Designing a Probiotic Skin Cream that Repels Mosquitoes

Team Mosquito
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Abstract

Over 700,000 people die annually from vector-borne illnesses. Vectors are living organisms that transmit diseases, with mosquitoes being some of the deadliest. For example, over 40% of people around the world are at risk of contracting malaria, a mosquito-borne disease, due to exposure to mosquitoes. Current methods to prevent mosquito-borne illnesses have limitations: mosquito repellents require frequent reapplication, resulting in the risk of low adherence rates and leaving people susceptible to mosquito bites, and protective covers such as bed nets are inconvenient and have spatial limitations. Therefore, a more effective, convenient, and accessible way to repel mosquitoes is needed to decrease the risk of contracting mosquito-borne diseases. The skin microbiome provides defense mechanisms against external pathogens and has been previously engineered to produce compounds for therapeutic purposes. We seek to prevent mosquito-borne diseases by engineering the skin microbiome to produce mosquito-repelling compounds. We propose using the compound fabclavine, found in the soil bacteria *Xenorhabdus budapestensis*, which exhibits mosquito feeding-deterrence against three deadly and common mosquito vectors: *Aedes aegypti*, *Anopheles gambiae*, and *Culex pipiens*. We aim to evaluate fabclavine's efficacy in repelling mosquitoes from the skin and characterize its safety for human skin and the skin microbiome (Aim 1), engineer the skin microbe *Staphylococcus epidermidis* to produce fabclavines (Aim 2), and create a safe probiotic cream that is effective at repelling mosquitoes and can be applied less frequently (every 2 weeks) (Aim 3 and Aim 4). This project aims to decrease the number of mosquito-borne diseases worldwide and has the potential to become a platform technology with therapeutic applications in combating additional vector-borne illnesses in humans and livestock.

Significance: Vector-borne diseases affect the global population and cause over 700,000 deaths annually, with mosquito-borne illnesses contributing to the majority of these deaths (World Health Organization, 2017). Current approaches to prevent mosquito bites are limited, requiring frequent application or restricting movement. Therefore, it is important to develop a mosquito repellent that will last longer, provide better protection, and be more accessible worldwide. We aim to accomplish these goals by developing an engineered probiotic cream containing bacteria capable of producing fabclavine, a compound three times more effective than the conventionally used DEET, that requires less frequent application. If successful, the development of this probiotic cream will advance the emerging field of skin microbiome engineering and provide a platform to repel vectors beyond mosquitoes and potentially decrease the incidence of vector-borne illnesses around the world.

Aim 1: Identify candidate fabclavine subtypes that effectively repel mosquitoes on skin and characterize their safety profiles. In order to develop a successful mosquito repellent, we need a compound that can be produced by bacteria at a concentration high enough to deter 90% of mosquitoes without affecting host cells. We will focus on fabclavines, peptide/polyketide hybrids produced by *fcl,* a hybrid Polyketide Synthase (PKS) and Non-Ribosomal Peptide Synthetase (NRPS), in the soil bacteria *Xenorhabdus budapestensis* that exhibit mosquito feeding-deterrence (Kajla et al. 2019). To identify the optimal mosquito-repelling subtype of fabclavines, we will first purify the fabclavine subtypes from *X. budapestensis* using high performance liquid chromatography (HPLC), then confirm the chemical structure of each fabclavine using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS), and finally evaluate their mosquito deterrence using artificial membrane feeding assays and olfactometer assays. We will characterize fabclavine's dermal safety on human skin through cell viability assays on human keratinocyte cell lines and its toxicity towards the skin's natural microbes using 16S rRNA sequencing following application of fabclavines on mouse ears. If safety concerns arise, we will modify domains of the *fcl* gene to engineer fabclavine with minimized toxicity on human skin and the skin microbiome. Through this aim, we will identify the optimal subtype of fabclavine that is not toxic to humans at a concentration necessary to deter mosquitoes.

Aim 2: Insert *fcl* gene into the skin microbe *Staphylococcus epidermidis* to produce fabclavine. We will engineer *S. epidermidis*, a prevalent skin microbe, to produce fabclavines by incorporating the *fcl* gene into its genome. In order to accomplish this goal, we must insert the *fcl* gene and a marker to test successful insertion and also make the bacteria more susceptible to antibiotics. To transfer the 61kb-long gene cluster, we will test two methods, transformation and transduction, and select the more efficient method. Each construct used will have an inducible fluorophores such as Green Fluorescent Protein (GFP) to determine successful gene transfer using fluorescence activated cell sorting (FACS), and it will also knock out existing antibiotic resistance genes to make the engineered strain more susceptible to antibiotics such that the genetically-engineered bacteria can be eradicated immediately if needed. This aim will allow us to engineer the bacteria *S. epidermidis* to produce mosquito-repelling compounds.

Aim 3: Develop an efficacious probiotic cream containing fabclavine-producing microbes that will remain stable. We will develop a cream containing the engineered microbes that can be directly applied to human skin. The cream should contain a high enough concentration of fabclavine-producing *S. epidermidis* to deter 90% of mosquitoes and maintain a stable composition. We will limit bacterial metabolism before application to the skin by lyophilization, and the bacteria will be reactivated by mixing with cream when used. We will identify components of the cream and optimal concentrations of these components required for reactivating the bacteria. To test the durability of the cream, we will measure the live bacteria count and the fabclavine production rate by sampling the cream after pre-defined intervals. In the case where creams are infeasible, we will switch to alternative products such as ointments.

Aim 4: Determine engineered probiotic cream efficacy and safety profile. To establish proof-of-concept efficacy of the cream, we will test the cream on guinea pigs *in vivo* to identify the cream concentration needed to reach 90% mosquito deterrence and the duration of that deterrence. We will then characterize the safety and pharmacokinetic profiles of the cream on pigs *in vivo* to determine the maximum tolerated dose (MTD) and the minimum effective dose (MED) that provides 90% deterrence for two weeks. This will allow us to predict pharmacokinetics of the cream on human skin to guide subsequent clinical trials.

Impact: A successful outcome of this project will be a probiotic cream capable of repelling mosquitoes for an extended period of time. Our goal is that the repellant will require less frequent application than current mosquito repellants. The use of the cream on a global scale, especially in developing countries, could decrease the number of mosquito-borne deaths by preventing mosquito bites. Furthermore, if successful, the engineered microbiome platform technology could be applied to various vector-borne diseases that affect humans and livestock.

Significance

Vector-borne diseases are a global issue, causing 700,000 deaths annually (World Health Organization, 2017). Vectors are living organisms that can transmit infectious diseases between humans or from animals to humans. The best known vector is the mosquito, which is responsible for a range of deadly diseases such as dengue, malaria, yellow fever, Zika virus and Eastern equine encephalitis. Approximately 4 billion people in over 128 countries are at risk of contracting dengue, and over 3 billion people globally are at risk of contracting malaria every year. Of these 3 billion people, 435,000 die each year, 60% of whom are children under 5 years old (World Health Organization, 2017).

Current methods of preventing mosquito bites can be effective but have limitations. For example, insect repellents, while able to deter mosquitoes, are only effective for a limited time frame and need to be reapplied multiple times a day. The most effective and widely used insect repellents contain DEET (N,N-diethyl-m-toluamide) and come in various forms (sprays, liquids, lotions, etc.) and concentrations (4-100%). Higher concentrations of DEET lead to longer repellence durability but do not impact the effectiveness of the product (Center for Disease Control). Commonly used repellents contain 20-25% DEET and need reapplication about every 4 hours, and even the most concentrated DEET repellents (98% DEET) require at least 3 rounds of application a day for all-day protection (Rodriguez et al., 2015). Picaridin, while newer than DEET, is another chemical that is capable of effectively repelling mosquitoes and comes in various forms such as lotions and sprays. Formulas of 20% picaridin provide maximum protection of up to 12 hours.

Other methods to prevent mosquito-borne illnesses are also suboptimal. Bed nets and insecticide-treated bed nets (ITNs), while able to provide physical barriers between humans and mosquitoes, are inconvenient and restrict movement. People are only protected within the net, leaving them susceptible to mosquito bites during the day when they are outside and moving around. Additionally, genetically modified malaria-transmitting mosquitoes designed to spread a sterile gene throughout the population and cause the species to crash raise concerns of unintended consequences on the environment if released into communities (Stein, 2019). Furthermore, there is still no licensed malaria vaccine on the market. Therefore, there is a clear need for a more effective product to address mosquito-borne diseases.

We aim to develop a probiotic cream that contains engineered bacteria from the skin microbiome that can produce a mosquito repellant compound more effective than DEET and requires less frequent application. In recent years, researchers have discovered that the skin naturally contains microbes that defend humans against external pathogens and are now realizing their potential to be engineered for therapeutic purposes. Our project will advance this emerging field by developing a method to engineer the skin's microbes to produce a useful compound. If we are able to successfully create this probiotic cream to repel mosquitoes, we will further develop our technology into a platform that can repel additional vectors such as ticks, fleas, and lice to prevent a broader range of infectious diseases affecting humans and animals.

Innovation

This project aims to develop a mosquito-repelling probiotic cream that is more effective, convenient, and accessible than current methods, ultimately decreasing the number of vector-borne deaths globally. The probiotic cream will aim to be better at repelling mosquitoes by providing 1) greater deterrence potency through the more effective microbe-produced compound, and 2) more consistent and longer-lasting protection with a reapplication period of ~2 weeks. This project will change the approach of combating vector-borne diseases, which is currently done by external measures such as chemical repellents and physical barriers, by instead harnessing products of the body's own natural defense mechanism to ward off vectors and prevent disease (Figure 1). Our project will be innovative in the following ways:

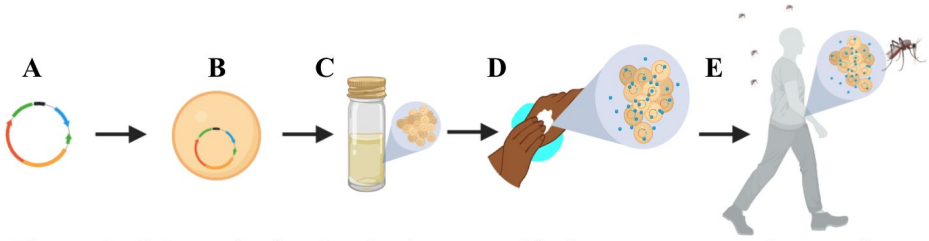


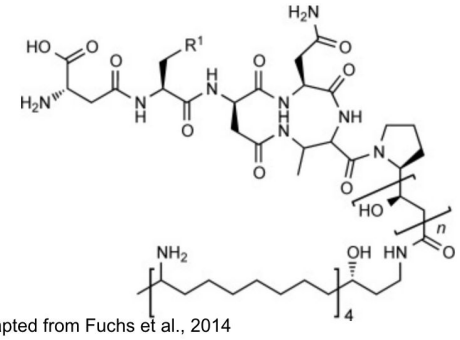
Figure 1: Schematic for developing a probiotic cream to repel mosquitoes. (A) The plasmid containing the mosquito repellent gene is inserted into (B) *S. epidermidis*, a prominent skin microbe. (C) A probiotic cream containing the bacteria is (D) applied to the skin (E) to repel mosquitoes for two weeks.

- Discover an optimal methodology to introduce recombinant genes into skin microbes like *S. epidermidis,* such that the skin can produce new and useful compounds.
- Engineer a probiotic mosquito-repellent cream that has much longer durability (2 weeks) than any existing repellants (several hours).

- Decrease vector-borne diseases not only in humans but also in livestock, which are similarly affected by vectors and present a billion-dollar market (Stuchin et al., 2016).
- Pave the way for research that uses biological engineering to repel disease-carrying vectors.

Background

We aim to produce a probiotic cream that will repel mosquitoes more efficiently, with higher accessibility, and longer dose duration than products used today. We will engineer the skin microbiome to produce a compound that can effectively repel mosquitoes. Companies such as MatriSys Biosciences and Azitra Inc. have engineered skin microbes to treat dermatologic conditions and are currently in Phase I clinical trials. In these therapies, skin microbes, such as *Staphylococcus hominis* and *Staphylococcus epidermidis*, produce compounds necessary to treat dermatologic diseases. We will use similar approaches to engineer microbes to produce a mosquito repellent compound.



Adapted from Fuchs et al., 2014

Figure 2: Structure of fabclavines. Subtypes identified will have a varying n according to the above structure.

A recent study suggests that compounds called fabclavines (Figure 2) exhibit three times more potent feeding-deterrent activity against three deadly mosquito vectors*, Aedes aegypti, Anopheles gambiae,* and *Culex pipiens*, than DEET (Kajla et al., 2019). Fabclavines are small molecules produced by a polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) hybrid gene, *fcl*. In our project, we aim to engineer the skin bacterium *S. epidermidis* to produce a subtype of fabclavine that is most effective at repelling mosquitoes. We will target *S. epidermidis* due to its abundance in the skin microbiome and other companies' success in engineering the microbe such as Azitra Inc. (Monk et al., 2012). After characterizing fabclavines' efficacy and safety profiles and inserting the *fcl* gene into the bacteria, we will develop a cream that contains the engineered bacteria and lasts for up to two weeks for use on human skin.

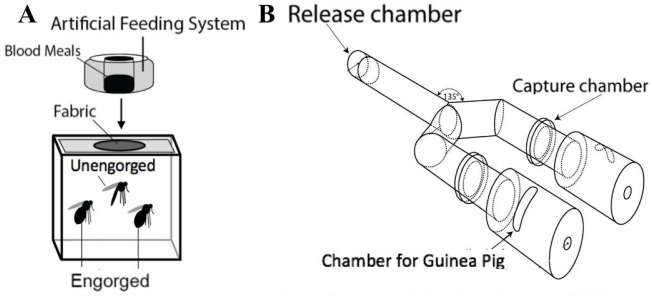
Specific Aim 1: Identify candidate fabclavine subtypes that safely and effectively repel mosquitoes on the skin.

Background:

We will first identify a mosquito repellant compound that can be feasibly produced by the skin microbiome. The compound must be able to repel mosquitoes with better efficiency than DEET-containing repellents that are most commonly used today. Furthermore, the compound must show minimal impact on the human body and the existing skin microbes. We will focus on fabclavine, a naturally-produced compound by the bacteria *Xenorhabdus* that exhibits three times greater feeding deterrence compared to DEET (Kajla et al., 2019). There are several subtypes of fabclavines produced by the *Xenorhabdus* species, *X. budapestensis and X. szentirmaii,* that show mosquito repellency (Kajla et al., 2019; Wenski et al., 2019). In this aim, we will purify three fabclavine subtypes from *Xenorhabdus* and characterize each subtype's safety and mosquito repellency profiles.

Approach

Aim 1.1: Identify potential mosquito-repelling compounds from fabclavines-producing bacteria. To confirm and expand upon previous findings (Kajla et al., 2019), we will first purify fabclavines from *X. budapestensis and X. szentirmaii*. Each species of bacteria will be streaked and cultured in nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) plates. Bacterial colonies will be cultured at 30°C at 120rpm in a rotary shaker for 72 hours. Because fabclavines are secreted from the bacteria, to enrich the active components, we will perform fast protein liquid chromatography (FPLC) on the cell-free culture supernatant after centrifugation of the bacteria. For this purification step, we will use an acetonitrile gradient of 50 to 100% to collect the mosquito deterrent active peak fractions on a C18 flash column. The active fractions will be further filtered using high-performance liquid chromatography (HPLC) on an analytical reversed-phase C18 column. Finally, we will confirm the structures of the active components in each



Adapted from the Hansen Lab Adapted from Fernández-Grandon et al., 2015

Figure 3: Artificial membrane feeding assays and olfactometer assays measure mosquito attraction. (A) Artificial membrane feeding assays determine mosquito feeding by providing a blood food supply and measuring feeding (engorged mosquito) vs no feeding (unengorged). (B) An olfactometer is a Y-shaped tube that measures mosquito preference.

fraction using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

Aim 1.2: Characterize the efficacy of fabclavine subtypes through artificial membrane feeding assays and olfactometer assays. Artificial membrane feeding assays and olfactometer assays are commonly used to determine mosquito attraction and/or deterrence (Figure 3). We will use both *A. aegypti* (Liverpool strain) and *A. gambiae* (G3) mosquitoes in our experiments (Kajla et al., 2019). Artificial membrane feeding assays are an *in vitro* method to measure feeding by mosquitoes in which blood in a warming chamber can be accessed through a membrane (Kajla et al., 2019). Artificial membrane feeding assays containing varying dilutions of each fabclavine subtype in water will provide IC50 and IC90 values for feeding deterrence. Olfactometer assays consist of a Y-shaped wind tunnel used to quantitatively investigate the preference of mosquitoes to host odors (Geier et al., 1999). In this assay, we will use a guinea pig model because guinea pigs attract comparable mosquito strains to humans (Gouck, 1972; McBride et al., 2014). Thirty seconds after releasing mosquitoes in a release chamber of the olfactometer and allowing mosquitoes to enter either side of the Y-shaped tube, each side of the tube will be closed and the number of mosquitoes on each side will be counted, allowing quantification of mosquito preference. We will perform olfactometer assays for fabclavine subtypes with low IC90 using guinea pigs on either side of the chamber. We will compare mosquito aversion between each subtype and identify the most effective subtypes for further studies. In the olfactometer assay, we will also compare mosquito-feeding deterrence to SC Johnson's OFF! Deep Woods containing 25% DEET in water and 7% picaridin in water as controls (Kajla et al., 2019).

Aim 1.3: Assess the safety of efficacious fabclavine subtype(s) on human keratinocytes *in vitro* and in mice *in vivo*. First, we will use N/TERT keratinocytes, a human keratinocyte cell line, to determine the safety profile of each fabclavine subtype on the human epidermal skin layer (Smits et al., 2017). We will treat the cells with varying concentrations of each fabclavine identified from Aim 1.2. To determine the toxicity of fabclavine on keratinocytes, we will measure cell viability by luminescence to determine the minimum toxic dose (MTD). For subtype(s) with MTD above IC90, we will test safety *in vivo*. We will apply each subtype at concentrations between IC90 and MTD on mouse skin over the entire body and observe mouse skin irritation. Because it is important that our compound of choice does not affect the skin microbiome, we will also take microbiome samples from mouse ears to evaluate the effects on the mouse's natural skin microbiome. To do this, we will perform 16S sequencing before and after fabclavine treatment to characterize each subtype's toxicity towards microbe strains. 16S rRNA sequencing allows for taxonomic identification of different microbial species based on the sequence of the 16S rRNA subunit of the prokaryotic ribosome (Patel, 2001). We will use this data to compare the abundance of each microbe present on mouse ears before and after treatment to assess the impact of the molecule on the skin microbiome.

Expected Results: We expect to isolate three subtypes of fabclavines previously described in the literature (Kajla et al, 2019; Wenski et al., 2019). We will determine a range of concentrations for each fabclavine subtype to identify a therapeutic window for the use of each subtype. We then expect to find an optimal fabclavine subtype that has the greatest mosquito repelling capability out of these subtypes through the olfactometer assays. The subtype with the greatest efficacy will be used in later experiments.

Potential Pitfalls and Alternative Approaches: If safety concerns arise, we will first modify the functional groups on fabclavine by selectively introducing or deleting modules from *fcl* to reduce its toxicity (Fuchs et al., 2014). This can be done because of the modular nature of PKS-NRPS and because the role of each module has been previously identified. When such modification attempts fail, we will perform a molecule screen to identify candidate compounds other than fabclavines that exhibit mosquito-deterring properties. Ideally, these candidates should be small molecules that are naturally produced by bacteria so that skin microbes can be feasibly engineered to produce them. These newly identified compounds will undergo the same efficacy and safety testing. We plan to use similar approaches should efficacy concerns arise by modifying the molecule and performing extra screenings when required. An alternative strategy is to produce DEET in the skin microbiome since it is well-characterized and has been approved as mosquito repellent for decades.

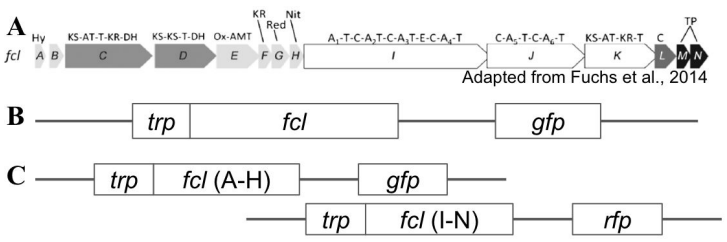


Figure 4: **Structure of the *fcl* gene and construct design.** (A) The *fcl* gene consists of subdomains A through N. (B) Construct contains *fcl* attached to a *trp* promoter and a *gfp* gene. (C) *fcl* will be subdivided into multiple plasmids to facilitate the transformation process.

Specific Aim 2: Insert *fcl* gene into the skin microbe *S. epidermidis* to produce fabclavine.

Background

We will engineer the skin bacteria to confer ability to produce the fabclavine subtype identified in Aim 1. We will focus on *S. epidermidis* as our target bacteria because of its prevalence across the body surface and precedence that the bacteria can be engineered (Grice & Segre, 2011; Monk et al., 2012). The gene transfer process must be carefully designed to account for two factors, the resistance of *S. epidermidis* to transformation and the 61kb size of the *fcl* gene. In this aim, we will design the gene construct and transfer the construct into the target bacteria *in vitro*.

Approach

Aim 2.1: Design the *fcl* gene construct and transfer it into *S. epidermidis*. We will first design the DNA construct to be introduced into the target bacteria (Figure 4). A *trp* promoter, which exhibits strong constitutive expression, will be used to regulate the previously identified *fcl* gene (Amann et al., 1983). The other component will be the *gfp* gene, which encodes the GFP protein and can be used to test the successful gene transfer through fluorescence-activated cell sorting (FACS) (Fu et al., 1999; Cormack et al., 1996). To transfer the construct, we will test two methods, transformation and transduction. *S. epidermidis* is typically resistant to transformation by conventional vectors, so we will use DC10B plasmid, a vector engineered to transform *S. epidermidis* (Monk et al., 2012). Since the DC10B plasmid is too small to fit the entire *fcl*, the subunits of *fcl* will be distributed amongst multiple DC10B plasmids, with *trp* promoters added upstream of each subunit gene and different fluorophores added to each construct (gfp and rfp) (Figure 4C). Previous studies have shown that the modular nature of PKS-class proteins would allow for such separation of subunits to achieve functional expression of the enzyme (Pfeifer et al., 2001). For transduction, we will use two strains of viruses, P1 virus and *S. epidermidis* phage 48. While P1 virus is an established transduction vector that can carry up to 100kb, its infection capability against *S. epidermidis* is unknown. On the other hand, *S. epidermidis* phage 48 has been used to transduce *S. epidermidis*, but its carrying capacity as a vector is not yet established (O'Connor & Zusman, 1983; Dybvig et al., 1988; Nedelmann et al., 1998). The bacteria will be co-incubated for 30 mins with one of the two viruses for transduction, and the cells will be collected through centrifugation (Kayser et al., 1972). In both transfer methods, we will knock out any existing antibiotic resistance gene in the *S. epidermidis* to increase the susceptibility of the engineered bacteria to antibiotics; this serves as a kill switch to selectively eradicate the engineered organism when needed. The two transfer methods will be performed in parallel to engineer *S. epidermidis* to compare their relative potentials for successful insertion of the construct.

Aim 2.2: Measure the gene transfer efficiency of transforming and transducing *S. epidermidis*. We will compare the efficiency of transformation and transduction procedures to determine the optimal way to insert *fcl*. FACS will be used to measure the amount of engineered fluorophore in the cells to determine the efficiency of gene transfer. The culture supernatant of the engineered bacteria will be analyzed using HPLC to measure the abundance of secreted fabclavine in the bacteria (Aim 1). The fabclavine production rate and the transfer efficiency rate will be used to assess the fabclavine production efficiency in transformation and transduction to choose the optimal method for later use.

Expected Results: We expect to see that both transformation and transduction successfully transfer the *fcl* construct into *S. epidermidis*, considering the precedence of engineering bacteria (Pfeifer et al., 2001; Monk et al., 2012). When the two methods are compared, we expect that the transduction method to have higher efficiency, since transformation requires recombination of the modules of the FCL protein inside the cell. We expect the more efficient method of the two to yield enough fabclavine to effectively repel mosquitoes.

Potential Pitfalls and Alternative Approaches: In the case that neither of the approaches produces a fabclavine yield sufficient for mosquito repellency, we will optimize the procedures for increased production. Different promoters will be tested to find the optimal promoter that induces expression of *fcl*. The transformation procedure will be optimized by varying the number of split constructs. The transduction procedure will be further tested using other established virus vectors with the size limit below our *fcl* construct. Similar to transformation, *fcl* subunits will be distributed into separate virus vectors and multiple transductions will be performed to introduce the entire construct.

Specific Aim 3: Develop an efficacious probiotic cream containing fabclavine-producing microbes.

Background

We aim to develop a cream containing fabclavine-producing *S. epidermidis* that can be applied to human skin. *S. epidermidis* will be engineered as outlined in Aim 2 to produce fabclavine or another mosquito-deterring compound determined in Aim 1. We identify three criteria that the cream must meet to provide competitive advantages over current available products such as DEET and Picaridin: 1) To guarantee effectiveness, the cream should contain a high enough

concentration of fabclavine-producing *S. epidermidis* to deter mosquitoes, 2) To ensure durability, the composition of the cream, especially the microbes, should remain relatively stable and should not degrade over time, 3) To make the product accessible to the general population, the storage and transportation of the cream should be made as easy as possible. We aim to achieve these goals by limiting bacterial metabolism before application to the skin through lyophilization and identifying additional compounds to be included in the cream that aid in durability and accessibility.

Approach

Aim 3.1: Lyophilize *S. epidermidis* to limit bacterial metabolism in the cream. Given the limited resources in the enclosed container, it is impractical to sustain a stable population of metabolically-active microbes. Therefore, we plan to minimize bacterial metabolism by lyophilizing *S. epidermidis* using a standard lyophilization protocol. The lyophilized *S. epidermidis* can be mixed with the rest of the cream ingredients for activation prior to application on the skin. The lyophilizing media, equipment, and hydrating fluids are available commercially (OPS diagnostics: Bacteria Freeze Drying Protocol). We expect to store the lyophilized *S. epidermidis* between 2°C to 25°C and potentially under room temperature with relatively good viability (Medline: Lyophilized or Dried Biological Material Preparations; Antheunisse, 1973). We will balance the viability and the temperature requirement by experimenting with different lyophilization methods and storage conditions, then examine *S. epidermidis* viability with the method outlined in Aim 3.3.

Aim 3.2: Determine the composition of the base cream for optimal stability. Besides lyophilizing *S. epidermidis*, we need to develop the base of the cream that can be mixed with the bacteria prior to application. The majority of the base cream will be the hydrating fluid, which hydrates and activates lyophilized *S. epidermidis* when mixed together. The base cream will potentially contain pH buffers, antioxidants, preservatives, and osmolality agents to help stabilize the cream, as well as fragrances and other additives to improve customer perception (Kulkarni & Shaw, 2016). The exact composition and concentration will be determined in conjunction with Aim 3.3.

Aim 3.3: Activate lyophilized *S. epidermidis* and measure fabclavine production rates in the cream. To validate the durability of our product, we will sample the cream after predefined intervals ranging from several hours to months and measure viable bacteria count upon activation with the base cream. We will measure viable cell counts, or equivalently, colony-forming units (CFU), in suspension using the 5-tube most probable number (MPN) test in liquid medium and colony counting (Taylor, 1962), and measure total DNA content using quantitative real-time PCR. To test the fabclavine concentration, we will perform FPLC on the cell-free culture supernatant, purify and filter using C18 flash column and HPLC, and measure the fabclavine content using MALDI-TOF MS, as described in Aim 1.

Expected Results: We expect that the cream will produce fabclavines efficiently and remain stable for a sustained period of time using lyophilization to limit metabolism and the cream base to rehydrate and thus activate the bacteria. The viable cell count and the fabclavine production after a prolonged period should sustain an equilibrium capable of repelling more mosquitoes according to predefined efficacy endpoints.

Potential Pitfalls and Alternative Approaches: Some protocols suggest that lyophilized bacterial culture should be kept in vacuum under 4°C in the dark (OPS diagnostics: Bacteria Lyophilization Overview). In the case that the cream does not meet our goals, we will turn to alternative media to transfer engineered *S. epidermidis* onto the skin. One such example is an oil-based ointment as opposed to water-based cream. To limit bacterial metabolism in the ointment, we can potentially facilitate *S. epidermidis* persister cell formation and dormancy by introducing environmental stressors (e.g. starvation) or selectively upregulating genes associated with dormancy proteins. Transformation from the dormant state back to the activated state can potentially be achieved by removing such stress or downregulating dormancy/sporulation genes prior to the application on the skin. Specifically, the design of an activating ointment/agent that the user can mix with the *S. epidermidis* containing ointment to "activate" the bacteria and the fabclavine pathway. Another idea is a patch with concentrated *S. epidermidis* that can be pasted on the skin such that the effective distance of fabclavine in repelling mosquitoes becomes more important.

Specific Aim 4: Determine engineered probiotic cream efficacy and safety profile.

Background

We aim to ensure that the probiotic cream repels 90% of mosquitoes for two weeks. The two-week application frequency is based on a previous study suggesting significant engraftment of microbes on the epidermis for about two weeks (Paetzold et al., 2019). We will test proof of concept efficacy in guinea pigs because guinea pigs attract similar mosquitoes to humans (Gouck, 1972; McBride et al., 2014). Cream safety and pharmacokinetics will be tested in pigs because pig skin is commonly used for dermal drug testing (Abd et al., 2016). For pharmacological characterization in

pigs, we will measure fabclavine half-life on skin, half-life of the engineered *S. epidermidis* cream, and the cream's protective radius. We will also measure *S. epidermidis* engraftment because this can affect cream durability. Based on pharmacological parameters from pre-clinical studies, we will model the pharmacokinetic profile of the cream to guide clinical development.

Approach

Aim 4.1: Test the efficacy of the probiotic cream in repelling mosquitoes on guinea pigs. We will apply the probiotic cream on guinea pigs to evaluate efficacy. On Day 1, the cream will be applied on guinea pigs at surface concentrations of 10^4, 10^6, 10^8, and 10^{10} CFU/cm^2 (n=15 per condition). Each day, from Day 1 to 14, 3 guinea pigs in each dose group will be subjected to an animal-in-cage feeding assay to measure mosquito deterrence by counting the number of bites (Bousema et al., 2012). Skin microbiome samples will be taken before each feeding assay to measure *S. epidermidis* engraftment via single-locus sequence typing (SLST) (Scholz et al., 2014). The feeding assay will identify the necessary cream concentration to achieve 90% mosquito deterrence and the durability of that deterrence. We will analyze the correlation between mosquito deterrence and *S. epidermidis* engraftment to guide modeling of fabclavine and *S. epidermidis* concentration over time.

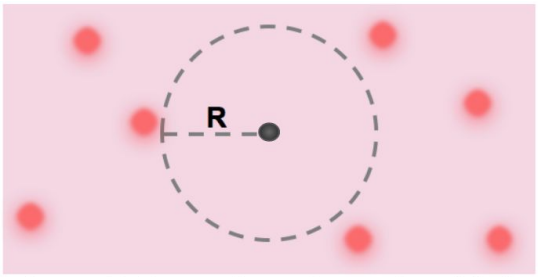


Figure 5. Protective sphere of probiotic cream. Example pig skin in direct feeding assay. Black: cream drop. Red: mosquito bites. R: protective radius.

Aim 4.2: Study the safety and pharmacokinetic profile of probiotic cream on pig skin. We will build upon the safety and efficacy of fabclavine established in Aim 1 for mice *in vivo* by characterizing the safety and pharmacokinetics of the probiotic cream on pig skin. First, we will apply I^{125}-labeled fabclavine alone to pig skin, to observe skin irritation and safety, and measure fabclavine chemical half-life on skin through radio-counting. We will then measure the protective radius of the cream. To do this, a 50μL drop of cream at varying concentrations will be placed on the pig's forelimb and feeding assays will measure the dose response of the protective sphere radius (Figure 5). Next, the cream will be applied on the pig's entire body at 10^4 to 10^{10} CFU/cm^2, and mosquito deterrence will be measured as described in Aim 4.1 to characterize the cream's safety and pharmacokinetics. Safety data including vital signs, adverse skin reactions, and other adverse events will be collected per FDA guidance (US Food & Drug Administration, 2019). This experiment will determine the minimum effective cream dose (MED) that provides 90% deterrence for at least 7 days and the maximum tolerated dose (MTD). Cream at concentrations between MED and MTD will be applied on pigs every 1, 2, or 3 weeks in a repeat-dosing experiment over 12 weeks, to study medium-term safety and efficacy via feeding assay.

Aim 4.3: Model probiotic cream pharmacokinetics on human skin. A recent study shows that 90% mosquito deterrence requires a concentration of 0.057 mg/cm^2 for fabclavine and 0.178 mg/cm^2 for DEET (Kajla et al. 2019). Based on DEET recommended dose of 1.7 g/day for malaria prevention (Koren et al., 2003), we estimate that a fablcavine concentration of 15 μg/cm^2 needs to be maintained for mosquito deterrence. To model the effective human dose, we will use fabclavine half-life, production rate, and engraftment percentage measured from pre-clinical studies in Aims 1, 2, and 4. Preliminary calculations show that assuming engraftment percentage of 5% for 2 weeks (Paetzold et al., 2019), fabclavine half-life of 24 hr, and bacteria fabclavine production rate of 0.43 pg/hr*CFU, the necessary dose is $2*10^7$ CFU/cm^2. This is comparable to natural skin microbe concentration 10^6-10^9 CFU/cm^2 (Ross et al., 2019), and corresponds to cream microbe concentration of $9*10^{10}$ CFU/mL, comparable to current probiotic formulations (Trinchieri et al., 2017).

Expected Results: For guinea pig and pig feeding assays, we expect higher mosquito deterrence with increasing cream concentration. By testing a range of concentrations, we expect to identify the concentration that maintains 90% deterrence for 14 days. For the pig protective sphere assay, we expect the protective sphere of the cream to be lower than that of DEET because fabclavine is nonvolatile, but higher than that of fabclavine alone due to microbe mobility. The cream safety and efficacy profiles will inform the optimal dosing schedule.

Potential Challenges and Alternative Approaches: If adverse reactions occur in guinea pigs or pigs, we will study the molecular mechanism of toxicity. If 90% deterrence cannot be maintained at a safe dosage, we will identify alternative mosquito repelling compounds such as DEET, and engineer skin microbes to produce the alternative compounds. Another potential challenge is microbe engraftment. Engraftment is necessary to maintain efficacy, and engraftment is likely different between animals and humans. We will measure engraftment on guinea pigs, but engraftment on human skin can only be tested in clinical trials. In clinical trials, if we do not observe sufficient engraftment for 2 weeks, we will sterilize the skin before cream application to improve engraftment, or we will increase dosing frequency to maintain deterrence.

Perspective

We envision that, if successful, our project will become a skin microbiome platform technology that can address multiple vector-borne infectious diseases in both humans and livestock. While we will first focus on mosquito repellency in humans, we will also explore repelling other disease-carrying vectors, including ticks, fleas and flies to prevent diseases such as Lyme disease, Rickettsiosis, and African sleeping sickness, respectively. Furthermore, we plan to expand into the agricultural industry since livestock can also contract vector-borne diseases. In fact, tick-borne diseases (TBD) are a major challenge to farmers, affecting 80% of the world's livestock holdings with an estimated economic cost of $14-$19 billion annually as calculated by Minjauw and McLeod (Stuchin et al., 2016). Thus, there exists another major market that our technology could address.

Translation and Commercialization

Clinical/Regulatory Planning:

Following efficacy and safety studies on the probiotic cream in rodents and pigs, we will pursue human clinical trials. We will determine whether the probiotic cream is categorized as a cosmetic or a therapy. If the cream is a therapy, we will file an investigational new drug (IND) application with the FDA. The IND will contain information on *in vitro* and *in vivo* preclinical studies of fabclavine alone and the probiotic cream determined in our previous experiments.

For the first in-human trial, we will perform a single-dose escalation study of fabclavine on healthy volunteers to evaluate fabclavine safety. Then, in the Phase Ib trial, we will apply probiotic cream with varying microbe concentrations on arms and legs of healthy volunteers, with dosing guided by preclinical data to characterize cream safety. In a Phase II trial, we will apply varying concentrations of the cream up to the maximum tolerated dose on the entire skin surface of healthy volunteers at an application frequency of 1, 2, or 3 weeks. Engraftment will be measured via single-locus sequence typing (SLST), and efficacy profile will be measured through repeat arm-and-leg-in-cage feeding assays with multiple mosquito species.

Intellectual Property (IP) Considerations

Our work presents a novel method to repel mosquitoes. Existing methods designed to repel mosquitoes rely on either a physical structure to prevent mosquitoes (e.g. bed nets), gene drive to eradicate mosquito populations (which has not yet been proven effective), or a chemical repellent that is sprayed periodically (Gantz et al., 2015). None of the existing solutions provide an effective, long-lasting and portable approach that combats mosquito-borne illnesses. Our idea of creating a probiotic cream provides a method that is different from competing methods in that it forms a long-lasting buffer zone around the body to repel mosquitoes. Moreover, the technical details for implementing the method combine traditional knowledge on the microbiome to recently characterized microbial small molecules, creating a novel method of interacting with the human skin microbiome.

The alteration of the human microbiome has been conducted in the field of pharmacology. Microbiomes have been administered therapeutically to treat metabolic disorders (PCT/US2015/058511), while fecal transplant has allowed for an effective treatment against Clostridium difficile infections and irritable bowel disease (Kassam et al., 2012). The skin microbiome has also been a target of therapeutic alterations such as the probiotic application of *S. epidermidis* to inhibit the growth of *P. acnes*, the main cause of acne (Grice, 2014).

Our probiotic cream to repel disease vectors, such as mosquitoes, is patentable. Neither the use of fabclavine nor engineering of *S. epidermidis* for therapeutic purposes have been patented. While the core methods do not require licensing, the lyophilization process requires licensing from an existing patent (US8793895B2).

Market Analysis and Commercialization Plan

Market Analysis. The global market size of mosquito repellent products was $3.2 billion in 2016 and is forecasted to be $5 billion in 2022 (Zion Market Research, 2016). In particular, the U.S. market is valued at $737 million in 2019 and is expected to increase to nearly $1.2 billion by 2025. Currently available insect repellents can be segmented into two categories: synthetic and natural. Among all product types, vaporizer repellents have the largest market share and account for around 30% of the value (KBV Research, 2019). Because of the innovative nature of our product, we hope to reach a broader customer base and open up new opportunities unreachable with the current solutions.

Application towards livestock. Our primary focus is to prevent mosquito-borne diseases in humans. Nonetheless, there is potential for extension of our product for agricultural purposes such as preventing vector-borne diseases carried by mosquitoes, ticks, fleas, and lice in livestock. For instance, managing ticks and tick-borne diseases (TBD) in the livestock industry is a major challenge both to individual farmers and on a global scale. The recent outbreak of African Swine Fever, which first appeared in China and later spread to other regions within East Asia, is transmitted by ticks of the genus *Ornithodoros* (Denyer et al., 1999). It has resulted in the death of over 10 million pigs and a $140 billion loss for China (The World staff, 2019; Sun et al., 2019). TBD affects 80% of the livestock holdings worldwide, resulting in an economic cost estimated between $13.9 billion to $18.7 billion annually (Minjauw and McLeod, 2003). Such economic burden can be substantial in resource-poor tropical and subtropical regions, particularly to small-scale livestock owners (Minjauw and McLeod, 2003). In this case, a spray containing engineered skin microbes rather than a cream would be better because it can be applied evenly and easily to a large area. We can also expect a faster approval process compared to human clinical trials from the FDA along with other foreign regulatory agencies.

Commercialization Plan. The first step towards commercialization is to establish the capabilities for large-scale manufacturing. This includes building biological reactors and sustaining stable *S. epidermidis* populations. We will sample daily from each reactor for quality control, using most probable number (MPN) tests for viable cell counting and quantitative real-time PCR to determine the species and concentrations within, as well as checking for mutations should they arise. We will also focus on building the production line, starting with extracting bacteria from reactors, lyophilizing the bacteria for storage, then creating single-use packets that contain the lyophilized bacteria on one side and cream base on the other side. The single-use packets will be designed such that when the product is ready to be applied to the skin, puncturing or squeezing the packet will cause the two sides to mix and turn into a cream.

Pilot testing. We plan to launch two pilot tests, one in Florida and the other one in Cambodia, before full-scale commercialization. The choice of Florida is due to its non-endemic setting, tropical climate thus higher demand for repellents, and the representativeness of the first world environment. We will launch in Cambodia because of its representativeness of the developing world and the prior success of another mosquito-repellent program (Heng et al., 2015). A group of international researchers implemented a distribution scheme of a topical repellent using the local public health system for a cluster-randomized trial, which is similar to our proposed experiments and ideal for launching a pilot test. We will focus on customer perception and receptiveness in the former and on testing the epidemiological efficacy and adherence of the product in the latter.

Full-scale distribution. At the late stage of commercialization, we plan to widely distribute our product into both emerging markets and developed markets. We will adjust the marketing strategy and distribution channel based on the target population. In developing countries in Africa and Southeast Asia, we will focus on the coverage rate and collaborate with various organizations including the government, the indigenous community, non-governmental organizations, as well as other public health programs. To ensure high coverage and adherence, we will establish programs to educate and train selected members of the community to act as ambassadors, similar to the training and developmental program utilized by Coca-Cola for their last-mile distribution (Yadav, 2012). By building a system based on information and trust rather than normative guidance and regulation, the incentives of the local distributors are aligned with the company goals and often dramatically increases the coverage and adherence rate. As an illustration, Project Last Mile is an initiative to help African governments supply vital medicines to hard-to-reach communities utilizing the aforementioned principles and has shown early-stage success in Tanzania, Mozambique, Ghana, and Nigeria (Jordin, 2016).

In developed economies such as North America and Eastern Europe, we will differentiate ourselves as a natural, probiotic cream that focuses on the convenience and effectiveness due to low reapplication frequency. Three target populations we identified include 1) outdoor enthusiasts (e.g. backpackers, hikers, and campers), 2) short-term travelers to areas where mosquito-borne diseases are prevalent, and 3) families with young kids who play outdoors for a significant amount of time. Advertisement campaigns will help shape customer perception, and relations with medical/healthcare suppliers and pharmacies/retailers will help increase the customer base.

Acknowledgments: We thank P. Bhargava, E. Choe, S. Clarke, J. Collins, and J. Essigmann for their guidance and feedback in the development of this project; T. Lieberman and T. Whitfill for their help in understanding the skin microbiome and its potential to be engineered; T. Whitfill for his feedback on our project design; and J. Niles for his expertise in mosquito-borne diseases and advice in manufacturing and consumer perception.

References

Abd, E., Pastore, M., & Telaprolu, K. (2016). Skin models for the testing of transdermal drugs. *Dovepress*, *2016*(8), 163–176. https://doi.org/10.2147/CPAA.S64788

Amann, E., Brosius, J., & Ptashne, M. (1983). Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in Escherichia coli. *Gene*, *25*(2), 167–178. https://doi.org/10.1016/0378-1119(83)90222-6

Antheunisse, J. A. van L. (1973). Viability of lyophilized microorganisms after storage. *Kluwer Academic Publishers*, *39*(243). https://doi.org/10.1007/BF02578856

Bacteria Freeze Drying Protocol. (n.d.). Retrieved November 26, 2019, from OPS Diagnostics website: https://opsdiagnostics.com/notes/ranpri/rpbacteriafdprotocol.htm

Bacteria Lyophilization Overview. (n.d.). Retrieved November 26, 2019, from OPS Diagnostics website: https://opsdiagnostics.com/notes/ranpri/bacteria_lyophilization_overview.htm

Bousema, T., Dinglasan, R. R., & Morlais, I. (2012). Mosquito Feeding Assays to Determine the Infectiousness of Naturally Infected Plasmodium falciparum Gametocyte Carriers. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042821

Center for Disease Control. (n.d.). *Fight the Bite for Protection from Malaria Guidelines for DEET Insect Repellent Use*. Retrieved from https://www.cdc.gov/malaria/toolkit/deet.pdf

Cormack, B. P., Valdivia, R. H., & Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, *173*(1), 33–38. https://doi.org/10.1016/0378-1119(95)00685-0

Denyer, M. S., & Wilkinson, P. J. (1998). African Swine Fever. In P. J. Delves (Ed.), *Encyclopedia of Immunology (Second Edition)* (pp. 54–56). https://doi.org/10.1006/rwei.1999.0015

Dybvig, K., Alderete, J., Watson, H. L., & Cassell, G. H. (1988). Adsorption of mycoplasma virus P1 to host cells. *Journal of Bacteriology*, *170*(9), 4373–4375. https://doi.org/10.1128/jb.170.9.4373-4375.1988

Fu, A. Y., Spence, C., Scherer, A., Arnold, F. H., & Quake, S. R. (1999). A microfabricated fluorescence-activated cell sorter. *Nature Biotechnology*, (17), 1109–1111. https://doi.org/10.1038/15095

Gantz, V. M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V. M., Bier, E., & James, A. A. (2015). Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proceedings of the National Academy of Sciences*, *112*(49), E6736–E6743. https://doi.org/10.1073/pnas.1521077112

Geier, M., & Boeckh, J. (1999). A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours. *Entomologia Experimentalis et Applicata*, *92*(1), 9–19. https://doi.org/10.1046/j.1570-7458.1999.00519.x

Gouck, H. K. (1972). Host preferences of various strains of Aedes aegypti and A. simpsoni as determined by an olfactometer. *Bulletin of the World Health Organization*, *47*(5), 680–683.

Grice, E. A. (2014). The skin microbiome: Potential for novel diagnostic and therapeutic approaches to cutaneous disease. *Seminars in Cutaneous Medicine and Surgery*, *33*(2), 98–103.

Grice, E. A., & Segre, J. A. (2011). The skin microbiome. *Nature Reviews Microbiology*, *9*, 244.

Heng, S., Durnez, L., Gryseels, C., Van Roey, K., Mean, V., Uk, S., … Sluydts, V. (2015). Assuring access to topical mosquito repellents within an intensive distribution scheme: A case study in a remote province of Cambodia. *Malaria Journal*, *14*. https://doi.org/10.1186/s12936-015-0960-4

Jordin, A. (2016, January 19). Project Last Mile Expands to 8 More African Countries: The Coca-Cola Company. Retrieved November 17, 2019, from https://www.coca-colacompany.com/stories/projectlastmile

Kajla, M. K., Barrett-Wilt, G. A., & Paskewitz, S. M. (2019). Bacteria: A novel source for potent mosquito feeding-deterrents. *Science Advances*, *5*(1), eaau6141. https://doi.org/10.1126/sciadv.aau6141

Kassam, Z., Hundal, R., Marshall, J. K., & Lee, C. H. (2012). Fecal Transplant via Retention Enema for Refractory or Recurrent Clostridium difficile Infection. *Archives of Internal Medicine*, *172*(2), 191–193. https://doi.org/10.1001/archinte.172.2.191

Kayser, F. H., Wüst, J., & Corrodi, P. (1972). Transduction and Elimination of Resistance Determinants in Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *2*(3), 217–223. https://doi.org/10.1128/AAC.2.3.217

KBV Research. (2019, October). North America Insect Repellent Market (2019-2025). Retrieved November 17, 2019, from Research and Markets website: https://www.researchandmarkets.com/reports/4852724/north-america-insect-repellent-market-2019-2025

Koren, G., Matsui, D., & Bailey, B. (2003). DEET-based insect repellents: Safety implications for children and pregnant and lactating women. *CMAJ: Canadian Medical Association Journal*, *169*(3), 209–212.

Kulkarni, V. S., & Shaw, C. (2016). Chapter 7—Preparation and Stability Testing. In V. S. Kulkarni & C. Shaw (Eds.), *Essential Chemistry for Formulators of Semisolid and Liquid Dosages* (pp. 99–135). https://doi.org/10.1016/B978-0-12-801024-2.00007-8

Lyophilized or Dried Biological Material Preparations. (n.d.). Microbiologics.

McBride, C. S., Baier, F., Omondi, A. B., Spitzer, S. A., Lutomiah, J., Sang, R., … Vosshall, L. B. (2014). Evolution of mosquito preference for humans linked to an odorant receptor. *Nature*, *515*(7526), 222–227. https://doi.org/10.1038/nature13964

Medline. (n.d.). Staphylococcus epidermidis ATCC 12228 by Microbiologics. Retrieved November 17, 2019, from https://www.medline.com:443/product/Staphylococcus-epidermidis-ATCC-12228-by-Microbiologics/Controlled-Organisms/Z05-PF126717

Minjauw, B., & McLeod, A. (2003). *Tick-borne diseases and poverty. The impact of ticks and tickborne diseases on the livelihood of small-scale and marginal livestock owners in India and eastern and southern Africa.* Retrieved from http://agris.fao.org/agris-search/search.do?recordID=GB2012100456

Monk, I. R., Shah, I. M., Xu, M., Tan, M.-W., & Foster, T. J. (2012). Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically Staphylococcus aureus and Staphylococcus epidermidis. *MBio*, *3*(2). https://doi.org/10.1128/mBio.00277-11

Nedelmann, M., Sabottke, A., Laufs, R., & Mack, D. (1998). Generalized Transduction for Genetic Linkage Analysis and Transfer of Transposon Insertions in Different Staphylococcus epidermidis Strains. *Zentralblatt Für Bakteriologie*, *287*(1), 85–92. https://doi.org/10.1016/S0934-8840(98)80151-5

O'Connor, K. A., & Zusman, D. R. (1983). Coliphage P1-mediated transduction of cloned DNA from Escherichia coli to Myxococcus xanthus: Use for complementation and recombinational analyses. *Journal of Bacteriology*, *155*(1), 317–329.

Paetzold, B., Willis, J. R., Pereira de Lima, J., Knödlseder, N., Brüggemann, H., Quist, S. R., … Güell, M. (2019). Skin microbiome modulation induced by probiotic solutions. *Microbiome*, *7*. https://doi.org/10.1186/s40168-019-0709-3

Patel, J. B. (2001). 16S rRNA Gene Sequencing for Bacterial Pathogen Identification in the Clinical Laboratory. *Molecular Diagnosis*, *6*(4), 313–321. https://doi.org/10.1007/BF03262067

Pfeifer, B. A., Admiraal, S. J., Gramajo, H., Cane, D. E., & Khosla, C. (2001). Biosynthesis of Complex Polyketides in a Metabolically Engineered Strain of E. coli. *Science*, *291*(5509), 1790–1792. https://doi.org/10.1126/science.1058092

Rodriguez, S. D., Drake, L. L., Price, D. P., Hammond, J. I., & Hansen, I. A. (2015). The Efficacy of Some Commercially Available Insect Repellents for Aedes aegypti (Diptera: Culicidae) and Aedes albopictus (Diptera: Culicidae). *Journal of Insect Science*, *15*(1). https://doi.org/10.1093/jisesa/iev125

Ross, A. A., Hoffmann, A. R., & Neufeld, J. D. (2019). The skin microbiome of vertebrates. *Microbiome*, *7*(79). https://doi.org/10.1186/s40168-019-0694-6

Scholz, C. F. P., Jensen, A., Lomholt, H. B., Brüggemann, H., & Kilian, M. (2014). A Novel High-Resolution Single Locus Sequence Typing Scheme for Mixed Populations of Propionibacterium acnes In Vivo. *PLOS ONE*, *9*(8), e104199. https://doi.org/10.1371/journal.pone.0104199

Smits, J. P. H., Niehues, H., & Rikken, G. (2017). Immortalized N/TERT keratinocytes as an alternative cell source in 3D human epidermal models. *Sci Rep*, *7*(118). https://doi.org/10.1038/s41598-017-12041-y

Stein, R. (2019, March 1). Your Questions About Italy's GMO Mosquito Experiment, Answered. Retrieved November 17, 2019, from National Public Radio website: https://www.npr.org/sections/goatsandsoda/2019/03/01/698708765/your-questions-about-italys-gmo-mosquito-experiment-answered

Stuchin, M., Machalaba, C. C., & Karesh, W. B. (2016). *VECTOR-BORNE DISEASES: ANIMALS AND PATTERNS*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK390438/

Sun, L., & Tang, Z. (2019, September 25). Deadly Pig Disease Has Cost China More Than $140 Billion: Professor—Caixin Global. Retrieved November 17, 2019, from Caixin website: https://www.caixinglobal.com/2019-09-25/deadly-pig-disease-has-cost-china-more-than-140-billion-professor-101466143.html

Taylor, J. (1962). The Estimation of Numbers of Bacteria by Tenfold Dilution Series. *Journal of Applied Bacteriology*, *25*(1), 54–61. https://doi.org/10.1111/j.1365-2672.1962.tb01119.x

Trinchieri, V., Laghi, L., & Vitali, B. (2017). Efficacy and Safety of a Multistrain Probiotic Formulation Depends from Manufacturing. *Frontier Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01474

The World staff. (2019, October 31). Pork prices soar as millions of pigs die due to African swine fever. Retrieved November 17, 2019, from Public Radio International website: https://www.pri.org/stories/2019-10-31/pork-prices-soar-millions-pigs-die-due-african-swine-fever

US Food & Drug Administration. (2019, May). Maximal Usage Trials for Topically Applied Active Ingredients Being Considered for Inclusion in an Over-The -Counter Monograph: Study Elements and Considerations | FDA. Retrieved November 26, 2019, from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/maximal-usage-trials-topically-applied-active-ingredients-being-considered-inclusion-over-counter

Wenski, S. L., Kolbet, D., Grammbitter, G. L. C., & Bode, H. B. (2019). Fabclavine biosynthesis in X. szentirmaii: Shortened derivatives and characterization of the thioester reductase FclG and the condensation domain-like protein FclL. *Journal of Industrial Microbiology & Biotechnology*, *46*(3–4), 565–572.

World Health Organization. (2017, October 31). Vector-borne diseases. Retrieved November 17, 2019, from World Health Organization website: https://www.who.int/en/news-room/fact-sheets/detail/vector-borne-diseases

Yadav, P. (2012, December 13). Coke is Everywhere: Why Aren't Medicines? Retrieved November 17, 2019, from NextBillion website: https://nextbillion.net/coke-is-everywhere/

Zion Market Research. (2019, August 9). Global mosquito repellent market value in 2016 and 2022 (in billion U.S. dollars). Retrieved November 17, 2019, from Statista website: https://www.statista.com/statistics/631760/mosquito-repellent-market-value-worldwide/

Appendix:

Pharmacokinetics Modeling of Probiotic Cream

We aim to calculate the required fabclavine concentration and the corresponding *S. epidermidi*s density using a standard single-molecule production-decay model.

I. Calculate the recommended DEET dose

The World Health Organization (WHO) recommends application of DEET twice a day for a total daily dose of 1.7g for malaria prevention (Koren et al. 2003). Based on this information, and assuming a skin surface area of 1.8m^2, we can calculate the recommended surface concentration of DEET (initial concentration upon 1st time application), $\mathbf{C_D}$, with the following formula:

$$C_D = 1.7g * \frac{1}{2} * \frac{1}{1.8m^2} * \frac{1m^2}{10^4 cm^2} * \frac{10^3 mg}{1g} = 0.047\ mg/cm^2$$

II. Obtain the necessary fabclavine dose

We assume that the WHO recommended DEET dose for malaria prevention is capable of repelling most mosquitos. We assume this concentration C_D can deter 90% of mosquitos.

In membrane feeding assays, 90% mosquito deterrence concentration is 0.057 mg/cm^2 for fabclavine and 0.178 mg/cm^2 for DEET (Kajla et al., 2019). Using this potency ratio, we can obtain the required EC90 for fabclavine, $\mathbf{EC90_F}$, from results in (I).

$$EC90_F = C_D * \frac{0.057 \frac{mg}{cm^2}}{0.178 \frac{mg}{cm^2}} = 15\ \mu g/cm^2$$

This estimated $EC90_F$ for human skin will be compared to experimentally measured $EC90_F$ from mouse model in Aim 1 to evaluate the likelihood that this estimation is physiologically relevant.

III. Fabclavine production-decay model

We use a standard production-decay model to determine the required fabclavine production rate such that the equilibrium concentration would match the result from (II).

Estimation of fabclavine production rate necessary to maintain $EC90_F$ on skin:

$$\frac{dC_F}{dt} = k_p - k_{deg} * C_F$$

Where C_F is fabclavine concentration, t is time, k_p is production rate, k_{deg} is fabclavine degradation rate. Solving the differential equation gives:

$$C_F = \frac{k_p}{k_{deg}}(1 - e^{-k_{deg} * t})$$

Assuming quasi-steady state of fabclavine production, $C_F = k_p/k_{deg}$. k_{deg} will be determined by measuring fabclavine half-life in Aim 2. The necessary k_p will be calculated for $C_F = EC90_F$. Assuming a fabclavine half-life of 24 hrs, a conservative estimate based on its low volatility (Kajla et al., 2019), production rate of $k_p = 0.43 \frac{\mu g}{hr * cm^2}$ is necessary to maintain $EC90_F$.

IV. Calculate the required bacterial concentration on human skin

The first in human dose will be estimated at:

$$D = \frac{k_p}{\alpha} * \frac{1}{E}$$

Where D is skin engineered microbe concentration in CFU/cm^2, k_p is the necessary fabclavine production rate on skin surface in $\frac{\mu g}{hr*cm^2}$, α is fabclavine production rate per microbe in $\frac{\mu g}{hr*CFU}$ measured in Aim 2, and E is engraftment percentage measured in pre-clinical animal models. We can calculate cream bacterial concentration from D as shown in (V).

V. Hypothetical calculations for cream bacterial concentration

Based on preliminary calculations and data from a related study, we set hypothetical parameter values: engraftment E = 5% (Paetzold et al., 2019), surface production rate k_p = 0.43 μg/hr*cm^2 based on (III), and per microbe production rate α = 0.43 pg/hr*CFU (or equivalently 0.32 fmol/hr*CFU). Using equation from (IV), the bacterial concentration to maintain 90% repellency for 2 weeks is 2*10^7 CFU/cm^2.

The 2*10^7 CFU/cm^2 skin surface concentration is comparable to natural skin microbiome concentration of 10^6-10^9 CFU/cm^2 (Ross et al., 2019). Assuming 4mL cream use per application, we calculate the corresponding cream bacterial concentration:

$$C_{microbe} = \frac{2*10^7 CFU}{cm^2} * \frac{10^4 cm^2}{m^2} * \frac{1.8 m^2}{4mL} = 9 * 10^{10}\ CFU/mL$$

The hypothetical cream bacterial concentration of 9*10^{10} CFU/mL is comparable to current probiotic formulations (Trinchieri et al., 2017).