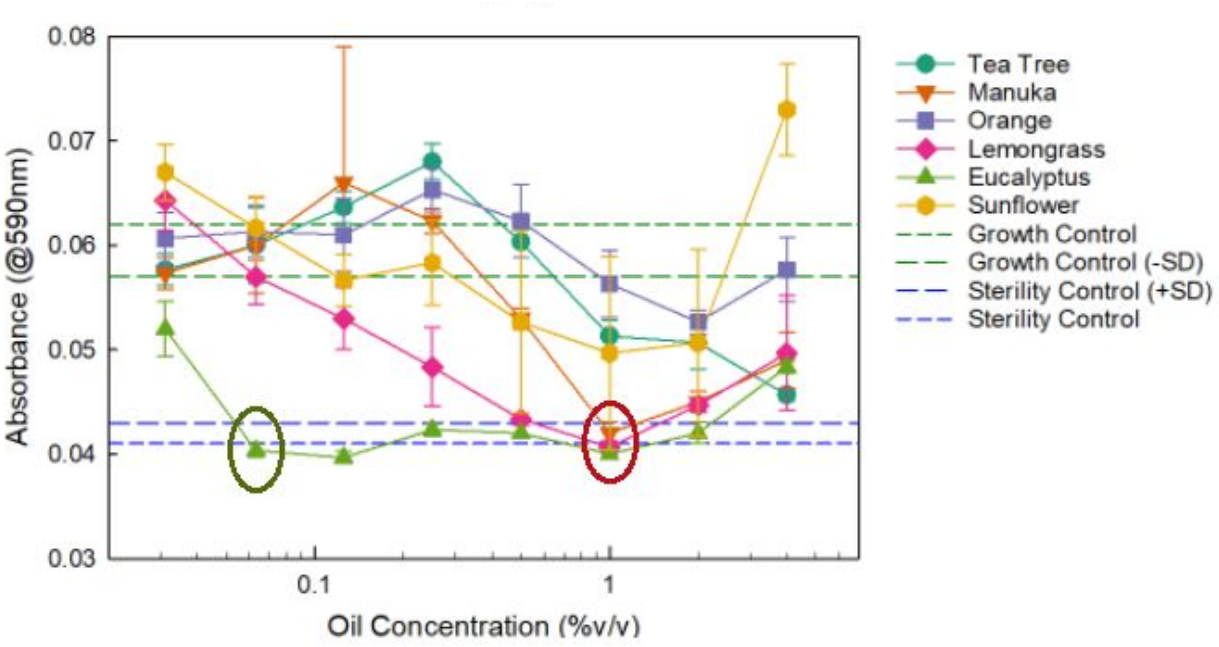

Fig. 11: **Six essential oils isolated antimicrobial activity *P. acnes.***

Table 6. **Results of Essential Oil Antimicrobial Activity Assays**

Essential Oil	MIC (% v/v)
Eucalyptus	**0.063**
Lemongrass	0.5
Manuka	**1**
Tea tree	4
Sunflower	>4
Orange	>4

Eucalyptus oil was chosen because it performed the best out of all six oils in requiring the lowest concentration to be effective against *P. acnes*. Manuka oil was chosen because, although

lemongrass oil performed slightly better, it has additional benefits for skin health, a vital component for a clinical skincare application [18].

5.2.3 Oil-Peptide Combination Activity

To determine whether or not the peptides retained their antimicrobial activity in combination with the essential oils in the delivery vehicle within a safe therapeutic window, a checkerboard dilution MIC assay was performed. Based on the MIC for *f*CBD-LL37 of 7.5 μM, the initial starting peptide concentration for both modified peptides in the serial dilutions was 7.5 μM. However, the team found that there was 100% visible growth reduction at all subsequent peptide concentrations. Due to this surprising result, the next assay instead began with 1 μM peptide to effectively determine the new MICs.

The results indicate that the combined activity of the peptide and oil significantly lowers the required concentration of peptide and oil to be active against *P. acnes*. For both sub-inhibitory concentrations of each oil, 100% killing was observed with all concentrations of the modified *f*CBD-LL37 peptide as low as 0.032 μM (*Fig. 12*). This means that, when paired with the essential oils, *f*CBD-LL37 experienced a near 250-fold decrease in its MIC.

A peptide-only control was used in conjunction with the growth and sterility controls to directly compare the antimicrobial activity of the modified peptide with and without the oil component. The peptide-only control resulted in MIC values that were consistent with the values found during the initial peptide-only MIC assays.

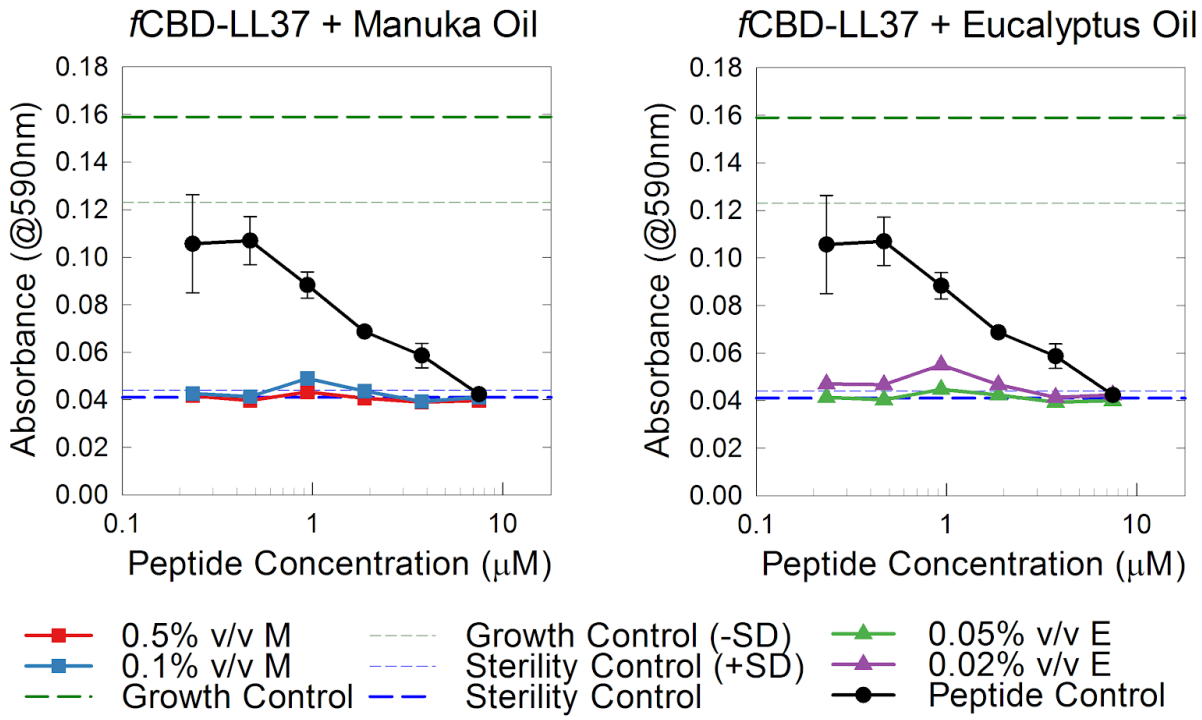

***Fig. 12*: Enhancement of *f*CBD-LL37 activity by inclusion of sub-inhibitory concentrations of manuka oil and eucalyptus**

5.2.4 Skin Diffusion

Results of the combined essential oil and *f*CBD-LL37 peptide demonstrated that eucalyptus 0.05% v/v performed the best out of the four formulations evaluated; therefore, this delivery vehicle was selected for use in the skin penetration studies. *Fig. 13* depicts the Eosin-stained, Hoechst-stained, and IHC-stained negative control slides, where no peptide was included in the assessed formulations. These Eosin-stained and Hoechst-stained negative controls allowed visualization of the hair follicle morphology, and the IHC slides provided a baseline fluorescence to compare with the images of the experimental formulation slides.

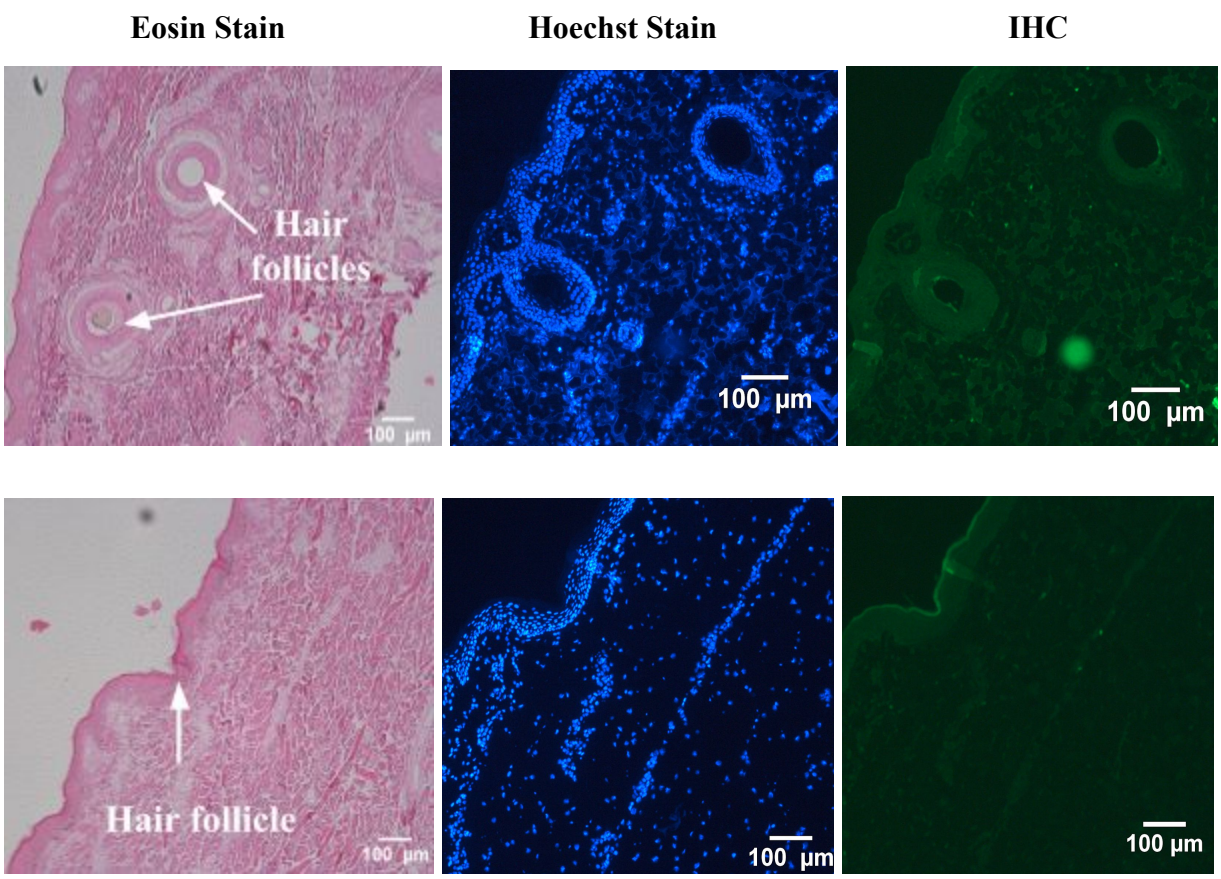

Fig. 13: **Eosin stain (Left), Hoechst stain (Middle), and IHC (Right) of both negative controls used - 0.05% Eucalyptus oil only (Top) and water + Tween® 80 only (Bottom). IHC results display baseline tissue autofluorescence for comparison to IHC with *f*CBD-LL37, and Eosin and Hoechst results display tissue morphology, including hair follicles, which are labeled.**

Experimental diffusion assay results comparing *f*CBD-LL37 solubilized in water versus in the delivery vehicle are shown below (*Fig. 14*). Increased fluorescence can be observed in the soluble *f*CBD-LL37 images compared to the negative controls portrayed above; the fluorescence on the epidermal layer and inside the hair follicle indicates that the peptide was able to diffuse into the hair follicle, following the path of least resistance. The additional increase in

fluorescence observed in the delivery vehicle formulation compared to the soluble *f*CBD-LL37 demonstrates that qualitatively, the delivery vehicle was able to enhance penetration and absorption of the peptide into the hair follicle.

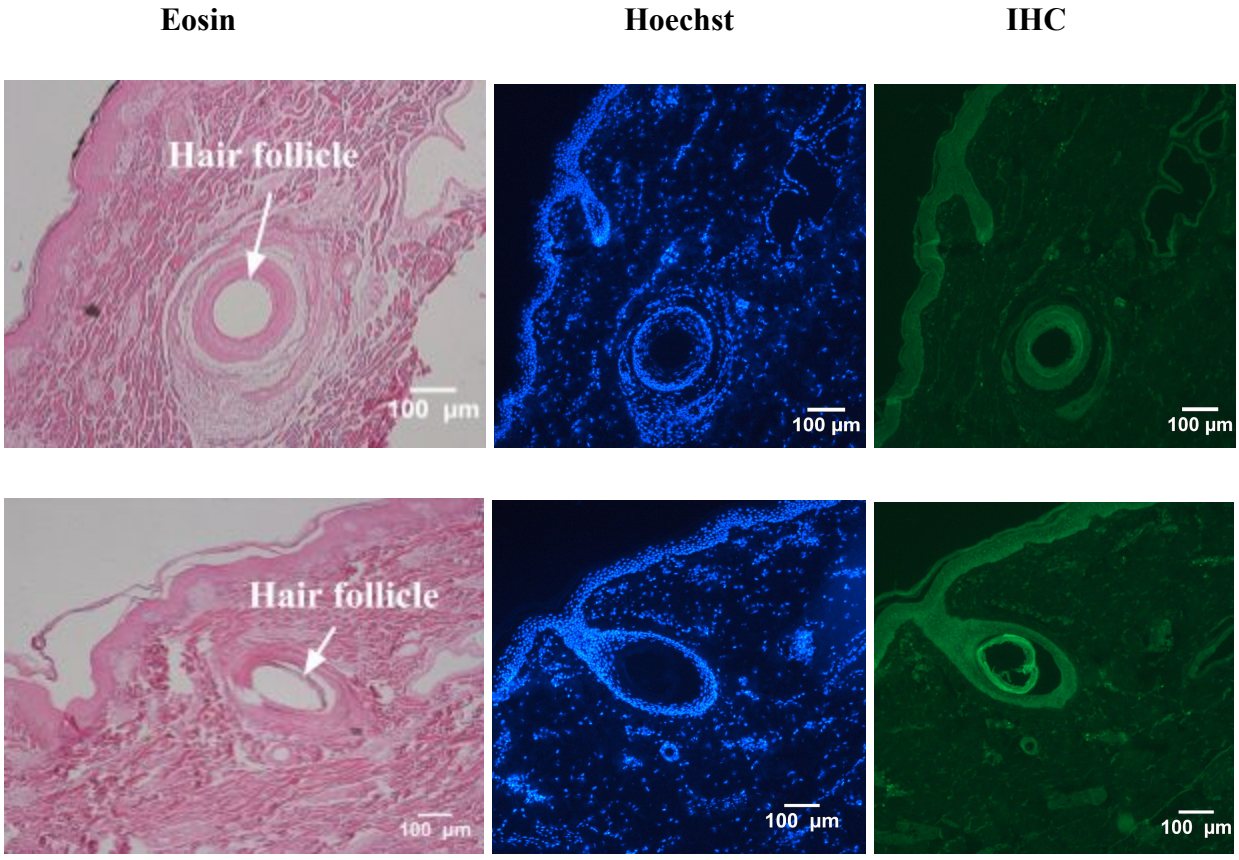

Fig. 14: **Eosin stain (Left), Hoechst stain (Middle), and IHC stain (Right) of *f*CBD-LL37 solubilized in water (Top) and in delivery vehicle formulation (Bottom). Qualitatively, the formulation treated samples showed the greatest fluorescent intensity within the hair follicles (labeled).**

5.2.5 IHC Pixel Intensity Analysis

A quantitative analysis of pixel intensity of the IHC images was performed. The average intensity of the three regions of interest within each skin sample show an increase of *f*CBD-LL37

within the 0.05% eucalyptus delivery vehicle, when compared to Tween® 80-only and *f*CBD-LL37 only controls.

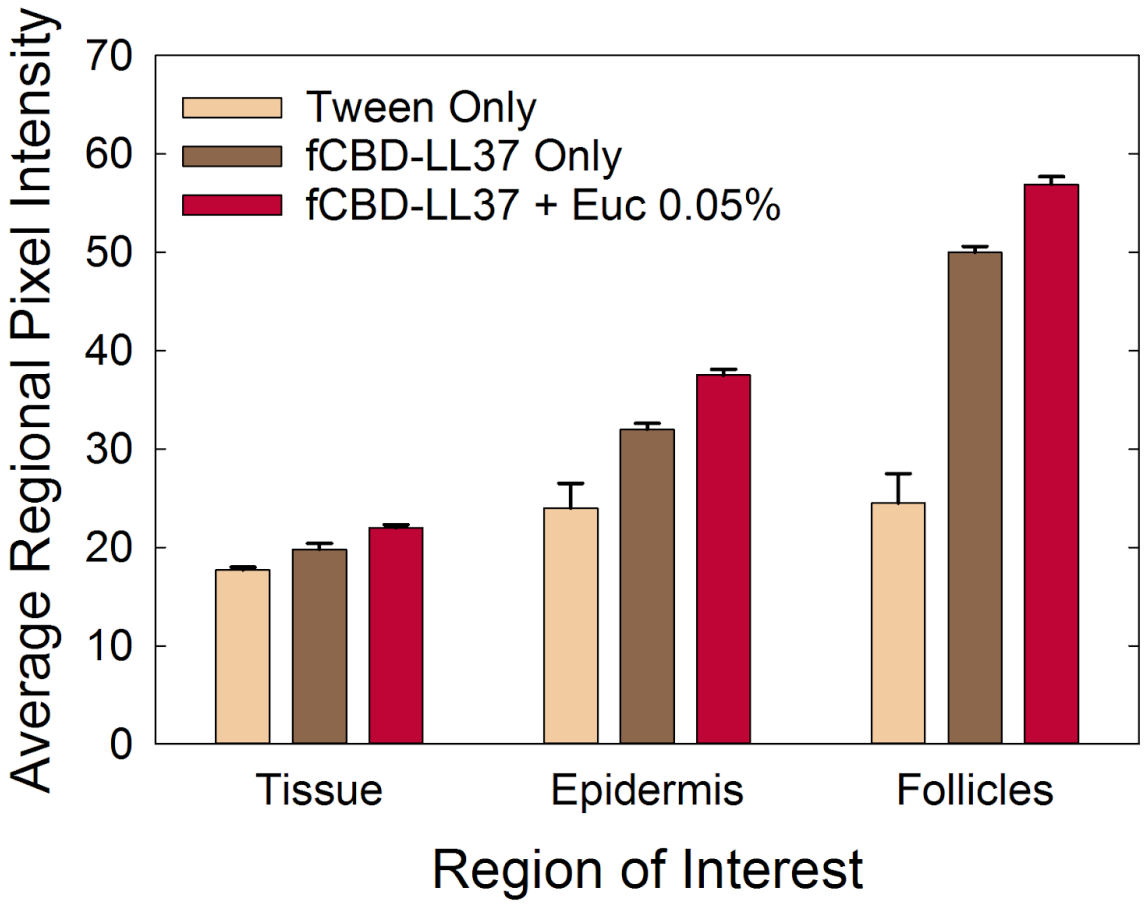

Fig. 15: **Pixel Intensity Analysis of the IHC images for the Tween® 80-only negative control, the *f*CBD-LL37 soluble peptide control, and the experimental *f*CBD-LL37 in the eucalyptus 0.05% delivery vehicle.**

The mean pixel intensities for each region and their associated variances were used to conduct a two-factor ANOVA. The ANOVA determined that the pixel intensities between the different regions were significantly different ($P < 0.05$) (*Table 7*).

***Table 7*. Two-factor ANOVA comparing the pixel intensity data of each tissue group**

Tissue Group	**N = ?**	**Mean (± S.D.)**	**P-value**
Epidermis	8	29.17 ± 6.362	0.000069
Follicle	8	38.96 ± 12.08	
Dermal tissue	8	20.38 ± 1.670	

A one-tailed t-test also determined that there was a statistically significant difference in the hair follicle fluorescence levels between the *f*CBD-only control and the essential oil delivery vehicle. The analysis revealed a significant difference between the *f*CBD-only control and the *f*CBD in the delivery vehicle (*Table 8*).

***Table 8*. One-tailed T-test of pixel intensity results between the *f*CBD-LL37-only control and the *f*CBD-LL37 in the delivery vehicle**

Tissue Group	***f*CBD-only Mean (± S.D)**	***f*CBD + delivery vehicle Mean (± S.D)**	**P-value**
Epidermis	32 ± 0.6	37.5 ± 0.6	0.0043
Follicle	50 ± 0.6	56.9 ± 0.8	0.0439
Dermal tissue	19.8 ± 0.6	22 ± 0.3	0.0245

6. Discussion

The goal of this project was to design a topical antimicrobial delivery vehicle and use it to evaluate the efficacy of *f*CBD-LL37 against *P. acnes* for the treatment of acne vulgaris in a safe and effective manner. This project set out to treat acne, the eighth most prevalent disease worldwide, with a novel design that eliminates the use of antibiotics while offering beneficial effects to the skin, rather than causing adverse reactions. *f*CBD-LL37 was chosen as the experimental target due to its low levels of cytotoxicity to mammalian cells compared to unmodified LL37. The critical objectives to be achieved in order for this novel design to be successful were that the delivery vehicle does not inhibit the antimicrobial activity of *f*CBD-LL37 against *P. acnes*, that the delivery vehicles allows for peptide activity within a safe therapeutic window, that the delivery vehicle facilitates penetration of *f*CBD-LL37 into the sebaceous glands, and that it must not be cytotoxic to human skin cells. Through numerous sets of experiments, including Minimum Inhibitory Concentration (MIC) assays and skin penetration assays, the team was able to demonstrate that the *f*CBD-LL37 peptide is antimicrobial and has enhanced effects in combination with manuka and eucalyptus essential oils. In addition, incorporation of essential oil carriers into the delivery vehicle improves the diffusion of the peptide into the hair follicles. Further experiments are required to demonstrate the cytotoxicity limits and diffusion characteristics of the formulations.

6.1 Analysis and Limitations of Experiments

The following sections include a detailed analysis of the results, placing them in context with past published studies, as well as presenting the potential limitations of each experiment. Overarching limitations included being trained and gaining proficiency in each procedure and time constraints for both conducting experiments and troubleshooting.

6.1.1 *f*CBD-LL37 Displays Antimicrobial Activity Against *P. acnes*

Establishing the antimicrobial effects of *f*CBD-LL37 against Gram-positive *P. acnes* was crucial to the success of an effective delivery vehicle design. This indicated effective performance against *P. acnes*, and that *f*CBD-LL37 was a plausible candidate for treating acne.

Using unmodified LL37 as a control in the soluble peptide antimicrobial activity procedure was essential because it ensured that the methods used to test antimicrobial activity were working as intended, since *f*CBD-LL37 had not been previously tested against *P. acnes*. This is because the modified LL37 peptides, including *f*CBD-LL37, were invented at WPI and had been tested against several bacterial strains, but not *P. acnes*. Our results found that unmodified LL37 performed more effectively, achieving compete killing at 1.88 μM, than shown by Harder *et al.* in 2013, who reported an MIC of 15 μM, which is likely due to the use of varying methods and lots of LL37 and different lots of *P. acnes* [46].

6.1.2 Essential Oils Kill *P. acnes* Independently

Identifying independent antimicrobial activity indicated that essential oils would likely not inhibit peptide efficacy within the delivery vehicle. Published studies report antimicrobial activity for tea tree oil, lemongrass oil, orange oil, sunflower oil, eucalyptus oil, manuka oil against various bacteria, including Gram-positive strains similar to *P. acnes* [69-75] (*Table 9*). The antimicrobial activity of essential oils is largely due to their hydrophobicity, which allow them to easily diffuse through the phospholipid bilayer of bacterial cell membranes and disturb cellular structures [74]. Altered permeability of bacterial cells leads to leakage of cellular contents, ultimately leading to microbial cell death [74].

However, three of the six essential oils that were evaluated (manuka, orange, and sunflower) had no published data regarding their MICs against *P. acnes* specifically. Of the remaining three oils, only the results from lemongrass oil were close to the published value. The MIC found for tea tree oil (4% v/v) was higher than the published value (0.25 - 1% v/v), and the MIC found for eucalyptus oil was lower than the published value (4% v/v) [75]. This discrepancy could be due to a number of factors, including differing levels of quality and purity between the oils used in this project and in the literature studies. The specific strains of *P. acnes* used in this study and in the literature studies could also have influenced the concentrations of oils required to kill the bacteria. Despite the differences between published and measured values, this does not affect the validity of the results because each oil-only MIC assay trial yielded consistent results.

Table 9. Essential oil MIC values compared to published values

Essential Oil	Experimental Value (% v/v)	Published Value (% v/v)	References
Eucalyptus	0.063	4	[75]
Lemongrass	0.5	0.125	[75]
Tea Tree	4	0.25 - 1	[70,71,72]

For a professional topical formulation development, further experimentation would be required with essential oils from multiple sources as well as different strains of *P. acnes* bacteria to determine which oils were most effective at inhibiting growth of the bacteria in general cases. However, this was outside the scope of this project.

6.1.3 Essential Oils Enhance *f*CBD-LL37 Activity Against *P. acnes*

When tested in combination, the effects of the combined essential oil and *f*CBD-LL37 treatment were significant compared to soluble *f*CBD-LL37 alone. Not only did manuka and eucalyptus oils allow for peptide efficacy within a safe therapeutic window, but it exhibited enhanced activity at concentrations up to 30-fold lower than *f*CBD-LL37's MIC, 7.5 μM.

Since the concentrations of the manuka and eucalyptus oils used in this assay were lower than the isolated MICs of the oils found through the essential oil activity assays, the oils alone did not kill as well as the combination formulations (*Table 10*). It has been shown that hydrophobic environments cause LL37 to gain a more helical structure, making it more active [61]. With this in mind, our team expected the modified *f*CBD-LL37 peptide to work similarly, thus forming the basis for the combination therapy incorporated into the delivery vehicle design.

Table 10. **Comparison of Isolated MICs and Combination Therapy MICs**

	***f*CBD-LL37**	***f*CBD-LL37 + 0.5% Manuka**	***f*CBD-LL37 + 0.1% Manuka**	***f*CBD-LL37 + 0.05% Eucalyptus**	***f*CBD-LL37 + 0.02% Eucalyptus**
MIC	7.5 μM	0.032 μM	0.032 μM	0.032 μM	0.23 μM

It is unlikely that these results are attributable to any factors other than the oils enhancing peptide activity. The exact mechanism behind how this occurs will be studied in the future, as both peptide structure (using circular dichroism spectroscopy) and membrane interactions (using quartz crystal microbalance analysis) can be evaluated.

6.1.4 Essential Oils Enhance Delivery of *f*CBD-LL37 to the Hair Follicles

Peptide diffusion studies using the *ex vivo* porcine ear skin model were successful, and results indicate that delivery vehicle may improve the ability of *f*CBD-LL37 to diffuse into the hair follicles and may therefore actively eliminate *P. acnes* where it grows.

The pixel intensity analysis data were analyzed for statistical significance using a one-tailed T-test and an analysis of variance (ANOVA). The one-tailed T-test was used to analyze the differences in pixel intensity, and thereby diffusion, between the *f*CBD-LL37 which was solubilized in DI water and the *f*CBD-LL37 which was in the delivery vehicle with 0.05% v/v eucalyptus oil. Within a 95% confidence interval, the one-tailed T-test revealed a statistically significant ($P<0.05$) difference between the pixel intensities for each of the epidermal layers, hair follicles, and dermal tissues of these two sample groups The t-test data quantitatively confirm

that the delivery vehicle significantly improves delivery of *f*CBD-LL37 not only through the skin, but also into the hair follicles.

The ANOVA test was used to analyze the differences between the pixel intensities of each tissue region using the values for every tissue sample. These data suggest that *f*CBD-LL37 diffuses most effectively into the hair follicles and the epidermis, while not entering a significant portion of the dermal tissue. This indicates that *f*CBD-LL37 in general will likely be effective at specifically targeting the *P. acnes* bacteria in the hair follicles and sebaceous glands while avoiding any potential off-target effects. This also indicates that *f*CBD-LL37 cannot non-specifically diffuse past the epidermis, and thus is not diffusing through the dermal tissue. Instead, *f*CBD-LL37 is following the path of least resistance to enter the hair follicles, which are open to the surface of the skin.

6.1.5 Cytotoxicity

Cell viability assays using adherent fibroblasts were attempted; however, the team was not able to directly measure the effect of the formulations on the skin cells, as either the oil components or the emulsifying components of the delivery vehicle prevented cellular adherence within the 96-well plate. It is important to note that with extensive literature consultation, the team found that all of the ingredients utilized in the final designed delivery vehicle have been shown to be safe for use. First, the concentration of the *f*CBD-LL37 peptide included in the delivery vehicle is significantly lower than the reported cytotoxic concentrations of both the developed *f*CBD-LL37 peptide and unmodified LL37, at a 1μM concentration versus the

cytotoxic concentrations of 20μM and 13μM, respectively [15,79]. In addition, both manuka oil and eucalyptus oil have been found to be non-cytotoxic or non-irritating up to 10% v/v [18,19].

6.2 Project Impact Analysis

6.2.1 Economics and Manufacturability

In the United States, over the counter (OTC) acne treatment manufacturing costs over $600 million, and prescription acne treatment manufacturing costs exceed OTC [80]. Since our designed product is a biologic, it would likely become part of the prescription acne treatment market once it becomes commercial. In that niche, it will have the potential to disrupt a market which is dominated by large, well-established companies, as the top two prescription products boast 44% of the total market share [81]. The team believes that the emphasis on minimal and natural ingredients to carry and assist the activity of *f*CBD-LL37 will ultimately drive adoption of the final product. In doing so, it would ideally gain a strong foothold in the prescription acne drug market.

Our design lowers the amount of peptide necessary for effective treatment, and therefore becomes more economically efficient. However, the manufacturability of the peptides is an area that needs to be investigated further (as is the case with AMPs in general), as the current cost/mg of peptide is high.

6.2.2 Environmental Impact and Sustainability

If the project progresses to a point where it succeeds in clinical trials and moves on to be commercialized, there will have to be consideration on how its essential oil components will be

sourced and extracted. It will be important to only purchase essential oils from reputable suppliers that emphasize care for the environment. This is because the distillation of essential oils from their corresponding plants requires a high amount of plant for a relatively small yield of oil. For example, da Costa et al. found that essential oils distilled from *Blepharocalyx salicifolius* and *Myracrodruon urundeuva* had yields of 0.045 - 0.069% and 0.08 - 0.13%, respectively [82]. The researchers calculated percent yield by dividing the mass (in grams) of the extracted oil by the initial plant biomass and multiplying by 100 [82]. These findings indicate that, to produce one gram of *B. salicifolius* essential oil, 145000 - 222000 grams (320 - 490 lbs) of the plant would be required, and to produce one gram of *M. urundeuva* essential oil, 76000 - 125000 grams (167 - 275 lbs) of the plant would be required [82]. While these oils are not directly applicable to our project, these findings indicate how important conscious sourcing will be in order to reduce the environmental footprint of the final product.

In addition to sourcing, Afzal et al. were able to utilize a solar-based system for essential oil distillation for eucalyptus and peppermint oils [83]. The solar energy steam receiver used for the eucalyptus and peppermint oil proved to be much more efficient than the biomass fire tube vertical boiler that was used to distill the pinus oil at 2.32 - 2.54 kWh of thermal energy required compared to 11.42 kWh of energy required [83]. The solar-based distillation system was not only more efficient than the biomass boiler, but it also produced a higher percent yield of oil than traditional methods described in previous literature [83]. By using clean, renewable energy and continuing to create techniques that maximize percent yield, essential oil distillation can become a much more sustainable and environmentally-friendly process [83].

Finally, it is important that the essential oils that are ultimately utilized in the product do not come from species that are listed as threatened by the International Union for Conservation of Nature (IUCN). Currently, neither *Leptospermum scoparium* (Manuka) nor *Eucalyptus globulus* are classified by the IUCN, so information regarding their conservation status is limited.

6.2.3 Societal Influence and Political Ramifications

As stated in section 2.1.3, acne is the most common skin disease among adolescents, with approximately 60% suffering from at least mild acne [23]. With our formulation working as intended, killing acne in the pilosebaceous unit with beneficial ingredients for the skin, the psychological problems associated with acne can be minimized. A decrease in acne will contribute to higher self-esteem and confidence.

Additionally, if the project succeeds and becomes a commercially-available product for prescription, it will likely cause a major increase in scientific research related to the potential beneficial effects of essential oils. There may also be greater emphasis in using essential oils as carriers for other biologics that are not always effective during *in vitro* and *in vivo* testing on their own. Overall, the concept of antimicrobial peptide and oil synergy has the potential to be incredibly influential in the scientific community, since it has not been widely investigated to date.

In the political sphere, product success would likely result in more specific regulations targeted toward essential oils, including a requirement that manufacturers make their material safety data sheets (MSDS) easy to find on their websites, and a provision that prevents

manufacturers from misrepresenting or exaggerating the health benefits of particular oils. Additionally, the success of this product would create precedent for the use of AMP therapeutics, and may assist in others being approved. Finally, the FDA may also include essential oils that are used for non-food applications in their GRASE lists.

6.2.4 Ethical Concerns

Our developed therapeutic formulation must be validated for its intended use, as well as confirmed to be safe for human use. As with other cosmetics and medicinal products on the market, our product will need to be validated through animal testing prior to proceeding to clinical trials. In animal testing, extensive lengths will need to be taken in order to ensure appropriate care and use of the animals. Each institution has an Institutional Animal Care and Use Committee whose role is to supervise and uphold scientific protocols and standards that must be followed with any animal trial. Such standards include humane living conditions for the animals and minimizing pain for the animals. Even with standards in place, sometimes ethical care is not present, and to avoid this, more and more companies are avoiding animal testing altogether.

Ideally, our product will be validated using an animal testing alternative. Cosmetic and skincare companies developing both common use products as well as clinical dermatology products are increasingly using three-dimensional, human cell derived skin model structures that are produced from mammalian cells. An example of this is MatTek's "EpiDerm Tissue Model", which replicates key traits of normal human skin. Such models can be used for skin irritation testing as well as skin diffusion and penetration assays. In addition, Tergus Pharma has

experience in developing acne-specific disease models to assist in designing topical formulations using effective surrogate disease models. Computer modeling of skin is also an up and coming animal testing alternative.

Additionally, because different essential oils need to be distilled from different crops grown all over the world, it will be imperative to make sure that the oil companies that we purchase from are not using crops harvested by exploited people in the global south that are paid low wages or exposed to unsafe working conditions. Fair trade essential oils will be of the utmost importance.

6.2.5 Health and Safety Concerns

In using essential oils as a carrier vehicle for the antimicrobial peptides, it is critical to identify any associated health or safety issues. Typically, essential oils are generally regarded as safe, having minimal adverse effects. The primary safety concern is extreme skin irritation or sensitivity, including contact dermatitis, due to a highly concentrated oil, such as a 100% pure oil composition [84]. Pure essential oils are generally not used at 100% v/v concentration for topical use or in aromatherapy [84]. In this project, the formulation involves significantly diluted concentrations of oil, and therefore does not pose a health or safety risk to potential users. Essential oils should also not be ingested as they can be toxic, producing adverse effects such as nausea, depression of respiration, and vomiting. Eucalyptus oil requires large amount of ingestion, approximately 30 mL to produce such symptoms [85].

Additionally, the modified AMP used in formulation has not been tested *in vivo* to date, and therefore should be conducted in clinical trials prior to commercial use in the designed

product. Any potential side effects are unknown and undocumented, requiring further investigation. It is important to note that AMPs pose challenges that limit their use, primarily their toxicity to mammalian cells when used in high concentrations. Because of this, they have a low therapeutic index, which is calculated by taking the ratio of the toxic dose over the effective dose. The modified AMP used in this project has shown to be non-toxic at higher concentrations through mammalian cell-based cytotoxicity assays [15]. As the essential oils synergize with the modified peptides at extremely low concentrations to kill *P. acnes*, it is likely that toxicity will not be a major concern with this formulated product.

7. Final Design and Validation

7.1 Description of Final Design

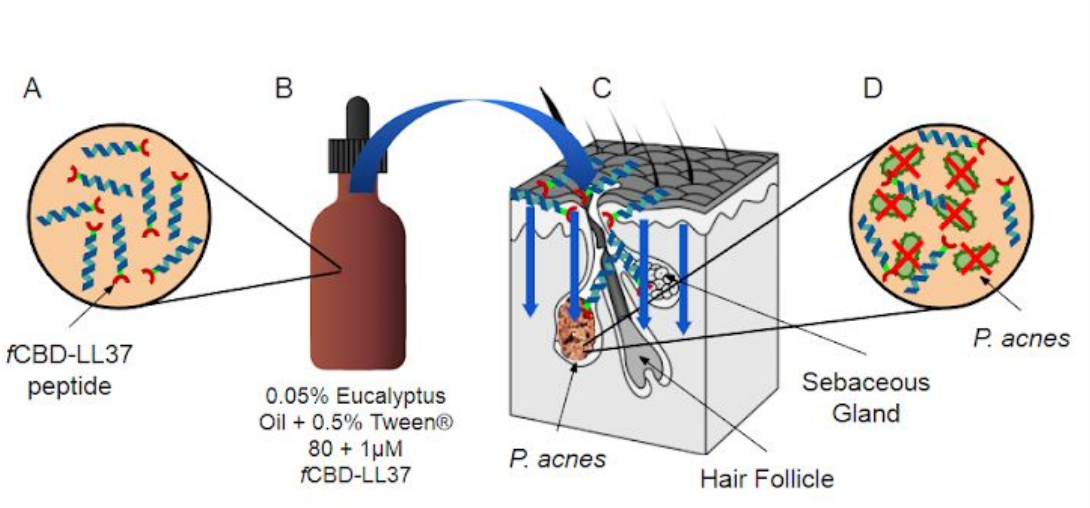

Fig. 16: **Schematic depicting the final delivery vehicle design. *f*CBD-LL37 (A) in the delivery vehicle (B) is being applied to the skin (C) to kill *P. acnes* within the sebaceous glands (D).**

Through repetitions of the experimental design process, the final design was developed and validated. The final delivery vehicle involves three finalized components: 1 μM *f*CBD-LL37 in an aqueous solution, 0.5% Tween® 80 emulsifier, and 0.05% v/v eucalyptus essential oil. While each experiment has used culture medium to dilute and suspend the peptides, the final formulation for preclinical and clinical trials will include *f*CBD-LL37 in deionized (DI) water. The final design comes together as an oil-water delivery vehicle carrying *f*CBD-LL37 to the target site for acne treatment: the sebaceous gland within the pilosebaceous unit.

The peptide was first prepared from 670 μM stock in a concentration double the desired final concentration. This is because the peptide solution was combined 50/50 with the diluted essential oil. To dilute the essential oils to a safe concentration in DI H_2O, the water was first supplemented with 0.5% v/v Tween® 80 as an emulsifier. To effectively mix the Tween® 80 with the water, it was warmed, pipetted in, and then the solution was vortexed to mix. Then, the oils were diluted in the water to double their final concentration for the same reason mentioned above. This means that eucalyptus oil was diluted to a concentration of 0.1% v/v. To ensure that the oils were fully mixed, they were vortexed as well.

7.2 Cost Analysis

Due to the low concentrations of essential oil required for the delivery vehicle to be effective, the *f*CBD-LL37 currently makes up the majority of the cost. Buying eucalyptus oil in bulk costs $67.50/pint (~67.50/450 mL), and Tween® 80 only costs $125/gallon, or ~$15.63/pint [86-88]. In contrast, *f*CBD-LL37 costs $21/residue/mg. When buying peptide products, a residue represents an amino acid. *f*CBD-LL37 contains 62 amino acids, thus costs $1302/mg. This is one of the major reasons why it was so important for the team to find a way to widen the therapeutic window, thus lowering the minimum peptide concentration needed for killing, so that the final product would not be prohibitively expensive. Thus, for this project to eventually be commercialized, it is essential that novel manufacturing techniques be used to scale-up peptide production in order to greatly lower the price per unit.

8. Conclusions and Recommendations

In conclusion, the team designed a delivery vehicle for the effective delivery of the modified LL37 peptide, *f*CBD-LL37, for the treatment of acne vulgaris. Through an extensive process of iterating experiments, we were able to show that the incorporation of essential oils into the delivery vehicle enhances the antimicrobial activity of *f*CBD-LL37, thereby widening the therapeutic window of the biologic. This would in turn lower the cost to manufacture this product, as AMPs are expensive to produce, and improve the safety profile. In addition, the essential oil carrier enhanced penetration of *f*CBD-LL37 into the pilosebaceous unit, delivering a large molecule through the skin, a significant challenge that has limited the therapeutic use of AMPs clinically.

A novel combination therapy approach was discovered, and the team alongside Professor Marsha Rolle, Professor Terri Camesano, and Dr. Lindsay Lozeau, have filed a provisional patent application for the the use of *f*CBD-LL37 in combination with the essential oils.

8.1 Future Work

The development of a viable topical biologic treatment leaves an extensive amount of work for teams to do in the future. Typically, the formulation design process on its own takes at least two years because it ultimately aims to optimize the product as much as possible. This project formed the basis for the beginnings of a potential formulation, but it will take more time

to truly have a final list of ingredients and an understanding of all the manufacturing processes necessary to transform this project into a marketable product. Additionally, after the formulation design process is completed, teams can work on determining the most optimal way to deliver the peptides, whether it simply requires rubbing the product onto the skin or applying the formulation to a face mask, among other options. The details on the future work that the team believes is possible are described below.

8.1.1 Cytotoxicity Testing

Additional cytotoxicity testing via cell-based assays or skin irritation models will be necessary to confirm the safety of the design. Current alternatives to animal testing include: skin-on-a-chip, engineered human tissue, and human skin cultures.

8.1.2 Formulations

In the future, it would be beneficial to test the formulation in a chronic acne infection model or living skin tissue model in order to validate the safety and efficacy of the delivery vehicle. Additionally, further formulation development for clinical use is recommended. The team recommends that in the future, two or more oils be tested together to see if there is any added combination effect that makes it even more effective than a single oil with *f*CBD-LL37.

8.1.3 Circular Dichroism Spectroscopy

In order to assess the mechanism of the peptide's enhanced activity in the essential oil delivery vehicle, circular dichroism (CD) spectroscopy should be used to analyze the structure of the peptide. Circular dichroism is a method that can be use to evaluate the secondary structure

and folding properties of proteins, which thereby can be utilized to evaluate whether or not the incorporation of the peptide into the delivery vehicle affects the conformation of the peptide [89]. Briefly, circular dichroism involves measuring the unequal absorption of left-handed and right-handed circularly polarized light interacting with molecules [89]. The team hypothesizes that the hydrophobic essential oil carrier alters the conformation of the peptide to be more α-helical. The conformational change induced in this hydrophobic environment may enhance the peptide's ability to attach and insert into cell membranes, thereby enhancing its activity [61].

8.1.4 Quartz Microbalance Analysis

In addition to the circular dichroism analysis of the peptide's structure in the delivery vehicle, quartz microbalance with dissipation (QCM-D) analysis can be used to model membrane interactions between the peptide and bacterial cell membrane [90].

8.1.5 Application Techniques

Future directions for this project include designing and evaluating different application techniques or devices to administer the designed delivery vehicle more efficiently. Preliminary suggestions, described below, could be evaluated based on performance.

8.1.5.1 Face Sheet Mask Topical Delivery Technique

Face sheet masks are face-shaped masks, usually made of a mixture of paper, fibers, or gels, and are typically soaked in a nutrient-rich serum [91].These masks are used once per sheet and are easy/convenient to use. The delivery vehicle designed in this project could be used to soak the face sheet mask. This would mean that the peptide is delivered to the skin uniformly

through the mask, as well as provide a convenient way for prolonged application, as these masks are normally worn for approximately 20 to 30 minutes. This conceptual design presents opportunity in a flourishing, up and coming market in the United States, with a study by the NPD Group in the USA reporting increased sale of masks by over 60% [91].

8.1.5.2 Mechanical Brush Design

Another design concept builds off of two existing skin cleansing techniques that have become fairly popular, skincare brushes and attachable pads with motorized vibration or oscillation. In current products, the brush design typically has soft bristles, or the device allows for attachable pads that contain a cleansing solution to be applied to the skin. These brushes deliver constant pressure to effectively exfoliate the skin, enabling skin care products applied after washing with the brush to be absorbed more easily [92].

The exfoliation from the brush combined with the therapeutic potential of the peptide formulation could facilitate absorption of the peptide into the sebaceous glands. A secondary design alternative within the realm of mechanical devices revolves around the mechanical device with an attachable pad soaked in solution. The peptide solution could be soaked into a pad that can be attached to a mechanical device, similar to the brush. The rotation of the brush would allow for exfoliation and better penetration of the drug into the skin.

8.2 Recommendations

Throughout the duration of this project, the team encountered a number of avoidable challenges that slowed progress down, as all of the procedures being conducted were new to us.

The following recommendations are provided to offer improved experimental design and validation testing in future projects.

For future experiments that involve *P. acnes*, we recommend growing the liquid culture in 5 mL glass tubes, covered with sterile aluminum foil, in a BD GasPak™ EZ chamber for anaerobic bacterial growth. The GasPak™ sachets should be changed every 2-3 days to ensure that an anaerobic environment is sustained within the chamber. Additionally, growth of liquid cultures should begin at least one week prior to experiments, to allow for an adequate number of bacteria for an effective McFarland standard. This is because *P. acnes* has a doubling time of five hours, which is much slower than more commonly used bacterial strains.

For mammalian cell culture, when using polysorbate 80, or Tween® 80, it is critical that the concentration used is not toxic to mammalian cells. We found that ~0.5%v/v Tween® 80 was too concentrated against CRL-2352 human fibroblast cells, and found in literature that formulations typically test 0.05% v/v.

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Appendix A. MIC Assay: Soluble Peptide Activity

Number of Experimental Replicates: 2 (MRSA), 2 (P. acnes)

Aim: Investigate the soluble activity of LL37, *f*CBD-LL37, and *c*CBD-LL37 against Methicillin-resistant *Staphylococcus aureus* (MRSA) or *Propionibacterium acnes*.

Purpose: For MRSA, ensure the peptides are active against an aggressive Gram-positive bacteria and confirm their isolated minimum inhibitory concentrations (MICs) with those found in the literature. For *P. acnes*, ensure our peptides are active against the bacteria linked to acne and determine their isolated MICs, as these values have never been experimentally determined before.

Materials:

- 96-well plate
- *f*CBD-LL37 (30µM, suspended in PBS)
- *c*CBD-LL37 (30µM, suspended in PBS)
- LL37 (30µM, suspended in PBS)
- MRSA or *P. acnes* liquid culture
- Bacterial medium (Mueller Hinton broth or tryptic soy broth, half-strength)
- Anaerobic incubation chamber (BD GasPak™ EZ)
- CO_2 gas sachets (BD GasPak™ EZ)
- Spectrophotometer/microplate reader
- Pipettes and tips

Setup: Performed in triplicate (n=3)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control		LL37 15uM	LL37 15uM	LL37 15uM	fCBD 15uM	fCBD 15uM	fCBD 15uM	cCBD 15uM	cCBD 15uM	cCBD 15uM	Growth control
B	Sterility control		LL37 7.5uM	LL37 7.5uM	LL37 7.5uM	fCBD 7.5uM	fCBD 7.5uM	fCBD 7.5uM	cCBD 7.5uM	cCBD 7.5uM	cCBD 7.5uM	Growth control
C	Sterility control		LL37 3.75uM	LL37 3.75uM	LL37 3.75uM	fCBD 3.75uM	fCBD 3.75uM	fCBD 3.75uM	cCBD 3.75uM	cCBD 3.75uM	cCBD 3.75uM	Growth control
D	Sterility control		LL37 1.875uM	LL37 1.875uM	LL37 1.875uM	fCBD 1.875uM	fCBD 1.875uM	fCBD 1.875uM	cCBD 1.875uM	cCBD 1.875uM	cCBD 1.875uM	Growth control
E	Sterility control		LL37 0.938uM	LL37 0.938uM	LL37 0.938uM	fCBD 0.938uM	fCBD 0.938uM	fCBD 0.938uM	cCBD 0.938uM	cCBD 0.938uM	cCBD 0.938uM	Growth control
F	Sterility control		LL37 0.469uM	LL37 0.469uM	LL37 0.469uM	fCBD 0.469uM	fCBD 0.469uM	fCBD 0.469uM	cCBD 0.469uM	cCBD 0.469uM	cCBD 0.469uM	Growth control
G	Sterility control		LL37 0.234uM	LL37 0.234uM	LL37 0.234uM	fCBD 0.234uM	fCBD 0.234uM	fCBD 0.234uM	cCBD 0.234uM	cCBD 0.234uM	cCBD 0.234uM	Growth control
H	Sterility control		LL37 0.117uM	LL37 0.117uM	LL37 0.117uM	fCBD 0.117uM	fCBD 0.117uM	fCBD 0.117uM	cCBD 0.117uM	cCBD 0.117uM	cCBD 0.117uM	Growth control

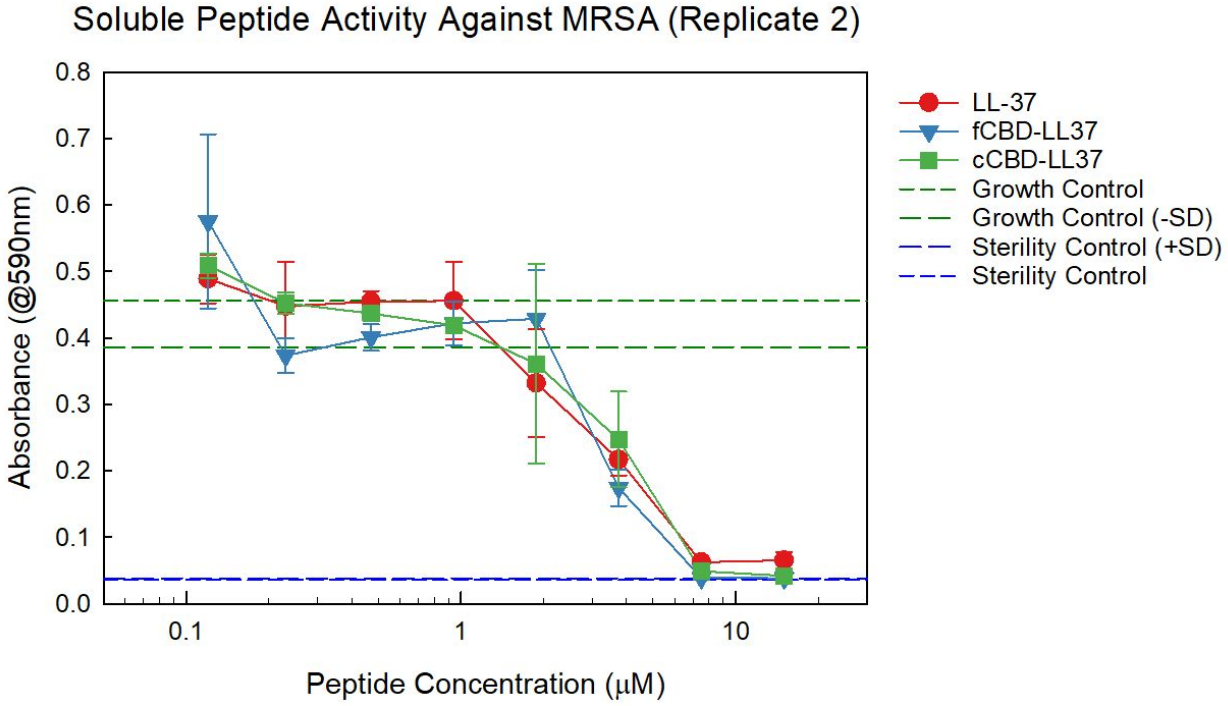
Controls: Bacteria only (growth control), broth only (sterility control)

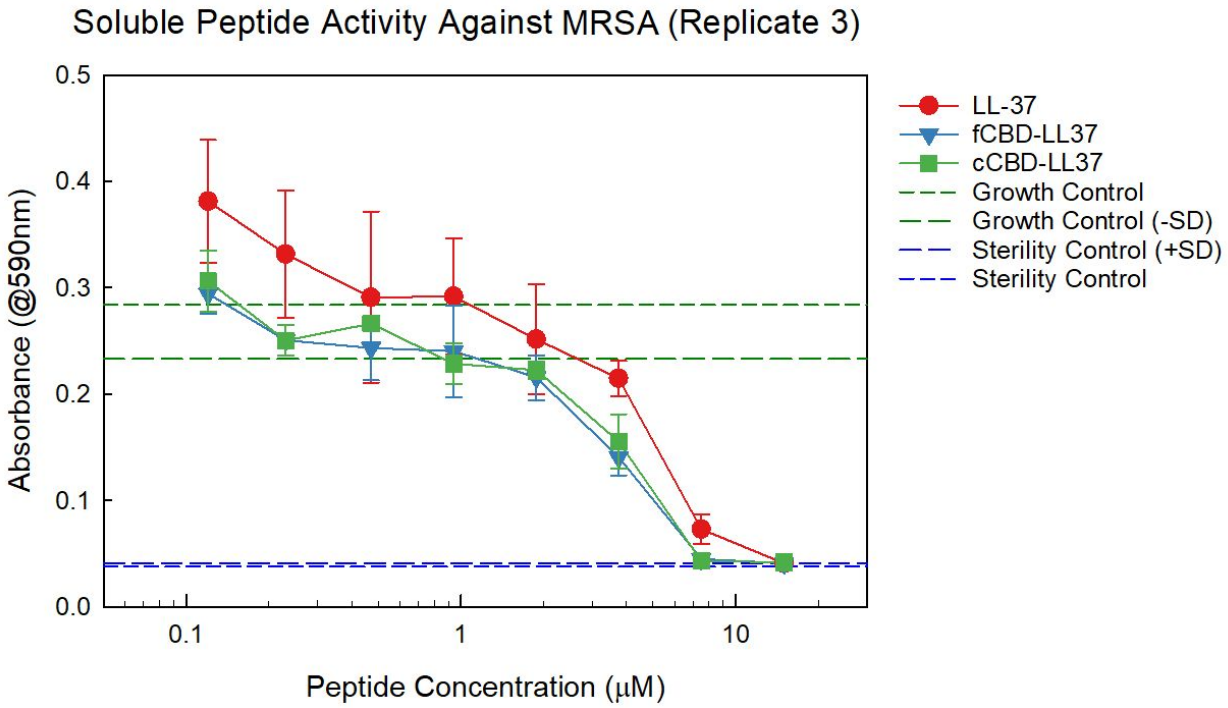
Controlled Variable: Type of peptide and their concentrations

Procedure:

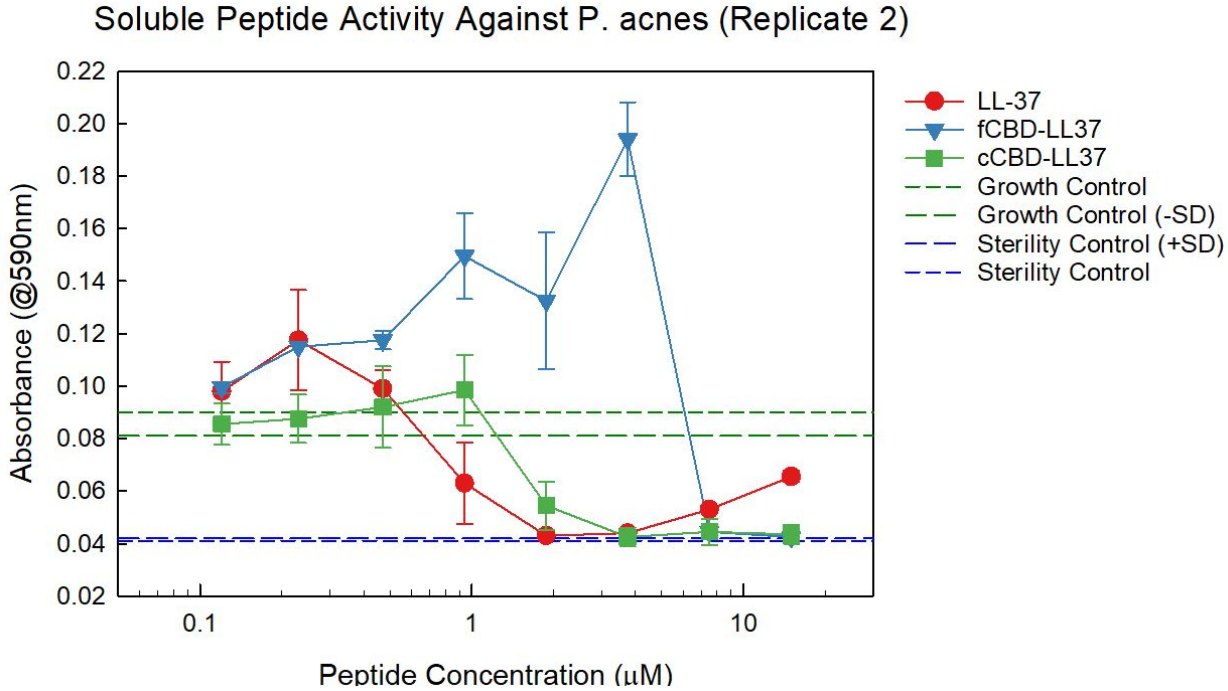
1. Open 96-well plate under the culture hood
2. Pipette 100µL of the peptide solutions to wells A3 through A11, following the plate diagram
3. Perform a 1:2 serial dilution of the peptide solutions through to row H of the plate such that every well contains 50µL when complete, using broth as the diluent
4. Pipette 100µL broth to all wells in column 1, and 50µL broth to all wells in column 12
5. Perform McFarland standard adjustment of bacterial liquid culture (see separate procedure)
6. Pipette 50µL bacterial culture to all wells in column 11
7. Pipette 50µL bacterial culture to all wells containing peptide solution
8. Incubate the 96-well plate (in the anaerobic incubation chamber for *P. acnes*) at 37ºC
9. Obtain a 16hr plate absorbance reading for MRSA, or a 72hr plate absorbance reading for *P. acnes*

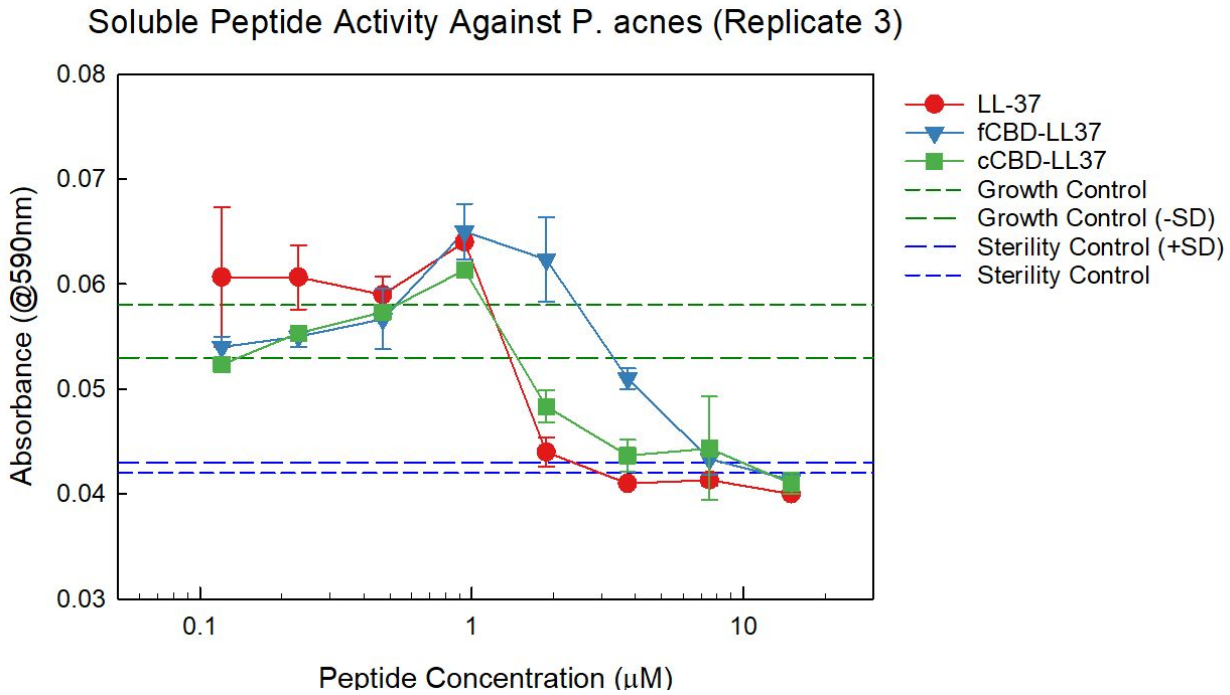
Results:





The first replicate of this assay against MRSA had poor results due to user error and poor technique, but the second and third replicates were consistent and confirmed the MIC values for the modified peptides found in the literature.





The two replicates of this experiment with *P. acnes* were consistent, and provided the first ever data about the minimum inhibitory concentrations for the modified peptides against this bacteria.

Appendix B. MIC Assay: Essential Oil Activity

Number of Experimental Replicates: 1 (MRSA), 2 (*P. acnes*)

Aim: Investigate the activity of 6 different essential oils diluted in an inert oil against Methicillin-resistant *Staphylococcus aureus* (MRSA) or *Propionibacterium acnes*.

Purpose: Determine which essential oils are most effective against MRSA or *P. acnes*, as well as the MICs for each of these oils against MRSA or *P. acnes*.

Materials:

- 96-well plates
- Tween® 80 (Sigma Aldrich, Lot # MKCC9801)
- Tea tree oil (Eve Hansen, UPC 5060339710099)
- Manuka oil (Earth Shield, UPC 733074273086)
- Orange oil (Kis Oils, UPC 030955728339)
- Lemongrass oil (Natural Acres, UPC 852709006254)
- Eucalyptus oil (Healing Solutions, UPC 853675006156)
- Sunflower oil (H & B Oils Center Co, UPC 765857859835)
- *P. acnes* or MRSA liquid culture
- Bacterial medium (Mueller Hinton broth or tryptic soy broth, half-strength)
- Anaerobic incubation chamber (BD GasPak™ EZ)
- CO_2 gas sachets (BD GasPak™ EZ)
- Spectrophotometer/microplate reader
- Conical tubes and microfuge tubes
- Pipettes and tips

Setup: Performed in triplicate (n=3)

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control		Tea Tree 4%	Tea Tree 4%	Tea Tree 4%	Manuka 4%	Manuka 4%	Manuka 4%	Orange 4%	Orange 4%	Orange 4%	Growth control
B	Sterility control		Tea Tree 2%	Tea Tree 2%	Tea Tree 2%	Manuka 2%	Manuka 2%	Manuka 2%	Orange 2%	Orange 2%	Orange 2%	Growth control
C	Sterility control		Tea Tree 1%	Tea Tree 1%	Tea Tree 1%	Manuka 1%	Manuka 1%	Manuka 1%	Orange 1%	Orange 1%	Orange 1%	Growth control
D	Sterility control		Tea Tree 0.5%	Tea Tree 0.5%	Tea Tree 0.5%	Manuka 0.5%	Manuka 0.5%	Manuka 0.5%	Orange 0.5%	Orange 0.5%	Orange 0.5%	Growth control
E	Sterility control		Tea Tree 0.25%	Tea Tree 0.25%	Tea Tree 0.25%	Manuka 0.25%	Manuka 0.25%	Manuka 0.25%	Orange 0.25%	Orange 0.25%	Orange 0.25%	Growth control
F	Sterility control		Tea Tree 0.125%	Tea Tree 0.125%	Tea Tree 0.125%	Manuka 0.125%	Manuka 0.125%	Manuka 0.125%	Orange 0.125%	Orange 0.125%	Orange 0.125%	Growth control
G	Sterility control		Tea Tree 0.063%	Tea Tree 0.063%	Tea Tree 0.063%	Manuka 0.063%	Manuka 0.063%	Manuka 0.063%	Orange 0.063%	Orange 0.063%	Orange 0.063%	Growth control
H	Sterility control		Tea Tree 0.031%	Tea Tree 0.031%	Tea Tree 0.031%	Manuka 0.031%	Manuka 0.031%	Manuka 0.031%	Orange 0.031%	Orange 0.031%	Orange 0.031%	Growth control

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control		Lemongr. 4%	Lemongr. 4%	Lemongr. 4%	Eucalyptus 4%	Eucalyptus 4%	Eucalyptus 4%	Sunflower 4%	Sunflower 4%	Sunflower 4%	Growth control
B	Sterility control		Lemongr. 2%	Lemongr. 2%	Lemongr. 2%	Eucalyptus 2%	Eucalyptus 2%	Eucalyptus 2%	Sunflower 2%	Sunflower 2%	Sunflower 2%	Growth control
C	Sterility control		Lemongr. 1%	Lemongr. 1%	Lemongr. 1%	Eucalyptus 1%	Eucalyptus 1%	Eucalyptus 1%	Sunflower 1%	Sunflower 1%	Sunflower 1%	Growth control
D	Sterility control		Lemongr. 0.5%	Lemongr. 0.5%	Lemongr. 0.5%	Eucalyptus 0.5%	Eucalyptus 0.5%	Eucalyptus 0.5%	Sunflower 0.5%	Sunflower 0.5%	Sunflower 0.5%	Growth control
E	Sterility control		Lemongr. 0.25%	Lemongr. 0.25%	Lemongr. 0.25%	Eucalyptus 0.25%	Eucalyptus 0.25%	Eucalyptus 0.25%	Sunflower 0.25%	Sunflower 0.25%	Sunflower 0.25%	Growth control
F	Sterility control		Lemongr. 0.125%	Lemongr. 0.125%	Lemongr. 0.125%	Eucalyptus 0.125%	Eucalyptus 0.125%	Eucalyptus 0.125%	Sunflower 0.125%	Sunflower 0.125%	Sunflower 0.125%	Growth control
G	Sterility control		Lemongr. 0.063%	Lemongr. 0.063%	Lemongr. 0.063%	Eucalyptus 0.063%	Eucalyptus 0.063%	Eucalyptus 0.063%	Sunflower 0.063%	Sunflower 0.063%	Sunflower 0.063%	Growth control
H	Sterility control		Lemongr. 0.031%	Lemongr. 0.031%	Lemongr. 0.031%	Eucalyptus 0.031%	Eucalyptus 0.031%	Eucalyptus 0.031%	Sunflower 0.031%	Sunflower 0.031%	Sunflower 0.031%	Growth control

Controls: Bacteria only (growth control), broth only (sterility control)

Controlled Variable: Type of essential oil and their concentrations

Procedure (Oil Emulsification):

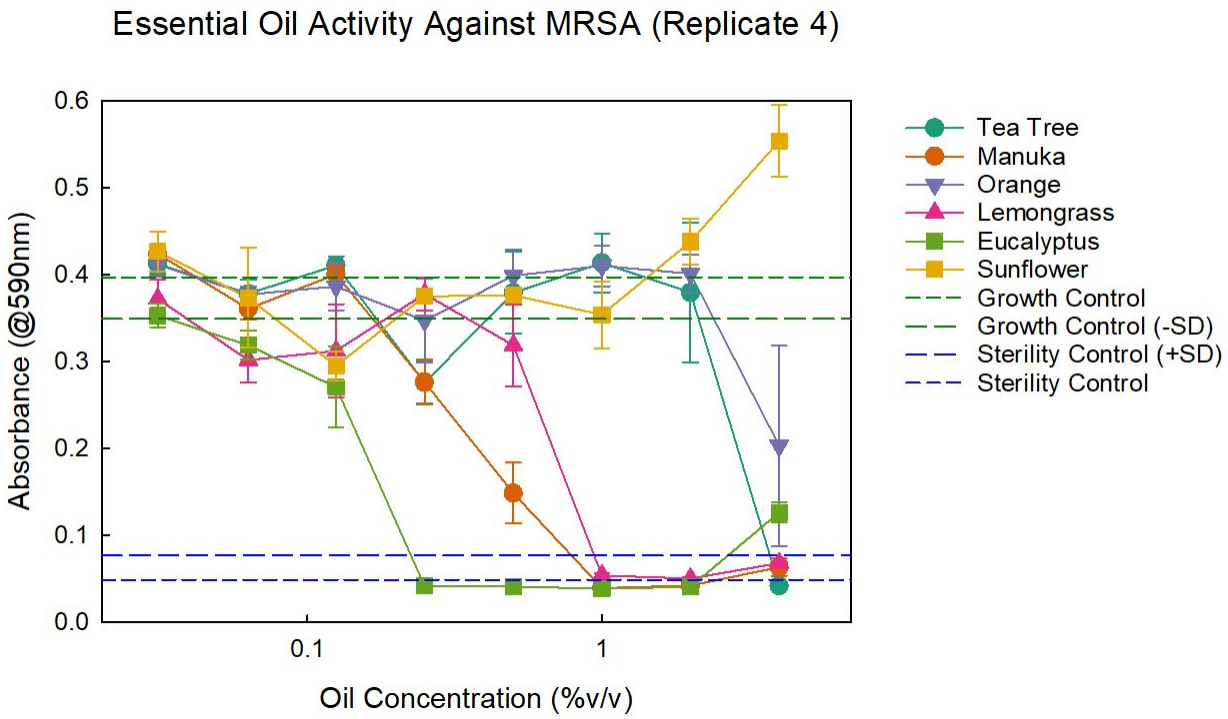
1. Prepare 0.5% v/v Tween® 80 + broth solution by transferring 40mL broth and 200µL Tween® 80 to two 50mL conical tubes and vortexing the tubes with a vortex mixer on highest speed setting for 30 seconds
2. Transfer 920µL of the tween® 80 + broth (Tween® 80 broth) solution to six microfuge tubes
3. Prepare 8% v/v essential oil emulsifications by pipetting 80µL of the six essential oils into the microfuge tubes and vortexing with a vortex mixer on highest speed setting for 30 seconds

Procedure (Assay):

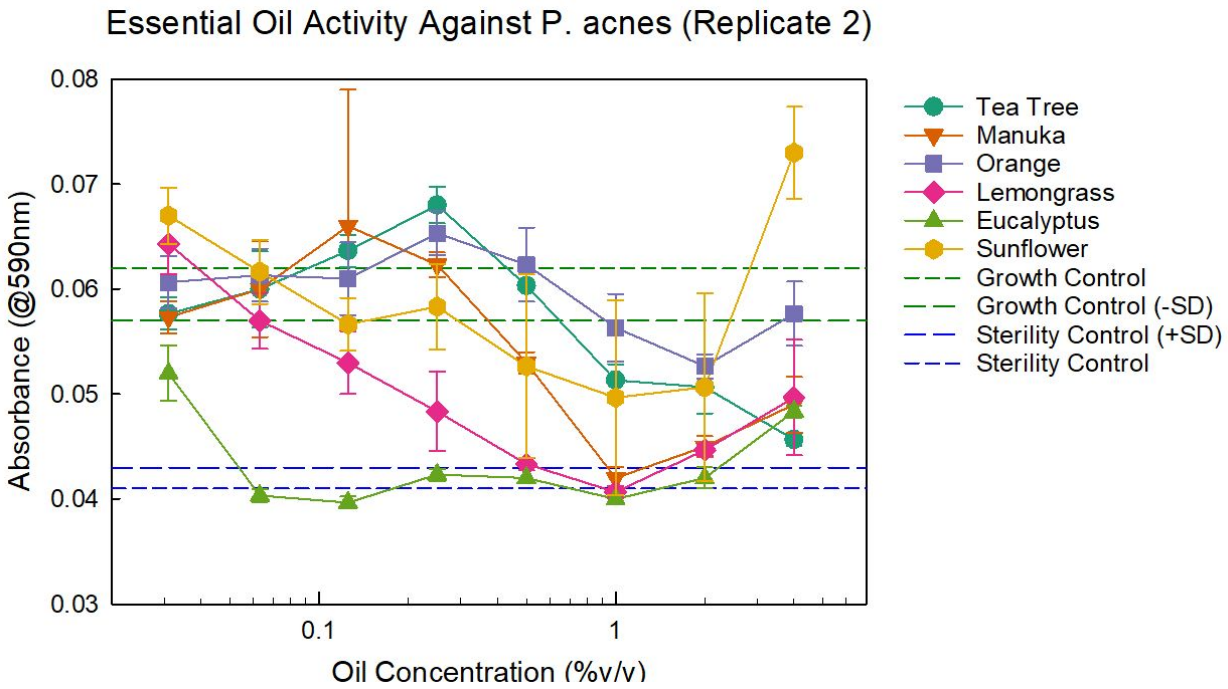
1. Open 96-well plates under the culture hood

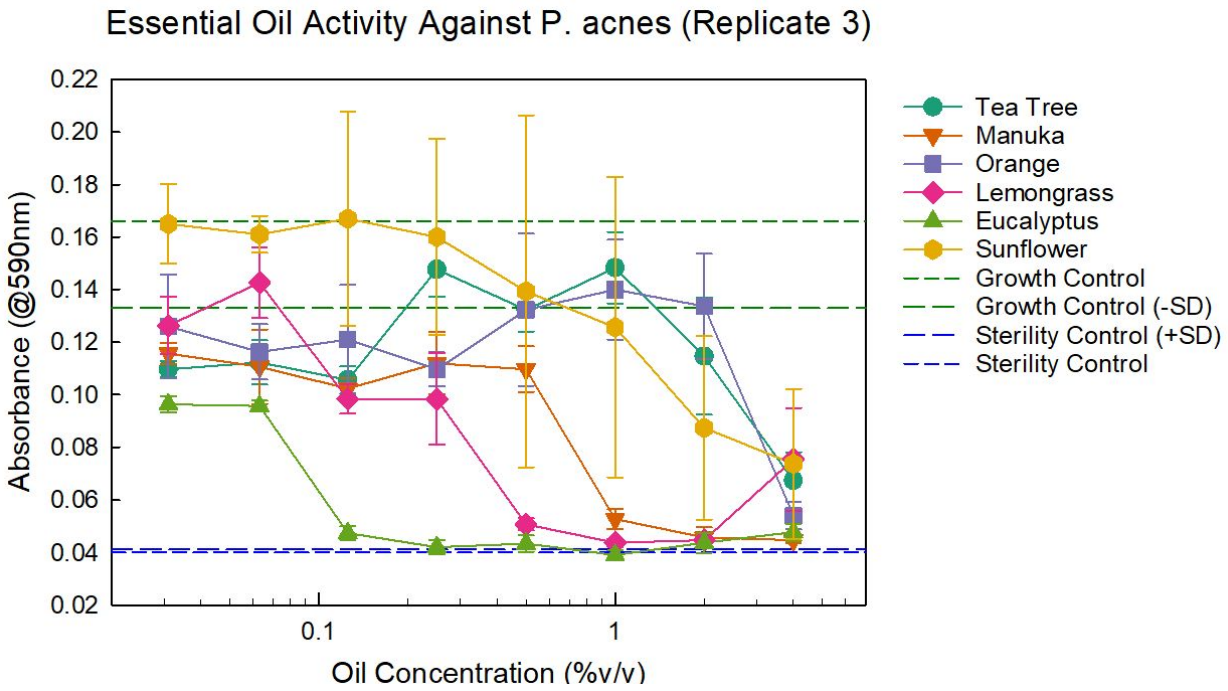
2. Pipette 100 µL of the essential oil emulsions to wells A3 through A11 in both plates, following the plate diagrams
3. Perform a 1:2 serial dilution of the oil solutions through to row H of the plates such that each well contains 50µL oil solution when complete, using Tween® 80 broth as the diluent
4. Pipette 100 µL Tween® 80 broth to all wells in column 12, and 50 µL Tween® 80 broth to all wells in column 11
5. Perform McFarland standard adjustment of bacterial liquid culture (see separate procedure)
6. Pipette 50 µL bacterial culture to all wells in column 12
7. Pipette 50 µL bacterial culture to all wells containing oil solutions
8. Incubate the 96-well plate (in the anaerobic incubation chamber for *P. acnes*) at 37ºC
9. Obtain a 16hr plate absorbance reading for MRSA, or a 72 hr plate absorbance reading for *P. acnes*

Results:



MRSA replicate 4 confirmed that the tween® 80 emulsification method was effective. Previous MRSA experimental replicates of this assay were inconclusive because the emulsification methods were not effective.





Some of the results from the two replicates of this assay with *P. acnes* do not match the values found in the literature, but the results of these two experimental replicates are consistent with every essential oil that was evaluated.

Appendix C. MIC Assay: Peptide-Oil Synergistic Activity

Number of Experimental Replicates: 4

Aim: Investigate the potentially synergistic antimicrobial activity of *f*CBD-LL37 and *c*CBD-LL37 with manuka and eucalyptus essential oils against *P. acnes*.

Purpose: Determine how the MICs of the peptides are changed by including low (below MIC) concentrations of manuka and eucalyptus essential oils

Materials:

- 96-well plates
- 610µL of 30µM *f*CBD-LL37
- 310µL of 15µM *f*CBD-LL37
- 610µL of 30µM *c*CBD-LL37
- 310µL of 15µM *c*CBD-LL37
- Tween 80 (Sigma Aldrich, Lot # MKCC9801)
- Tea tree oil (Eve Hansen, UPC 5060339710099)
- Manuka oil (Earth Shield, UPC 733074273086)
- Orange oil (Kis Oils, UPC 030955728339)
- Lemongrass oil (Natural Acres, UPC 852709006254)
- Eucalyptus oil (Healing Solutions, UPC 853675006156)
- Sunflower oil (H & B Oils Center Co, UPC 765857859835)
- *P. acnes* liquid culture
- Bacterial medium (tryptic soy broth, half-strength)
- Anaerobic incubation chamber (BD GasPak™ EZ)
- CO_2 gas sachets (BD GasPak™ EZ)
- Spectrophotometer/microplate reader
- Conical tubes and microfuge tubes
- Pipettes and tips

Setup: performed in triplicate (n=3)

Peptide Control Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	sterility control -->						sterility control-->					
B	7.5 uM	7.5 uM	7.5 uM				7.5 uM	7.5 uM	7.5 uM			
C	3.75 uM	3.75 uM	3.75 uM				3.75 uM	3.75 uM	3.75 uM			
D	1.88 uM	1.88 uM	1.88 uM				1.88 uM	1.88 uM	1.88 uM			
E	0.94 uM	0.94 uM	0.94 uM				0.94 uM	0.94 uM	0.94 uM			
F	0.47 uM	0.47 uM	0.47 uM				0.47 uM	0.47 uM	0.47 uM			
G	0.23 uM	0.23 uM	0.23 uM				0.23 uM	0.23 uM	0.23 uM			
H	growth control -->						growth control -->					

fCBD Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	sterility control -->						sterility control -->					
B	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM
C	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM
D	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM
E	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM
F	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM
G	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM
H	growth control -->						growth control -->					
	0.5% oil			0.1% oil			0.05% oil			0.02% oil		
	Manuka						Eucalyptus					

cCBD Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	sterility control -->						sterility control -->					
B	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM	7.5 uM
C	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM	3.75 uM
D	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM	1.88 uM
E	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM	0.94 uM
F	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM	0.47 uM
G	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM	0.23 uM
H	growth control -->						growth control -->					
	0.5% oil			0.1% oil			0.05% oil			0.02% oil		
	Manuka						Eucalyptus					

Controls: bacteria only (growth control), broth only (sterility control), peptides only (peptide control plate)

Controlled variables: Type of peptide and their concentrations, type of oil and their concentrations

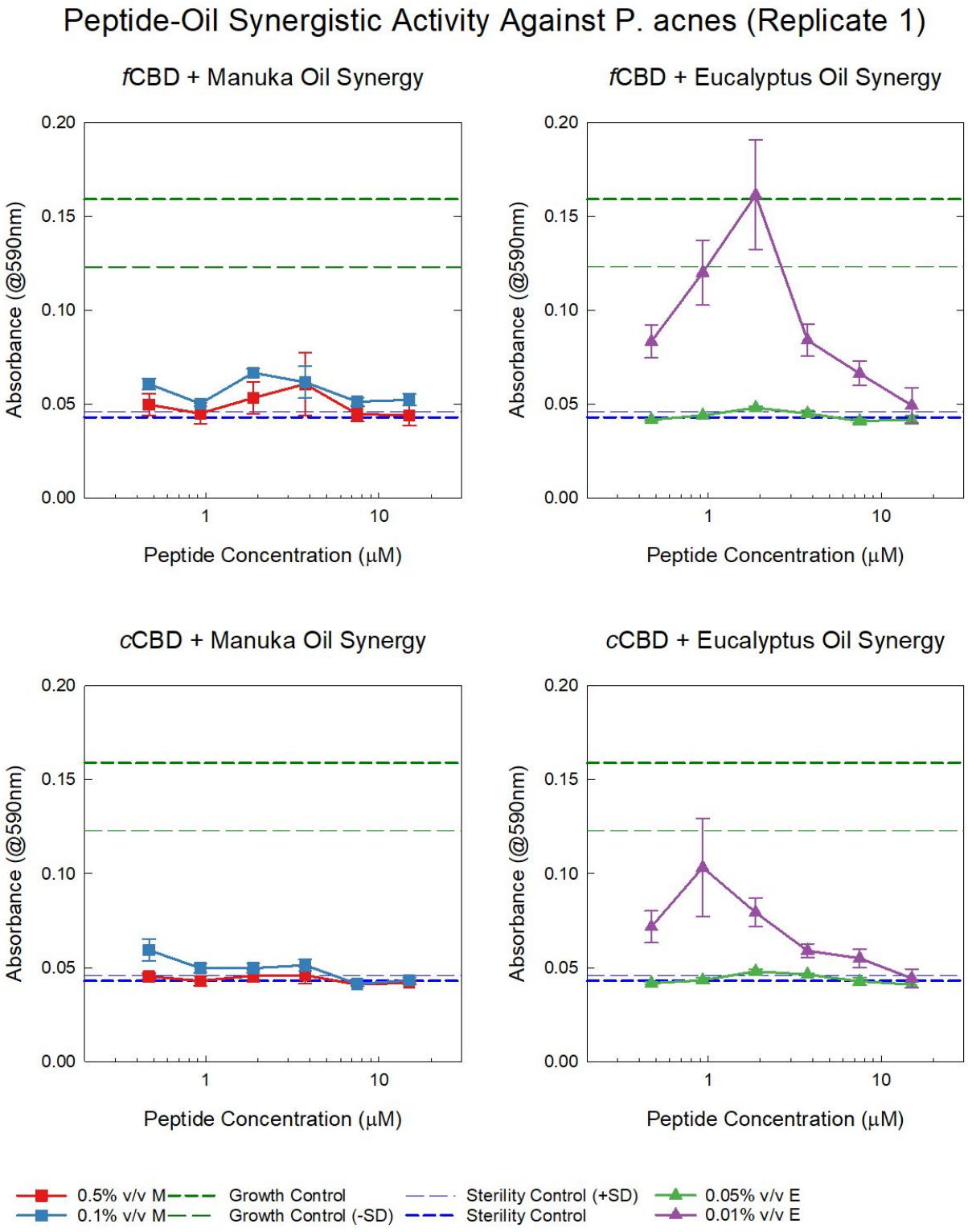
Procedure (Oil Emulsification):

1. Prepare 0.5% v/v Tween® 80 + broth solutions by transferring 40mL broth and 200µL Tween® 80 to two 50mL conical tubes and vortexing the tubes with a vortex mixer on highest speed setting for 30 seconds
2. Prepare the following essential oil emulsifications by pipetting the given amounts of essential oil and Tween® broth to 15mL conical tubes and vortexing the tubes for 30 seconds to mix:
    a. 2% manuka (200µL manuka oil + 9800µL Tween® broth)
    b. 0.4% manuka (40µL manuka oil + 9960µL Tween® broth)
    c. 0.2% eucalyptus (20µL eucalyptus oil + 9980µL Tween® broth)
    d. 0.08% eucalyptus (8µL eucalyptus oil + 9992µL Tween® broth)

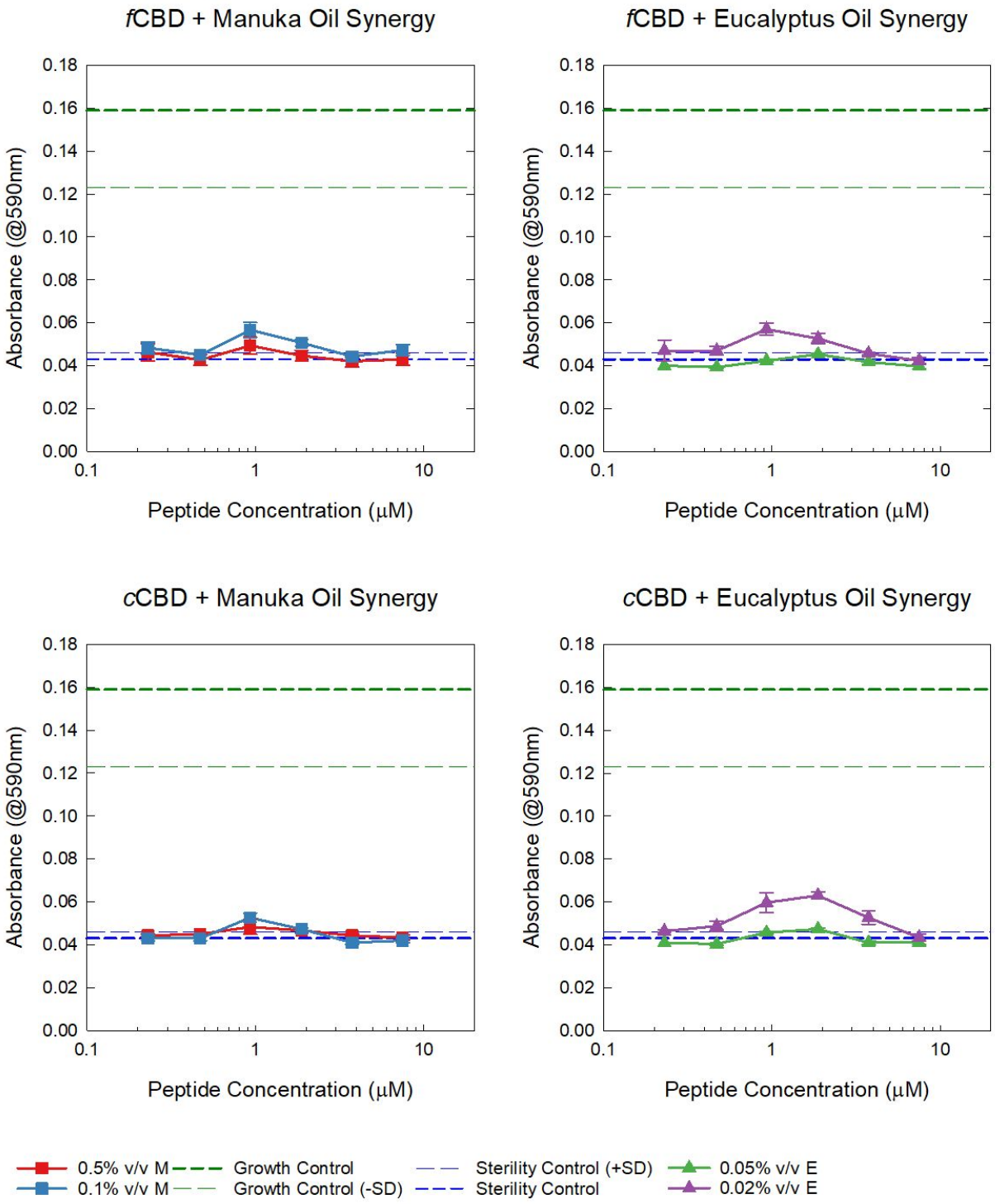
Procedure (Assay):

1. Open 96-well plates under the culture hood
2. Pipette 50µL of the 30µM peptide solutions to wells B1-B12 in the assay plates, following the plate diagrams
3. Perform a 1:2 serial dilution of the peptide solutions through to row G such that each well contains 25µL peptide solution when complete, using Tween® 80 broth as a diluent
4. Pipette 100µL of the 15µM peptide solutions to wells in row B in the peptide control plate, following the plate diagram
5. Perform a 1:2 serial dilution of the peptide solutions through to row G such that each well contains 50µL peptide solution when complete, using broth as a diluent
6. Pipette 100µL Tween® 80 broth to wells A1-A12 in all plates, and 50µL Tween® 80 broth to wells H1-H12 in all plates
7. Pipette 25µL of:
    a. 2% manuka emulsion to wells B1-G3 in both plates
    b. 0.4% manuka emulsion to wells B4-G6 in both plates
    c. 0.2% eucalyptus emulsion to wells B7-G9 in both plates
    d. 0.08% eucalyptus emulsion to wells B10-G12 in both plates
8. Perform McFarland standard adjustment of bacterial culture (see separate procedure)
9. Pipette 50µL adjusted bacterial culture to all wells containing liquid in all plates, except sterility control wells
10. Incubate plates in anaerobic incubation chamber with CO_2 gas sachet at 37ºC for 72 hours
11. Obtain a 72hr plate absorbance reading

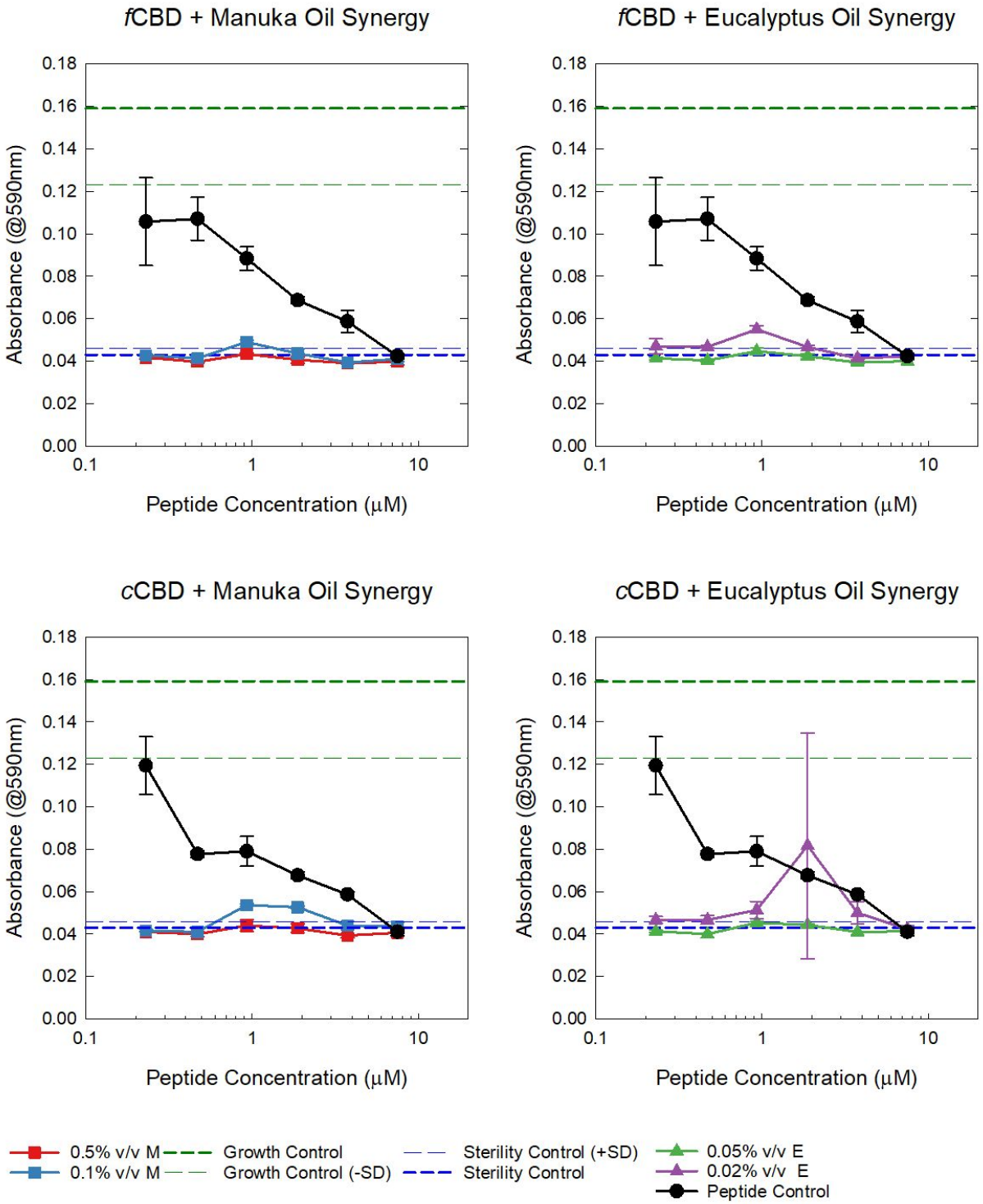
Results:



Replicate 1 displayed synergy, but growth was observed with 0.01% v/v eucalyptus oil. For subsequent assays, the low eucalyptus concentration was changed to 0.02%.

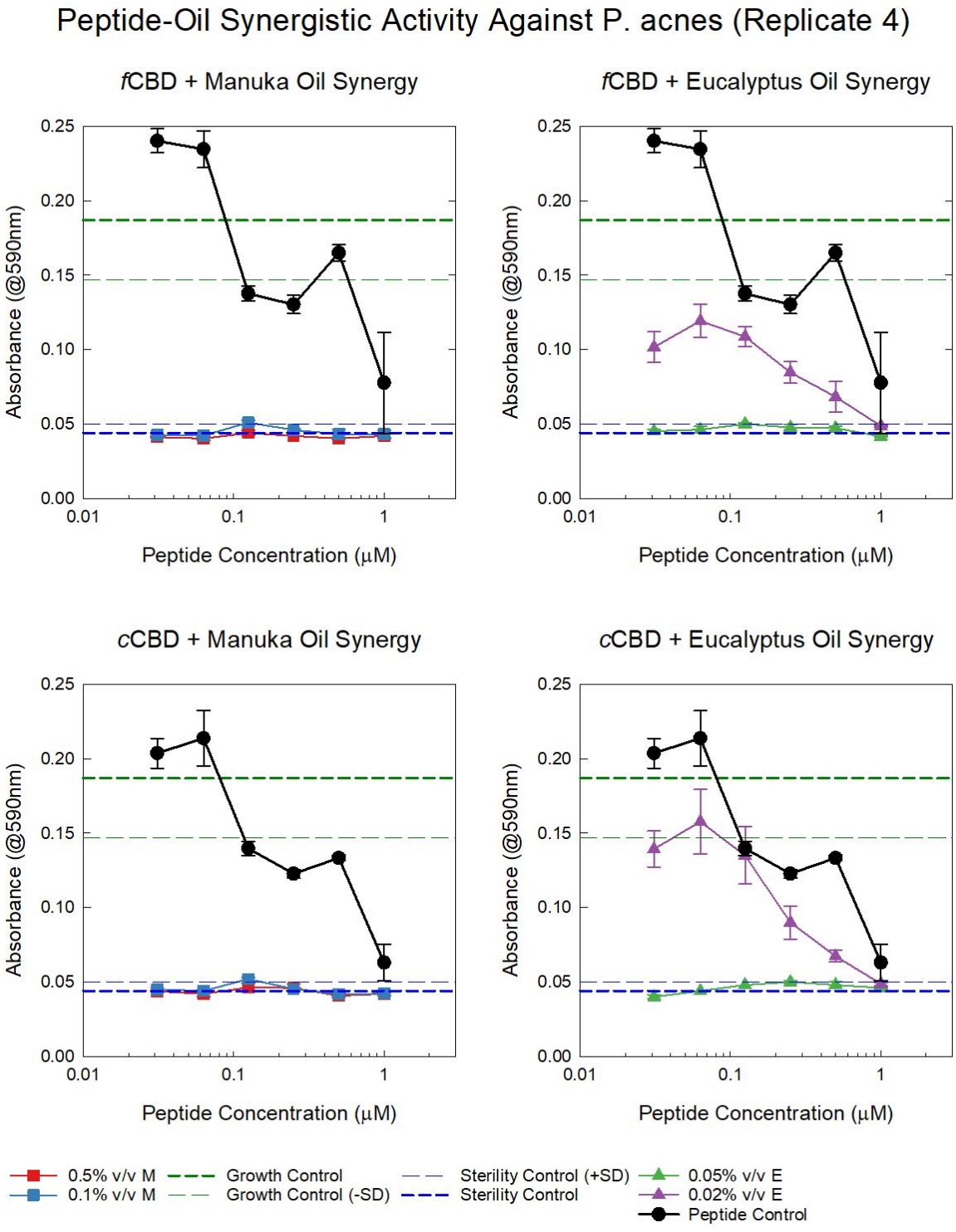


Replicate 2 confirmed the results from the first experiment, and the altered low eucalyptus oil concentration demonstrated complete killing. For subsequent assays, peptide-only controls were added to further confirm that the killing was due to peptide-oil synergy and not other factors.



Replicate three confirmed the results of the previous two experiments, with the added evidence of the peptide-only controls. For the next assay, the highest peptide concentration in the dilutions

was reduced from 7.5µM to 1µM with the goal of observing the point at which the peptide concentration with the oils was too low to inhibit growth.



Replicate 4 showed how aggressive the synergy between the peptides and the oils is, with manuka oil and the high concentration of eucalyptus oil demonstrating complete killing with the peptides as low as 0.032 µM.

Appendix D. Histology and Immunohistochemistry

Number of Experimental Replicates: 1

Aim: Characterize the diffusion and penetration of the formulation and peptides within the formulation on a porcine skin model.

Purpose: Verify the efficacy of the formulation for acne treatment by demonstrating that the active ingredients (peptide and essential oil) can penetrate into the sebaceous glands in a porcine skin construct after being applied to the top of the skin. Verify the safety of the formulation for acne treatment by demonstrating that the active ingredients do not diffuse far enough into the skin to reach blood vessels in the epithelial tissue.

Materials:

- Tween 80 (Sigma Aldrich, Lot # MKCC9801)
- Tea tree oil (Eve Hansen, UPC 5060339710099)
- Manuka oil (Earth Shield, UPC 733074273086)
- 500µL LL37 (10µM)
- 500µL *f*CBD-LL37 (10µM)
- DI water
- Porcine ear
- Black grossing dye (Ted Pella)
- Histology cassettes
- Formalin
- Histological equipment (tissue processor, tissue embedding machine, sectioning device)
- Surgical scalpel, forceps, and scissors
- Xylene
- Ethanol (100%, 95%, 70%)
- Rabbit anti(human)-LL37 polyclonal antibody (Anaspec)
- Alexa Fluor 488 goat anti-rabbit secondary antibody (LifeTechnologies, Carlsbad, CA: cat. No. A11008)
- Phosphate buffered saline (PBS)
- Normal Goat Serum (NGS; Vector labs 5-5000, diluted in PBS)
- 10X Tris-EDTA Buffer Antigen Retrieval Solution pH 9.0
- Pressure cooker
- Hoechst (Invitrogen H3750)
- Prolong Gold (Invitrogen P36930)
- Microscope slides and cover slips

- Microscope and fluorescent microscope

Procedure (Oil Emulsification and Dye Addition):

1. Transfer 40mL DI water and 200µL Tween® 80 each to 2 50mL conical tubes
2. Vortex conicals w/ a vortex mixer on highest setting for 1min each
3. Transfer the following to 15mL conical tubes to create experimental and control formulations:

Formulation	Tween® 80 Water (µL)	DI Water (µL)	Oil (µL)	10µM Peptide (µL)
*f*CBD-LL37, 0.5% Manuka	4875	0	25	100
*f*CBD-LL37, 0.1% Manuka	4895	0	5	100
*f*CBD-LL37, 0.05% Eucalyptus	4897	0	2.5	100
*f*CBD-LL37, 0.02% Eucalyptus	4899	0	1	100
*f*CBD-LL37 only control, +Tween® 80	4900	0	0	50
*f*CBD-LL37 only control, -Tween® 80	0	4900	0	50
LL37, 0.5% Manuka	4875	0	25	100
LL37, 0.1% Manuka	4895	0	5	100
LL37, 0.05% Eucalyptus	4897	0	2.5	100
LL37, 0.02% Eucalyptus	4899	0	1	100
LL37 only control, + Tween® 80	4900	0	0	50
LL37 only control, -Tween® 80	0	4900	0	50
0.05% Eucalyptus, no peptide control	4897	0	2.5	0
Tween® 80 only control	5000	0	0	0

4. Vortex oil solutions w/ a vortex mixer on highest setting for 30sec each
5. Add 30uL black tissue marking dye to each formulation for visualization

Procedure (Skin Preparation, Processing, and Sectioning):

1. Wash pig ears prior to use to remove excess dirt/oil
2. Use surgical scissors to trim the perimeter of the pig ear to expose the layers of skin and cartilage in the ear
3. Use surgical forceps to carefully separate the pig ear skin from the cartilage, using a scalpel to severe the connective tissue, until an entire flap of skin can be removed
4. Shave the hair off the top of the skin flap using the scalpel or an electric razor
5. Further dissect the skin sample into 2cm x 0.5cm sections for histology
6. Apply 25µL of each formulation to skin sections in duplicate (2 sections per formulation) and incubate the skin sections for 30 minutes at 37℃
7. After incubation, place each skin section into a histology cassette (labeled appropriately in pencil)
8. Insert cassettes into 10% neutral buffered formalin to fix the samples overnight
9. After fixing overnight, process samples in Sakura's Tissue-Tek VIP 6 AI Vacuum Infiltration Processor
    a. Processor dehydrates samples by displacing water in the samples with ethanol, and then displacing ethanol with xylene (x3)
10. Embed processed tissue samples in paraffin wax and let wax cool
11. Prepare a water bath up to 42℃
12. Prepare an ice bath and insert samples in an ice bath prior to sectioning
13. Section tissue samples into 5µm thick slices and mount onto glass microscope slides for staining and immunohistochemistry

Procedure (Eosin Staining):

1. Load glass microscope slides into a slide holder rack
2. Submerge slides completely into the following solutions for the following times:
    a. 100% xylene I, 3 min
    b. 100% xylene II, 3 min
    c. 100% xylene III, 3 min
    d. 100% ethanol I, 2 min
    e. 100% ethanol II, 1 min
    f. 95% ethanol I, 2 min
    g. 70% ethanol I, 2 min
    h. 95% ethanol II, 1 min
    i. Eosin stain, 1 min
    j. 95% ethanol III, 1 min
    k. 95% ethanol IV, 1 min
    l. 100% ethanol III, 2 min
    m. 100% ethanol IV, 1 min
    n. 100% xylene IV, 3 min

o. 100% xylene V, 2 min
p. 100% xylene VI, 2 min

3. Coverslip using Permount (xylene based) mounting medium, avoiding bubbles
4. Dry slides before imaging

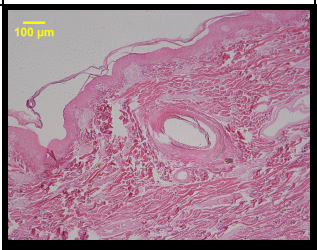
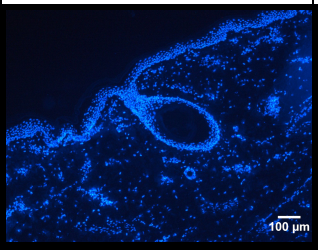
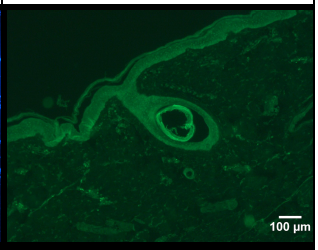
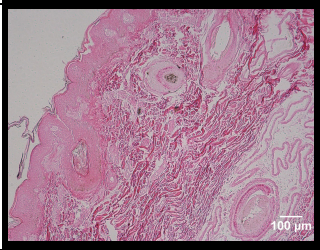
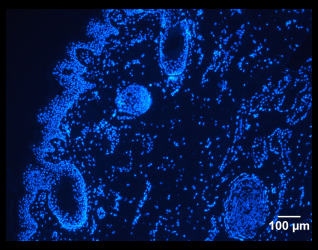
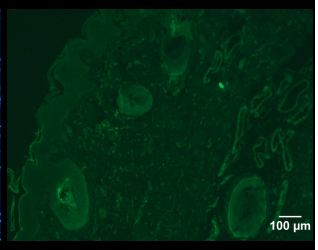
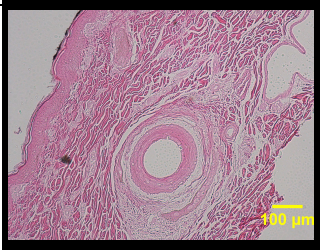
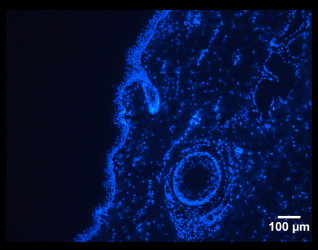
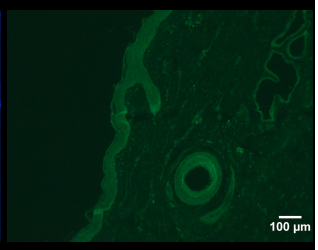
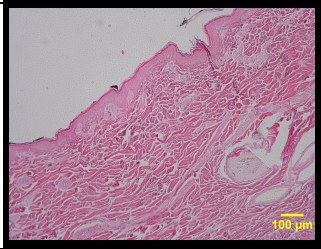
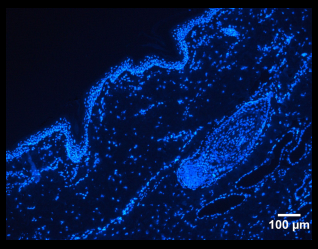
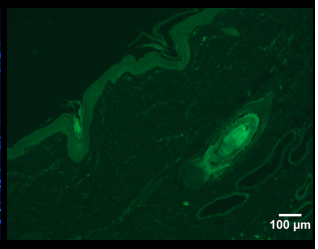
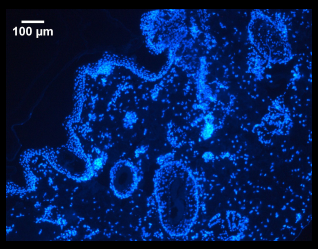
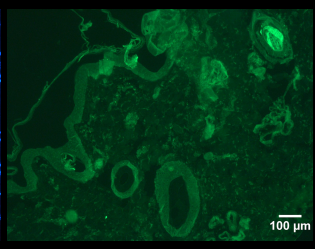
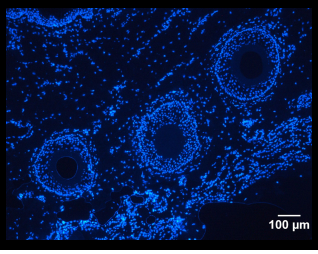
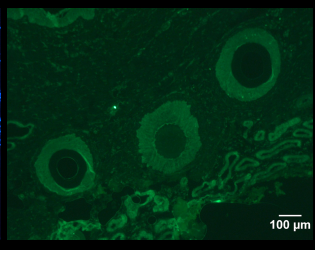
Procedure (Immunohistochemistry):

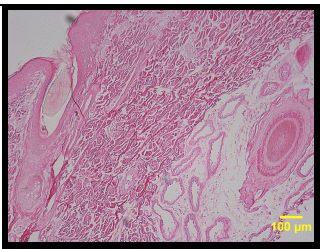
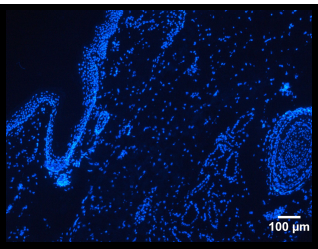
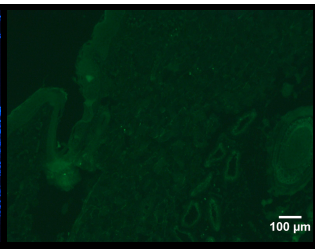
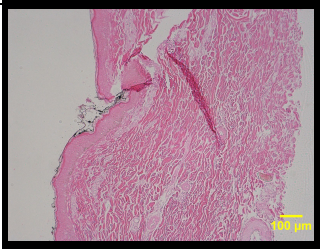
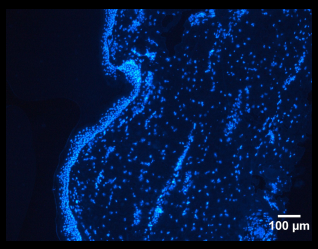
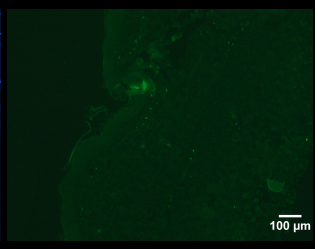
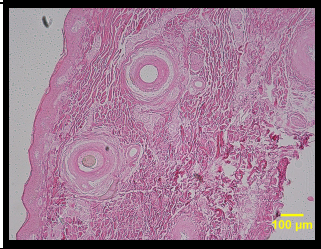
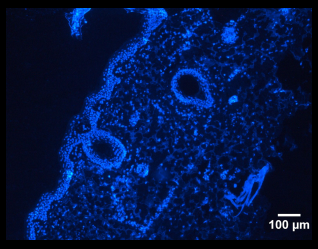
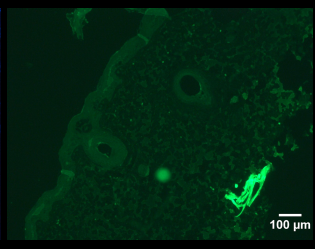
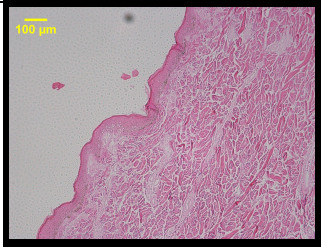
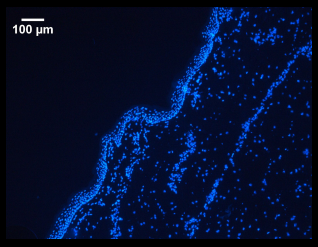
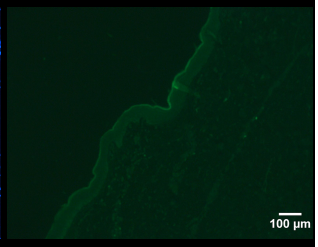
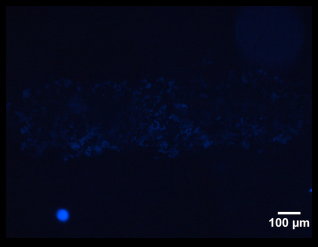
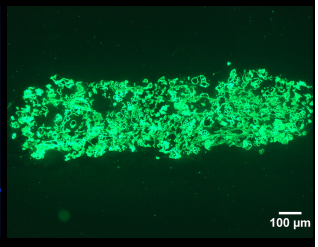
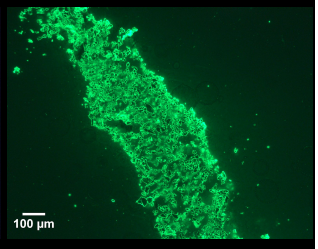
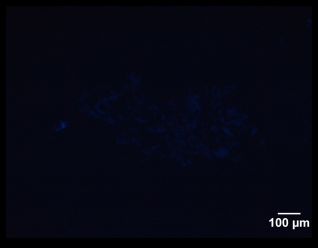
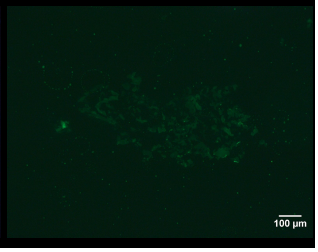
1. Perform antigen retrieval on slides:
    a. Dilute antigen retrieval solution 1:10 in DI water to make 1L of solution (100mL 10X solution + 900mL DI water)
    b. Add the stock solution to the pressure cooker and place it on a hot plate on high (no cover)
    c. Once the solution is boiling, move the slides from running water and place them in a metal rack and in the pressure cooker with the cover on. Move quickly so the slides do not dry out.
    d. Put the lid on the pressure cooker and turn the red switch to the "lock" position, and set the pressure kob to 2
    e. Once the pressure cooker has reached full pressure (red pin pops up), time 5 minutes.
    f. Remove the pressure cooker from heat and carry to the sink. Make sure the vent is facing away from you.
    g. Run cold tap over the pressure cooker to cool
    h. Push the red knob to the "unlock" position and remove the lid. Let settle for 5 min
2. 3x PBS washes (5 min each)
3. Block with 5% normal goat serum (NRS) in PBS for 30 minutes at room temperature
4. Add primary antibody, diluted 1:500 in 3% NGS overnight at 4℃
5. 3x PBS washes (5 min each)
6. Add secondary antibody, diluted 1:1000 in 3% NGS for 1 hour at room temperature, protecting samples from light
7. 3x PBS washes (5 min each)
8. Counter stain with Hoechst nuclear dye (1:3000), keeping samples in the dark
9. 3x PBS washes (5 min each)
10. Use Prolong Gold to mount glass coverslips over samples (use approximately 10μL per sample)
11. Image slides as soon as possible or store protected from light at 4℃

Results:

Manuka formulation samples were rendered unusable because of an organizational error that resulted in these skin samples being placed in incorrectly labelled tissue cassettes. Images were still gathered without issues from the unaffected eucalyptus and control samples, shown below.

Previously prepared collagen scaffold slides loaded with *f*CBD-LL37, LL37, and PBS were used as positive controls and an additional negative control. They were subjected to the same IHC procedure as the pigskin slides.

Sample	Eosin	Hoechst	IHC
*f*CBD-LL37, 0.05% Eucalyptus			
*f*CBD-LL37, 0.02% Eucalyptus			
*f*CBD-LL37 only control, +Tween® 80			
*f*CBD-LL37 only control, -Tween® 80			
LL37, 0.05% Eucalyptus	N/A		
LL37, 0.02% Eucalyptus	N/A		

LL37 only control, + Tween® 80			
LL37 only control, -Tween® 80			
0.05% Eucalyptus, no peptide control			
Tween® 80 only control			
*f*CBD-LL37 Collagen (+) control	N/A		
LL37 Collagen (+) control	N/A		
Collagen (-) control	N/A		

Appendix E. Cytotoxicity Assay

Aim: Determine the cytotoxic effects of each formulation with and without peptides by evaluating cell viability with CRL-2352 fibroblasts.

Point of Experiment/Expected Results: Ensure that *f*CBD-LL37 in the formulations does not display any significant levels of cytotoxicity towards human fibroblasts over the course of 24 hours. This length of time was chosen because a typical application of the topical product should not be on the skin for longer than 24 hours. This experiment will also validate the chosen essential oils at the chosen concentrations for use in the formulation without harming the cells.

Materials:

- 96-well plates, seeded with CRL-2352 fibroblasts 16hrs prior
- AlamarBlue® reagent
- 900µL *f*CBD-LL37 (22.2µM)
- 900µL *c*CBD-LL37 (22.2µM)
- Eucalyptus Oil (Healing Solutions, UPC 853675006156)
- Manuka oil (Earth Shield, UPC 733074273086)
- Tween® 80 (Sigma Aldrich, Lot # MKCC9801)
- Cell growth medium (45% DMEM, 45% Ham's F-12, 10% FBS)
- Conical tubes and microfuge tubes
- Pipettes and tips
- Fluorescence microplate reader

Setup: Four replicates (n=4)

	1	2	3	4	5	6	7	8	9	10	11	12
A									Sterility	Sterility	Sterility	Sterility
B				fCBD	fCBD	fCBD	fCBD		fCBD + oil	fCBD + oil	fCBD + oil	fCBD + oil
C	manuka*			fCBD	fCBD	fCBD	fCBD		fCBD + oil	fCBD + oil	fCBD + oil	fCBD + oil
D	manuka*			fCBD*	fCBD*	fCBD*	fCBD*		fCBD + oil*	fCBD + oil*	fCBD + oil*	fCBD + oil*
E	manuka*			cCBD	cCBD	cCBD	cCBD		cCBD + oil	cCBD + oil	cCBD + oil	cCBD + oil
F	manuka*			cCBD	cCBD	cCBD	cCBD		cCBD + oil	cCBD + oil	cCBD + oil	cCBD + oil
G				cCBD*	cCBD*	cCBD*	cCBD*		cCBD + oil*	cCBD + oil*	cCBD + oil*	cCBD + oil*
H	Growth	Growth	Growth	Growth	Growth	Growth	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80

10 uM	10 uM
5 uM	5 uM
1 uM	1 uM

	1	2	3	4	5	6	7	8	9	10	11	12
A									Sterility	Sterility	Sterility	Sterility
B									fCBD + oil	fCBD + oil	fCBD + oil	fCBD + oil
C	Euc.*								fCBD + oil	fCBD + oil	fCBD + oil	fCBD + oil
D	Euc.*								fCBD + oil*	fCBD + oil*	fCBD + oil*	fCBD + oil*
E	Euc.*								cCBD + oil	cCBD + oil	cCBD + oil	cCBD + oil
F	Euc.*								cCBD + oil	cCBD + oil	cCBD + oil	cCBD + oil
G									cCBD + oil*	cCBD + oil*	cCBD + oil*	cCBD + oil*
H	Growth	Growth	Growth	Growth	Growth	Growth	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80	Growth + Tween80

Controls: Fibroblasts only w/o Tween® 80 (growth control), fibroblasts only w/ Tween® 80 (growth + Tween® 80 control), medium only (sterility control), oils w/o peptides (essential oil controls), peptides w/o oils (peptide controls)

Controlled Variables: peptide type and concentration, oil types and concentrations

Procedure (Oil Emulsification):

1. Transfer 40mL medium and 200µL Tween® 80 each to 2 50mL conical tubes
2. Vortex conicals w/ a vortex mixer on highest setting for 1min each
3. Transfer the following to 15mL conical tubes to create 0.5% manuka and 0.05% eucalyptus solutions:
    a. 9995µL medium + 5µL eucalyptus oil
    b. 9950µL medium + 50µL manuka oil
4. Vortex oil solutions w/ a vortex mixer on highest setting for 30sec each

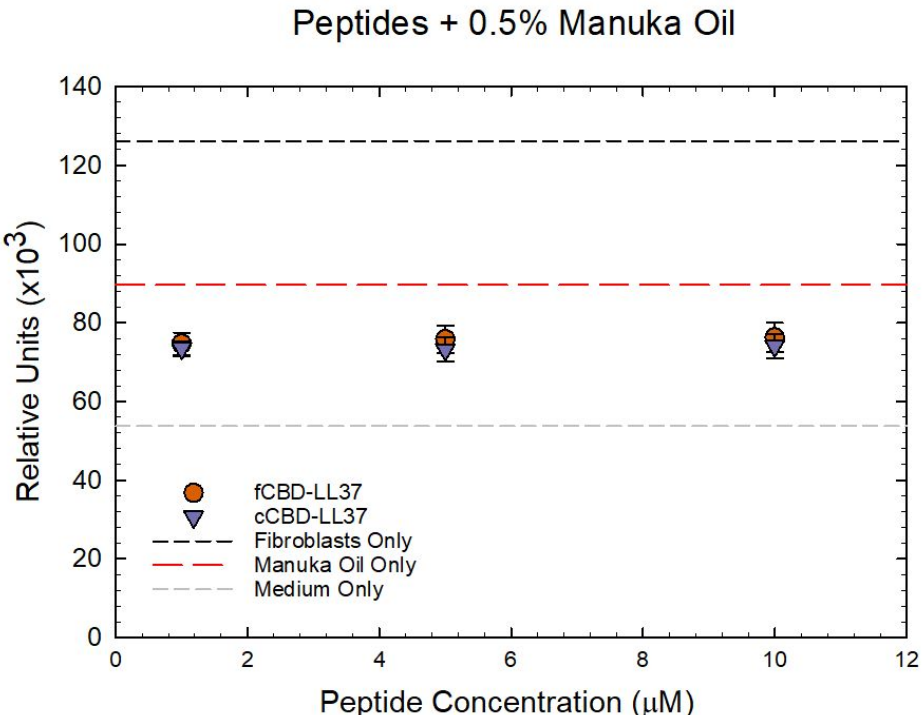
Procedure (Peptide Preparation):

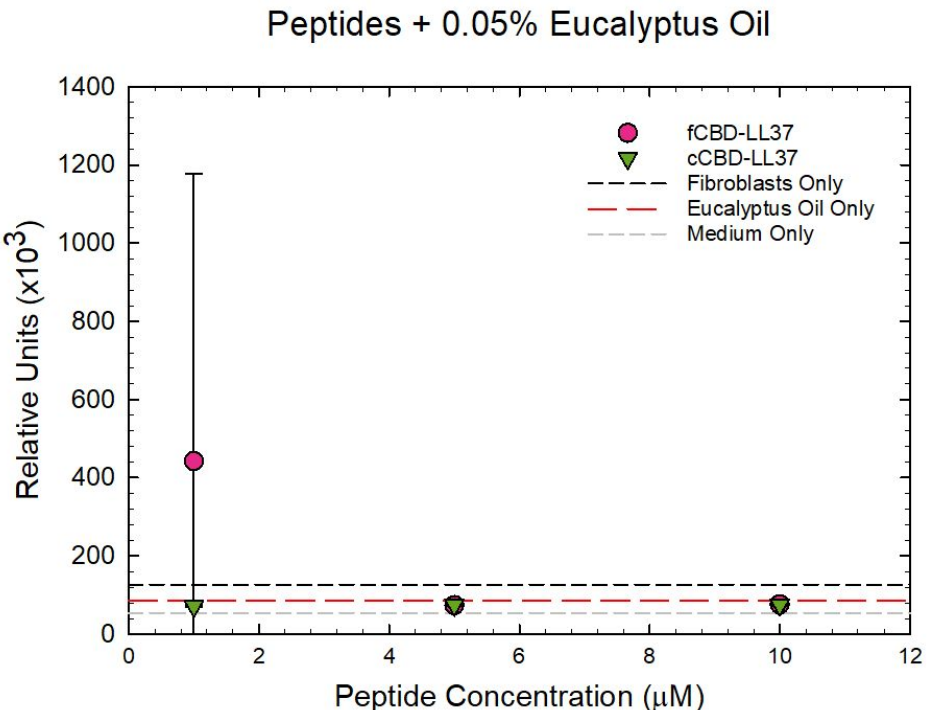
1. Prepare 550µL of 11.1µM peptide solutions by pipetting 275µL medium and 275µL of the 22.2µL peptide solutions to microfuge tubes
2. Prepare 550µL of 2.2µM peptide solutions by pipetting 495µL medium and 55µL of the 22.2µL peptide solutions to microfuge tubes

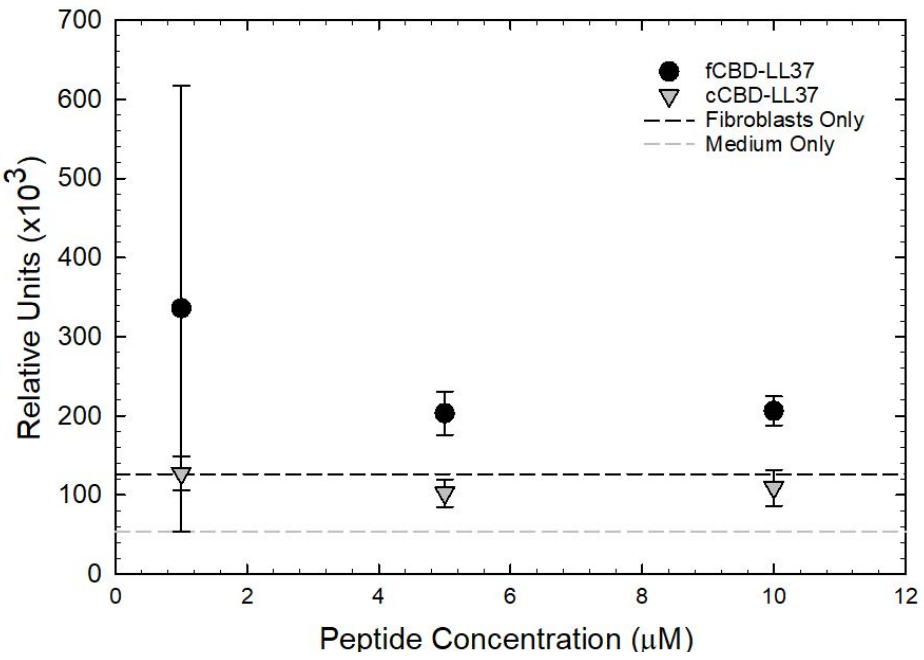
Procedure (Assay):

1. Open the plates seeded with fibroblasts under the hood
2. Aspirate the medium from the wells containing fibroblasts
3. Rinse wells containing fibroblasts with 100µL DPBS(-)
4. Aspirate the DPBS(-) from the wells containing fibroblasts
5. Transfer 45µL of the peptide solutions to the appropriate wells in both plates, following the plate diagrams
6. Transfer 45µL of the essential oil solutions to wells C1-F1 and B9-G12 in both plates, following the plate diagrams
7. Transfer 45µL Tween medium to wells C1-F1 and 90µL to wells H7-H12 in both plates, following plate diagrams
8. Transfer 45µL medium to wells B4-G7 in plate 1, and 90µL to wells A9-A12 and H1-H6 in both plates, following plate diagrams
9. Cover plates and incubate w/ 5% CO_2 at 37ºC for 8 hours
10. Open the plates under the hood
11. Pipette 10µL AlamarBlue® reagent to all wells containing liquid
12. Cover plates and incubate w/ 5% CO_2 at 37ºC for an additional 4 hours
13. Obtain fluorescence and absorbance readings of the plates

Results:







The first replicate of the cytotoxicity assay was inconclusive because there was an extremely small difference between the fluorescence of the sterility and growth controls, and the growth control should have displayed a much larger fluorescence based on the cytotoxicity assay data from Lozeau et al. A troubleshooting assay was performed without any essential oils or peptides to determine whether or not phenol red in the medium had affected the AlamarBlue®

reagent. The results of that assay showed that growth control wells without phenol red had fluorescence readings that were close to those achieved in the cytotoxicity assays performed by Lozeau et al. Three subsequent troubleshooting assays were performed using a control plate without peptides.

Control Plate Setup:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC	Media SC
B	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC	Media w/ Tween SC
C	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells	Manuka w/o cells
D	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells	Euc. w/o cells
E	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells	Manuka w/ cells
F	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells	Euc. w/ cells
G	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC	Media w/ Tween GC
H	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC	Media GC

In an attempt to overcome the previous challenges with this assay, the protocol was changed to reflect procedures found in the literature involving AlamarBlue® reagent and oil emulsification. The cells were incubated for 20 hours with the formulations instead of 8, and the wells were washed twice with DPBS(-) before adding the reagent and 90µL media. This was done so that the reagent would not interact with the Tween® 80 or the oils. The results of this replicate were not an improvement, as there was no observed change in the fluorescence for any wells except the growth control without Tween® 80. When visually examining the wells, no cells were observed in the wells containing Tween® 80 solutions. To ensure that user error had not accidentally washed the cells out of the wells, this troubleshooting assay was repeated, and the results were the same. This indicated that some part of the delivery vehicle was causing cell death or inhibiting cell adherence, and they were being washed out during the DPBS rinse step. For the last troubleshooting experiment, the concentration of Tween® 80 was reduced to 0.1% and 0.05% in order to see if the high concentration (0.5%) of Tween® 80 in the delivery vehicles was proving to be cytotoxic. Two control plates were made, one for each new concentration of Tween® 80. This troubleshooting replicate was not completed because it was observed that the cells were washed out of the wells during the DPBS rinse step. This indicated that even these lowered concentrations of Tween® 80 were still toxic to the fibroblasts, despite literature supporting its use at the lowest concentration, or that the delivery vehicle was preventing adherence to the plate. For the next planned troubleshooting assay, the emulsification method was changed, and DMSO was used instead of Tween® 80 based on a procedure found in the literature. Unfortunately, this alteration did not prove to be any more successful, and no further attempts were made due to time restrictions.