ANTIMICROBIAL PROPERTIES OF POLYPEPTIDE BASED MATERIALS

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2023

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Master's Committee, we certify that we have read the thesis prepared by: Lauren Grace Melcher, titled: Antimicrobial Properties of Artificial Polypeptide Based Materials and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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Acknowledgements

The author is incredibly thankful for their mentor and former principal investigator, Dr. Anne Wertheimer, for her support, teaching, guidance, and mentorship over the last four years of laboratory work and the last year of this master's program and thesis preparation. The author would also like to thank committee members Dr. Lonnie Lybarger, Dr. Minkyu Kim, and Dr. Adam Buntzman for giving their time and effort to help guide and polish the research project, edit writing, and provide feedback. The author would also like to thank and acknowledge the contributions of Wertheimer laboratory members: Logan Beers, Haerin Park, MS, Jerry Carr, Ingrid Peterson, MS, Karen Sanchez-Perez, Samantha Faria, Micah Hiner, Evelyn Zhaoyinqian, MS, and Dayanira Paniagua, PhD. The author would also like to acknowledge Drs. David Armstrong, and Dr. Karen D'Hyvetter and the patients from their clinic at USC Keck School of Medicine for their enrollment as de-identified study subjects- for this project. The author would also like to acknowledge the BIO5 Institute at the University of Arizona for providing space, the TRIF fund for support, and the UArizona Genetics Core for extracting and sequencing samples. The author would also like to thank Dr. Minkyu Kim and his laboratory at the University of Arizona for providing materials and troubleshooting issues with antimicrobial peptide materials tested for the project, and for laboratory members, Fathima Doole, Zhu Zhao, Bumjoon Kim, and Dr. Chris Camp. Last, the author would like to thank her partner David, mother Deborah, brothers Cody and Adam, grandmothers Jewell and Mary, friends and roommates for the endless support and encouragement.

Land Acknowledgement

We respectfully acknowledge the University of Arizona is on the land and territories of Indigenous peoples. Today, Arizona is home to 22 federally recognized tribes, with Tucson being home to the O'odham and the Yaqui. Committed to diversity and inclusion, the University strives to build sustainable relationships with sovereign Native Nations and Indigenous communities through education offerings, partnerships, and community service.

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ABSTRACT

Foot ulcers develop in 9.1 to 26.1 million diabetics annually worldwide, and 1 to 3 million in the U.S. alone have a history of foot ulcerations (22). More than 50% of foot ulcers become infected, many with antibiotic resistant infections, and of those infected ulcers, 20% lead to some level of amputation (3). Our novel matrix, a biosynthetic, elastin-like polymer (ELP) hydrogel that can be either a liquid, or semi-solid gel chip, offers a non-invasive potential therapeutic for these patients. We aimed to evaluate the safety and impact of the ELP matrix ("gel-chip") itself both in vitro using cell culture, and when placed on the soles of human feet. The "gel-chip" also called "hydrogel" is a biosynthetic platform with a variety of uses in biomaterials applications, like stabilizing antimicrobials, particularly antimicrobial peptides (AMPs) that otherwise rapidly degrade, for topical application and drug delivery. We proceeded to both evaluate the ability of the ELP matrix to alter the microbiome of healthy human feet and, then tested the ELP matrix in its liquid form biosynthetically engineered with AMPs, to determine if it could alter the diabetic foot infection (DFI) microbiome in vitro. We demonstrated that the ELP matrix in solid form, itself captured a unique microbiome distinct from that obtained by the current standard of care, a dry culture swab. Of crucial importance was our finding that our ELP matrix ("gel-chip") itself was well tolerated for over eight hours when placed on intact inner soles of human feet (n=11) without any notable skin irritation. Our studies revealed changes in the type and total amount of bacterial genera, as illustrated by number of reads, indicating how much genetic material was picked up and the diversity, as well as relative amounts of those genera, indicating if a sample is homogenous with a large amount of one or few types of bacteria, or heterogenous with smaller or more equal amounts of several types of bacteria. Further, we performed growth curve assays which allowed us to calculate growth inhibition of the DFI wound microbiome in the presence of

an AMP either free or tethered within the ELP. Our studies revealed that the free AMP, LL37 (50uM) reduced the overall microbiome from two of the three patient samples we tested ("USC 1" and "USC 7") between 45-65% after 12 hours of exposure. We also demonstrated that in liquid phase, 12.5 mg/ml ELP-Pex, our liquid matrix synthesized with an AMP, reduced bacterial growth of these same two subjects by 26-34%. These results show the ability of our novel biosynthetic ELP material, based on naturally occurring AMPs, to alter clinical sample microbial profiles, with varying levels of bacterial growth suppression and composition alteration. Our results suggest that our antimicrobial ELP matrix has potential as an alternative DFI therapeutic and may be preferable over antibiotics for the reduced risk of multi-drug resistance development, natural occurrence in a wide range of organisms, and little to no toxic side effects.

Portions of this work have been previously published by Melcher et al., 2020 at the American Society for Microbiology '*Microbe 2020'* Conference.

INTRODUCTION

Every 30 seconds, a person with diabetes undergoes a lower limb amputation somewhere in the world (23). Most of these amputations, approximately 85%, are due to an uncontrolled diabetic foot ulcer (DFU) (19). Foot ulcers are a common complication for diabetic patients, with an estimated 25% lifetime risk of developing an ulcer, and of those that develop an ulcer, 50% become infected (20). Once infected, a DFU can become a diabetic foot infection (DFI). Up to 20% of all diabetes related hospitalizations in the U.S. are due to a DFI (20) caused by microorganisms, namely bacteria. DFIs are diagnosed and described based on clinical presentation, using criteria from one of several established classification systems. Classification systems include the Braden Scale for Predicting Pressure Sore Risk, the National Pressure Ulcer Advisory panel staging system, the Wagner DFU Grade classification system, the University of Texas DFU classification system, and the International Working Group on the Diabetic Foot (IWGDF) classification system. Each classification system varies, so for example, the National Pressure Ulcer Advisory panel staging system includes stages one through four, unstageable, and deep tissue pressure injury (15). Classification is an initial assessment of the wound stage and progression. Characterization is then needed to understand the wound environment and determine appropriate treatment. Increasing antibiotic resistance, a complex wound environment, and exacerbating comorbidities make DFU and DFI difficult to treat. The prevalence and severity of this clinical condition necessitates testing alternative treatments and methods to improve characterization of a wound environment and to identify effective treatments.

Pathophysiology of DFU

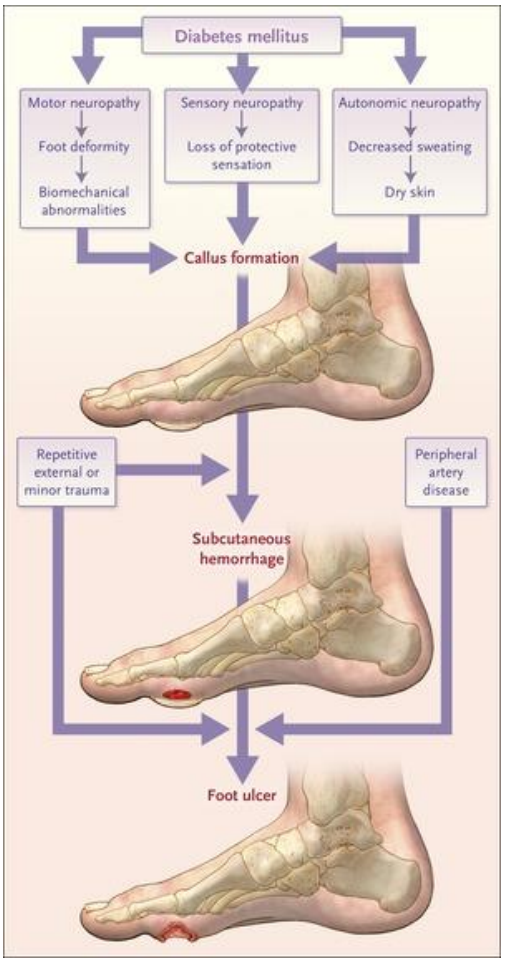

Figure 1:
From Armstrong et al., 2017. "Figure 1. Common Pathway of Diabetic Foot Ulcer Occurrence and Recurrence". Reproduced with permission from Armstrong et al., 2017, Copyright Massachusetts Medical Society.

Diabetic patients suffer from a host of conditions affecting the skin, often neuropathy and poor circulation or ischemia (13). These concomitant conditions contribute to a weakened natural skin barrier and reduced sensitivity reflexes. A weak barrier and insensitivity create an environment susceptible to ulcer formation and subsequent infection by pathogenic bacteria. A diabetic patient may not feel pain or normal sensation that alerts us to a wound, sore, or ulcer, allowing that break in the skin to go untreated and progress. Infected ulcers are increasingly common in diabetic patients due to neuropathy of the legs and feet associated with poor blood glucose management, reduced vascular health, and altered immune profiles, including high levels of proinflammatory cytokines (6). DFUs foster unique environments, highly susceptible to infection. These environmental alterations, including hypoxemia, loss of epithelial integrity, reduced vasculature, inflammation, neuropathy, and hyperglycemia interfere with normal wound healing and tissue repair processes, exacerbating the condition (7). In addition to a complex wound environment, increasing antibiotic resistance creates another challenge for targeting and treating DFIs. An estimated 25% of bacteria isolated from DFIs have multidrug-resistant phenotypes (40), reducing feasible and effective treatment

options. Additionally, diabetes is associated with an increased risk of infection, most commonly skin and soft tissue (including DFIs, sepsis, pneumonia, and urinary tract infections, as well as community-acquired infections, rare infections, and recurrent infections, all of which are often difficult to treat (47. Antibiotics are the main drug treatments for infections and for DFIs and are becoming less effective due to continually developing microorganism resistance (25. Thus, there is an increasing demand for new and effective therapies because current therapies are not effective. One potential therapy, antimicrobial peptides (AMP, are gaining popularity as an alternative to antibiotics (25. Recent literature shows that many of these peptide treatments have broad spectrum activity against bacteria and are less susceptible to the development of bacterial resistance (14) which makes them favorable over antibiotics.

DFI Characterization

To treat a DFU or DFI, clinicians first assess and characterize the wound to establish a baseline and measure progression or regression. DFUs are characterized by the size and appearance, drainage, stage, presence of pathogenic bacteria, as well as normal skin flora, and the duration which informs chronic versus acute or simple (37. According to a 2015 review, Noor et al. found top genera include *Staphylococcus, Streptococcus, Proteobacteria, Pseudomonas*, and coliform bacteria. Some genera, including *Staphylococcus* are classified based on their thick peptidoglycan wall as gram-positive, while others like *Pseudomonas* are gram negative with a thinner peptidoglycan wall and outer membrane. The top two isolated species from infected DFUs are *Staphylococcus aureus* (*S. aureus),* a gram-positive bacterium, and *Pseudomonas aeruginosa* (*P. aeruginosa* a gram-negative bacterium (34. The depth and progression of the wound, the surface area, and the environment (i.e. hypoxemic can influence the organisms that proliferate in the wound. Various identification techniques exist, including

culturing, sequencing, and biopsy. Two recent studies noted that from severe forms of DFIs, as many as three to five different organisms can be cultured (11, 29) which our laboratory was able to replicate. Historically, culturing a wound sample to determine the organisms present is done before treatment, so providers can prescribe antibiotics tailored to those microorganisms identified (34). However, culturing of patient infected wounds has been shown to capture only part of the full spectrum of microorganisms present, as many sampling techniques select against a wide range of organisms, and DFU wounds are deep, varied, and host relationships between commensal and opportunistic bacteria, biofilms, and create microenvironments within the wound that a swab may not reach or pick up (25). Though culturing from a wound swab is still the primary method to characterize organisms present, synthesis from recent literature has shown that swab culturing has drawbacks, including inhibition of pathogen growth, difficulty detecting small quantities of microorganisms, limited types of selective media which falls short of the immense number of possible microorganisms present, risk of contamination, and the inability to quantify the amount of each species (2). Additionally, swabs are collected from a superficial wound environment, which provides incomplete and limited information on the reality of the wound composition, as they can be deep and vary from one layer to the next. Treating these infections without knowledge of which organisms are present adds an additional challenge (34). As antibiotic resistance continues to expand and create challenges for providers and patients across the spectrum of healthcare, there is a need for improved sampling techniques, and new targeted therapies to address polymicrobial infections and particularly antibiotic-resistant infections.

Normal Skin Flora -implications and impact

Many bacteria normally colonize human skin and contribute to the barrier and immune functions. Certain top species found in DFUs, including *S. aureus,* naturally colonize human skin, but can become pathogenic under different environmental conditions like a wound environment, also known as dysbiosis (8. Another commensal, *P. aeruginosa*, exists naturally on the skin and in the colon. Under opportunistic conditions, however, it is well-known for intrinsic antibiotic resistance and is especially difficult to treat due to its production of biofilm and the presence of efflux pumps which can quickly remove foreign substances from within the cell (32. Other species including *S. epidermidis, S. hominis,* and *Propionibacterium acnes* exhibit commensal relationships with *S. aureus*, some of which include inhibition of biofilm formation and synergistic effects with human AMP LL37 to control *S. aureus* colonization (8. These interactions and unique proportions of microorganisms, along with an intact skin barrier, help sustain a normal microbial flora and aid in combatting opportunistic colonization and infection. In healthy individuals, our polymicrobial skin environment contributes to the overall barrier and protective function of the skin and innate immunity (42, while in a disease state, altered skin flora composition can contribute to reduced barrier function, leaving individuals more susceptible to an imbalanced microbiome and infection. Keeping pathogenic microorganisms out involves a delicate balance of beneficial bacteria which help discourage propagation of pathogenic bacteria.

AMP Classifications

As bacteria rapidly mutate and acquire resistance genes, first line antibiotic treatments are becoming less effective, and patients continue to acquire infections needing treatment. Therefore, alternative treatments like AMPs are needed to help suppress, inactivate, and control increasing

patient bacterial burden. AMPs are small chains of amino acids, typically from five to 50, but can be up to 100, that can interact with bacterial cells (14). AMPs have unique properties that enable them to broadly interact with a variety of molecules and cells. Some of these properties include their amphipathic nature, variety of folding structures, presence of a charge, and their ubiquitous presence in the animal kingdom. Most AMPs are cationic, with a positive charge, which bind to negatively charged structures, for example, common bacterial membrane components like lipopolysaccharides (LPS), though some AMPs are anionic and have a negative charge, which can bind to positively charged structures embedded within the cell wall (14). AMP structures include alpha helix and beta sheets most commonly, as well as extended, loop, cyclic, and lasso peptide structures (14). The amphipathic nature also aids in broad interaction in different environments, with both hydrophobic and hydrophilic properties, and enables these peptides to cross membranes like that of bacteria. AMPs can be divided into several categories to target a variety of microorganisms, including antiviral, antifungal, antiparasitic, anticancer, and antibacterial (14). Our focus is on antibacterial AMPs, as bacteria are the principal microorganism found in DFIs (24). AMPs are also a safer potential treatment because they are found throughout the animal kingdom, including in human digestive tract cells, in mucous membranes of other animals and insects, and even in other bacteria (14). For this project, two AMPs were tested: a human host defense peptide, LL37, and an amphibian derived peptide, Pexiganan, on human DFU clinical samples. LL37 is a cationic (net +6 charge), amphipathic, 37 amino acid chain peptide that is part of the innate immune system in humans and other animals. In humans, it is stored in neutrophil granules, and can be found in macrophages, parietal cells, and epithelial cells (12, 27). LL37 is cleaved from the C-terminal end of the human CAP18 protein, where it can then interact with other cells, pathogens, and the environment. Because it is

amphipathic, in hydrophilic environments it forms a coil shape while in hydrophobic environments it forms an alpha-helical shape, which allows for broad interaction, like binding with gram-negative bacterial membrane protein lipopolysaccharide (LPS or inserting into cell membranes and disrupting cell integrity or binding with other intracellular components (48. Pexiganan is a cationic, 22 amino acid chain peptide that is found in the skin and mucus of African clawed frogs or *Xenopus laevis.* This host defense peptide is like human enzymes in our saliva, sweat, and tears. The cationic properties allow Pexiganan to disrupt microorganism membranes via a toroidal pore mechanism where it inserts into the membrane in an alpha-helical shape, and inside the cell, can bind to or disrupt protein synthesis, causing cell death (49. These two AMPs have been identified in previous literature as promising treatments for DFU and DFI (5, 14, 21, 26, 35, 36, 44. We aimed to characterize our clinical DFU samples, assess alterations in the microbiota after treatment, and identify microbial targets for each AMP based on the alterations. Peptides from other sources like insects or microorganisms were not tested, though they are being explored in the literature.

AMP Platform

AMPs interact with a variety of microorganisms and cells via cell surface receptors, and cell membrane structures, which makes them broadly desirable and potentially effective. These small peptides are also highly susceptible to degradation and subsequent inactivity. To help reduce degradation, we can stabilize an AMP, which our collaborators, biomedical engineers at the University of Arizona did using an elastin-like polypeptide (ELP to tether AMPs (9. An ELP can maintain a micelle shape, in liquid form, or can become a hydrogel, in semi-solid form. There are benefits to both forms, and for our purposes the liquid forms of ELP tethered to AMPs were used for our initial tests in this study to address their antimicrobial activity prior to adding

AMPs to a solid material which may alter AMP structure or function. We also examined the liquid state of the free AMP with no ELP tethered to evaluate AMP ability to impair microbial growth cultures initially of specific microbes, and progressed to test actual human DFI patient wound samples. In the future, we plan to test AMPs tethered to the solid ELP hydrogel chip. The semi-solid hydrogel chip ("gel chip") provides a stable platform to tether AMPs, which allows for successful delivery of AMPs and a more feasible application form for patient use. The gel chip provides dual functions as a physical barrier to the outside world, and a topical source of AMP treatment to a clinical wound, rather than the traditional use of oral antibiotics which circulate systemically, and are often prescribed. These oral antibiotics can be ineffective in some patients due to reduced blood flow to lower extremities and antibiotic resistance. By stabilizing our AMP treatments, we can help prolong the degradation process, and provide a more continuous and topical treatment source.

As the hydrogel chip is a novel biosynthetic material, we performed initial application tests on 'healthy' human skin, to address any adverse effects like skin irritation or discomfort. Initial application tests are necessary to establish ease and comfort of use, before attempting to use the hydrogel chip and AMP materials on a wound environment, where skin irritation or discomfort are common (1). Previous work by Camp et al., tested the novel hydrogel chip on neonatal foreskin fibroblast cells (ATCC CRL-2076), which are highly sensitive and the cell type recommended by the FDA for testing of topical applications, to address changes in cell growth or cytotoxicity of the biosynthetic material (9). Prior to tethering AMPs to our hydrogel chip, we confirmed that the hydrogel matrix itself was non-toxic to human foreskin fibroblast cells (9), and non-irritating when worn on a human foot for 8 +/- 2 hours. In this study we have gone on to examine the hydrogel chip after being worn by healthy subjects to address whether the hydrogel

alters the microbial environment and composition, in preparation for adding AMP treatments in the future.

Rationale, Hypothesis and Objectives

Rationale: Due to growing antibiotic resistance, increasing prevalence rates of diabetes, and increasing rates of associated comorbidities like severe infection and amputation, there is a need for expanded and improved treatment alternatives for DFUs. AMPs are a promising therapeutic target for non-healing and infected DFUs due to the established antimicrobial activity and synergistic effects when used in combination with other treatments, a reduced risk of antibiotic resistance development, and a wide range of AMPs produced in a variety of cells and organisms with broad targets. Patients with a DFU are at a higher risk for development of additional DFU, so wound healing and infection treatment are top clinical priorities. Testing our hydrogel chip matrix will provide dual benefits of establishing the safety of a potential AMP treatment stabilizer and delivery vehicle, and as a protective, physical barrier on a wound. With a library of thousands of potential AMPs to choose from, testing two initial AMP targets identified as effective in the literature on clinical samples will provide valuable information about AMP use on a realistic and clinical sample. Testing two different methods of skin sample collection, swab compared to hydrogel chip, will also provide valuable information on bacteria identified and reveal differences between, which informs treatment selection for DFUs and can promote antibiotic stewardship. These findings can inform clinical practice and protocols for improved DFU treatment and characterization, and antibiotic treatment alternatives.

Hypothesis: The hypotheses are that one, when culturing bacteria, the presence of an AMP treatment alters bacterial growth; two, increasing treatment concentrations, both commercially purchased and laboratory synthesized therapy, correlate with reduced bacterial growth in controls

and patient samples; and three, control and commercially purchased bacteria samples are more susceptible to growth inhibition with AMP treatment compared to clinical specimens from patients.

Objectives:

1. To test the hydrogel chip matrix on a healthy skin cohort and determine safety and potential for clinical use.
2. To use whole genome sequencing (WGS) to determine hydrogel chip matrix impact on a healthy skin cohort's foot microbiome by comparing bacterial genera picked up from our hydrogel compared to a swab in a healthy skin cohort.
3. To test AMPs on control and clinical bacterial samples to assess alterations in growth and diversity.
4. To use 16S sequencing to identify bacterial species present before and after AMP treatment and identify organisms and/or characteristics sensitive to AMPs.

MATERIALS AND METHODS

Antimicrobials

Commercial antimicrobial peptide (AMP) LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES was purchased from Ana-Spec REF# AS-61302. Stock concentrations of 1 mM, 200 uM, and 50 uM. Sterile diH_20 and MH broth were both used for reconstitution, for different experiments. Once generated materials were either used immediately or aliquoted and stored at -20. Once frozen, stocks were limited to one use.

Biosynthetic elastin-like polypeptide (ELP platforms with either LL37 or Pexiganan AMPs in

two different configurations, AYA-A_{48} and AYA-S, were expressed via plasmid in *E. coli* by our collaborators Fathima Doole and Zhu Zhao. After sufficient protein yield, cells were lysed, and the ELP-AMPs were purified, dialyzed, characterized for micelle formation and thermos-responsiveness (Doole et al., 2021), and then reconstituted in phosphate buffer (PB). After reconstitution, materials were sterile filtered, and concentration was measured via UV-VIS. Hydrogel 'chips' were made from the same ELP platform though no AMP was included and the ELP was crosslinked to create a tissue-like material (9). These gel chips were used for our human subject's study to assess human skin tolerance of the hydrogel chip and any changes to the skin microbiome after sequencing genetic material picked up by the chips. Gel chips were prepared by our collaborator, Dr. Chris Camp. 12-18 hours before skin application, chips were washed in 70% EtOH and soaked overnight in sterile PB to re-hydrate the chip and prepare it for placement on human skin.

Bacteria

ATCC Strains

Lyophilized *Staphylococcus aureus* or *S. aureus* ATCC strain (ATCC® 29213™): lot numbers: 70009636, was purchased from American Type Tissue Culture Collection. *S. aureus* is gram-positive and beta-hemolysis positive (28). This bacterium is sensitive to oxacillin, which is helpful information for when providers and pharmacists are choosing the antibiotic or drug a patient will receive. *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 29213).

Lyophilized *Pseudomonas aeruginosa* or *P. aeruginosa* ATCC strain (ATCC® 25619™): lot number: 70000545 was purchased from American Type Tissue Culture Collection. Gram-negative, non-hemolytic, fluorescein positive, and pyocyanin positive. (28). *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC 25619). ATCC Strains were propagated as described in

the product instructions. Briefly, lyophilized material was resuspended and cultured shaking overnight at 37C in 3mL Luria broth. The next morning 800 ul of the 3mL overnight suspension was transferred into a sterile glass vial each containing 200 ul glycerol (5 glass vial per isolate). All glass vials containing the resulting 1 ml stock bacterial suspensions were labeled and stored at -80^{o}C for future use. We were unsuccessful in determining the antibiotic sensitivity of commercially purchased ATCC bacterial strains, potentially due to confidentiality issues and unavailability of information.

Sequencing (UAGC and Cosmos ID)

UA Genetics Core (UAGC) Sequencing

The UAGC performed sequencing on bacterial pellets provided to them post 12-hour growth in a 96 well plate assay. Overnight (16-18hr) cultures from each clinical sample were made, by adding a 1 ul loopful of bacteria from the frozen glycerol cultures to 3 ml trypticase soy broth, and shaking at 37^{o}C, medium, constant speed with a loosened and tape secured cap for oxygen exchange. The 96 well plate contained 50 ul Mueller Hinton broth in each test well, and 50 ul of overnight culture diluted down to an O.D. 600 of 0.005. Test wells and controls were set up and plated in duplicate, with additional un-inoculated background media and material wells. The plate was then placed in a BioTek Synergy 2 microplate reader for a 12-hour assay, with medium constant shaking, at 37^{o}C, and the O.D. 600 read every 10 minutes over 12 hours. When the 12-hour assay finished, the plate was ejected from the Synergy 2 plate reader. Samples in the wells were observed with the naked eye for cloudiness, indicating positive bacterial growth in test and control wells, and clear in control broth and background un-inoculated material wells. Duplicate wells were compared to each other, to observe if the samples had the same color and opacity, indicating similar conditions and reducing suspicion of contamination. Each clinical

isolate culture had a slightly different color, USC 1 had a light-yellow appearance, USC 2 a green appearance, and USC 7 a white appearance. The clinical isolates in duplicate were removed from the wells with a sterile micropipette and added to separate 1.25 ml sterile microtubes and labeled. Microtubes were then spun at 7,000 g for 5 minutes to create a pellet, supernatants were removed, and then 1 mL of lysis buffer was added to each. These pellets were then given to the UAGC to perform extractions on each sample to prepare for sequencing. Extractions were performed using the ZymoBIOMICS DNA Miniprep Kit. Samples were transferred by a technician to bead bashing tubes and tubes were bashed three times in a Tissue Lyser II machine for five minutes at 30 h/s with apx at 30 seconds pause in between cycle. Then samples were eluted with 50 ul water from the kit and 1 ul was taken out for quantification, and the rest stored at 4^{o}C and then -20^{o}C. Samples were then sequenced using the Illumina '16S Metagenomic Sequencing Library Preparation' protocol. Samples were prepared for sequencing by performing PCR with primers to amplify specific variable regions (V3 and V4) and then purified. Sample fragments then had adapters and dual indices or barcodes added for later identification and genome reconstruction and were quantified and then pooled. Then, samples were sequenced and genomes reconstructed and compared to a reference library for taxonomic classification and identification. Sequence data was also used to confirm the ATCC control bacterial species used, *S. aureus* (ATCC 29213) and *P. aeruginosa* (ATCC 25619). *S. aureus* control data revealed 86.97% relative abundance, and *P. aeruginosa* control data revealed 95.26% relative abundance, which indicates the purity of our sample and that the tested sample matched our control information.

Cosmos ID Sequencing

Swabs and chips from our healthy cohort were mailed to Cosmos ID, a commercial company, who performed DNA extractions and shotgun WGS, and then provided WGS data

and analysis results. WGS was performed by first extracting the DNA from the swab and chip samples. The DNA extractions were then denatured and sheared or cut into fragments. Fragments were read and bar codes were attached to identify each piece. Fragments were then combined and added to the Illumina NextSeq 550 platform 2x150bp for sequencing. After sequencing, samples were analyzed and quantified. Genomes were recreated using the bar codes identifying fragments that together make up a sample, and compared to a reference library so samples could be taxonomically identified and classified.

Human subject study design

We designed and conducted a human subjects' study to compare flora from healthy/non-diabetic subjects with the flora from diabetic foot ulcer patients. The study was designed in two arms; Arm 1 aimed to evaluate the foot microbiome of a healthy non-diabetic cohort, and Arm 2 aimed to characterize the flora obtained from diabetic foot wounds and to test our novel treatment on these foot wound samples ex vivo. This study was approved by the University of Arizona (UA) and Institutional Review Board (IRB) (IRB # 2012309920). (see attached consenting documentation) Arm 2 which involved diabetic foot ulcer patients was also approved by the University of California Keck Institute IRB. The specifics of each study arm will be detailed within the results section.

RESULTS

Healthy Foot Microbiome and Tolerance of the ELP Matrix "gel chip" Study - Arm 1

Healthy subjects (n=11) were recruited at the University of Arizona for Arm 1 of our human subjects' study. Due to limited funding and resources, we focused on five of the 11 subjects with the most genetic data collected from swab and chip samples, for further analysis. To evaluate the effect of the ELP scaffold itself upon the non-diabetic foot, study participants were pre-screened and consented prior to gel chip application. For safety regarding Covid-19 exposure, the study was conducted outside the BIO5 Institute building, to reduce airborne exposure as participants were within 6 feet of study coordinator during the swab, application, check, and removal of the gel chip. Participants were asked to not wash their feet for 24 hours prior to participation in the study, as washing feet can alter the normal skin flora, and our aim was to assess bacteria present on the sole of the foot before gel chip application and after gel chip removal. Participants were then given a unique number and identifier, and asked to remove their shoes and socks, if wearing. Study coordinators swabbed the bottom of the foot with a dry, sterile swab, provided for sequencing use later, near the border between the pad of the foot and the top of the inner arch, where pressure is high and many foot ulcers develop. Figure 2 indicates other areas of pressure where ulcers commonly develop. The area was swabbed for 1 minute over an estimated 1-inch square which outlined the adjacent spot for gel chip placement. The swab was then placed in a commercial tube designed to preserve genetic material (Zymogen) and stored at 4C for up to 1 week before being provided to the core sequencing facility. Sterile forceps were then used to remove one gel chip from a sterile container, and place it on the

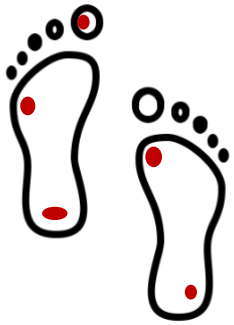

Figure 2:
Typical pressure points on the foot plantar surface.

participant's pad of foot, with a clean band-aid placed on top to secure. Additional self-adhesive bandages were wrapped around the foot and covered the band-aid to help prevent the gel chip from moving or falling out. The subject's skin underneath and around the hydrogel chip was evaluated at 1 hour for any signs of irritation, typically seen as redness, rash, or other noticeable skin changes. If no irritation or changes were noted, the chip was replaced and left on for 6 +/- 2 hours. After gel chip removal, participants completed the study, and the swabs and hydrogel chips were sent to Cosmos ID for WGS. WGS data was then uploaded to the Cosmos-ID Microbiome Hub where dataset results were visualized and organized, see Figures 3 and 4. The hydrogel chip and swab allowed us to collect and sample the microbiome of our healthy control, and then WGS the DNA collected on them to identify, quantify, and characterize genera and species present in our controls. Figure 3 (below) shows the top five bacterial genera in each swab sample (3a top chart) and each chip sample (3b bottom chart). Notable genera from WGS of the healthy skin cohort include *Pseudomonas* which is labeled in dark green (Figure 3a and 3b) and appears in all five swab samples and in three of the five chip samples; *Staphylococcus* labeled in yellow, appears in the same three of the five chip samples; and *Cellulosimicrobium* labeled in bright green, appears in four of the five swab samples and all chip samples, though in much smaller quantities compared to *Pseudomonas* and *Staphylococcus* and not easily visible in the figure. Because *Pseudomonas* was a top genus identified from sequencing in both materials and most samples, we then looked at the relative abundance of that genus, or what percentage that genus made up the whole sample, and whether *Pseudomonas* was in the top five genera or lower down on the list. Figure 4 (below) shows *Pseudomonas* as one of the most abundant genera in the swab samples (dark green), between 30 and 60% for four of the five swab samples.

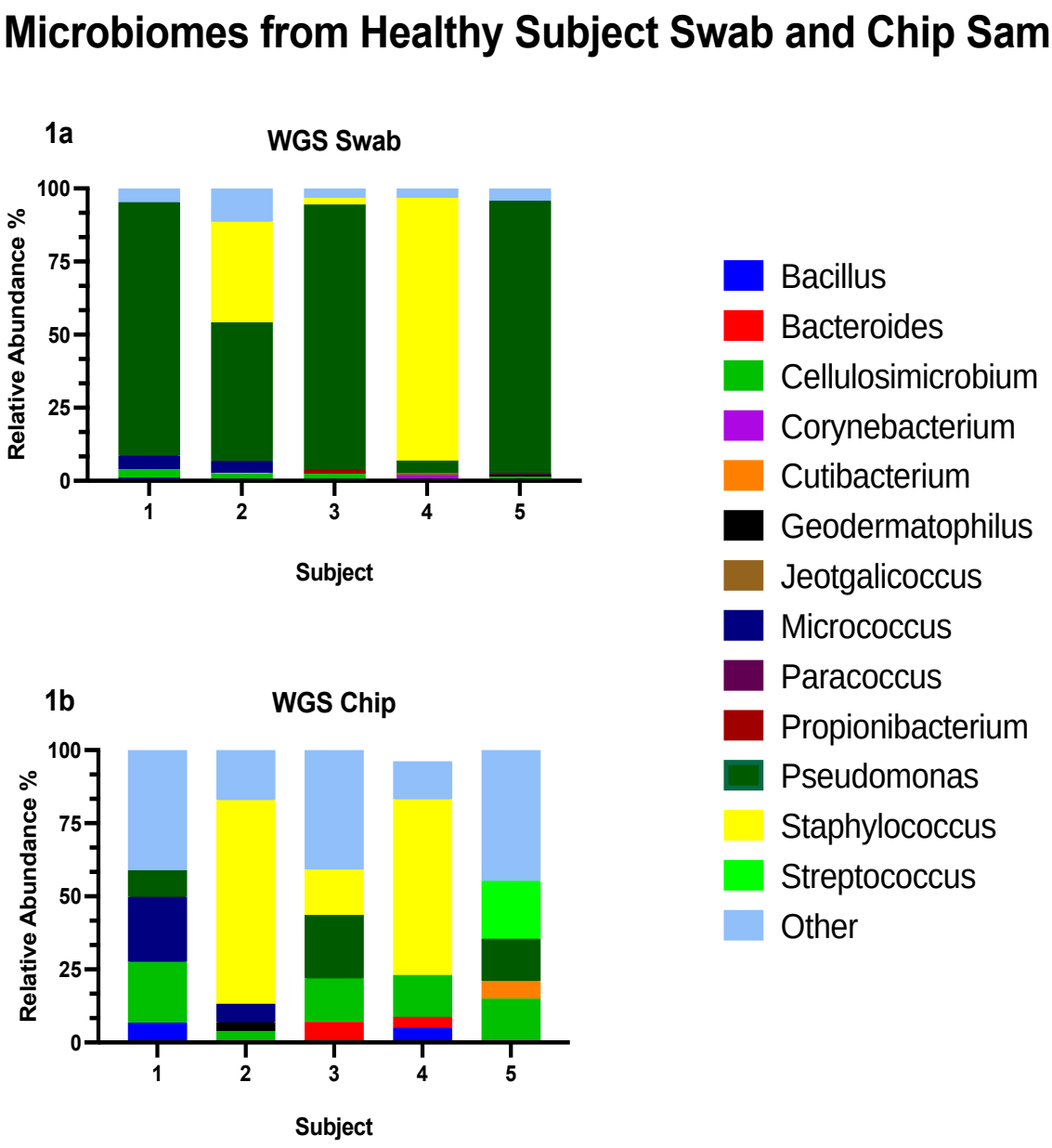

Figure 3a (WGS Swab) and 3b (WGS Chip):

Top 5 bacterial genus identified from a 'healthy cohort' of individuals. Each subject was assigned a number, 1-5, and had a region of their foot swabbed, and then wore a hydrogel 'chip' near the swabbed region for 8 +/- 2 hours. The swab and hydrogel 'chip' both underwent whole genome sequencing (WGS) to identify the microbial flora collected from the different materials. WGS was performed by Cosmos ID. The figure shows each subject, ie. 1, and the five most abundant bacterial genus obtained from the swab (in panel a) and the chip (in panel b). This human subject's study was the first test of this hydrogel 'chip' on a human cohort and was approved by the University of Arizona (IRB #2012309920).

This genus was identified in all five of the swab and chip samples, in varying amounts, and was highlighted as abundant in several of our samples in both materials. This indicates that *P. aeruginosa* is a common genus found on healthy human skin, which previous literature has also identified (6, 13, 17, 19, 30, 35, 38) and a reassurance that our collection methods and sequencing techniques were robust.

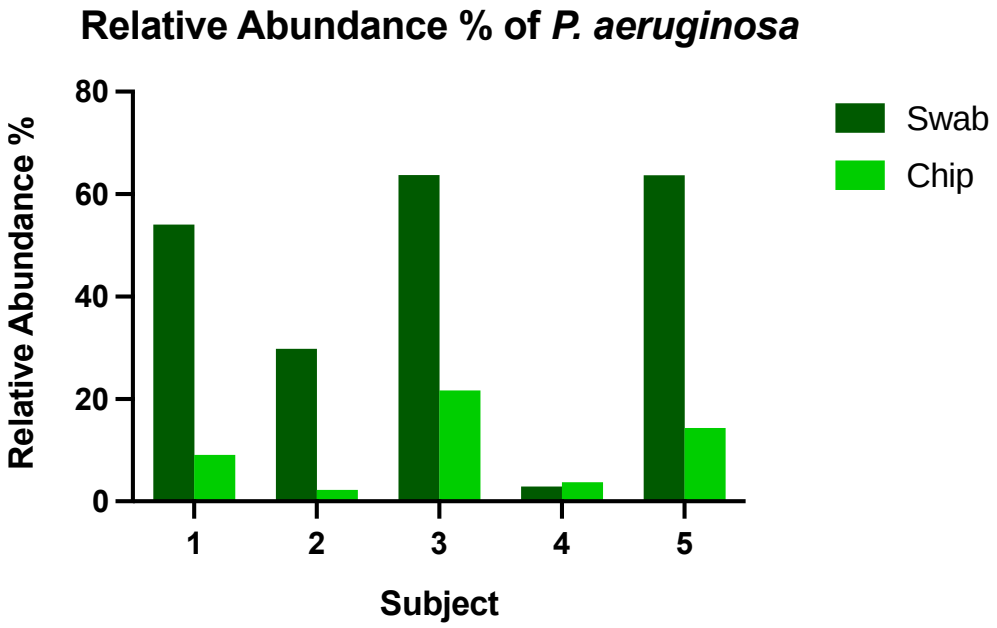

Figure 4:

Healthy Microbiome (Cosmos ID - WGS). *Pseudomonas aeruginosa* in top 5 relative abundance of species in 4 of 5 chip (C) sequencing data. *Pseudomonas aeruginosa* in top 5 relative abundance of species in 5 of 5 swab (S) sequencing data.

After exploring differences between genera identified on the swab versus the chip in our healthy cohort, we wanted to identify differences between sequencing methods: 16S and WGS. Figures 5a and 5b show the top 5 genera identified in each hydrogel 'chip' and swab sample, by the two methods 16S and WGS, with the relative abundance percentage of the genus on the Y-axis. The relative abundance percentage is helpful for visualization of how homogenous or diverse the polymicrobial samples are. For example, WGS of swab 3 (S 3) in figure 5b shows a bar graph dominated by dark green, *Pseudomonas* at 90% relative abundance, with other colors

representing different genera barely visible. In figure 5a, the WGS of chip 1 (C 1) bar shows a more even dispersion of the top genera with each color visible and representing between 7 and 41% of the top genera. Repeat colors, for example dark green for *Pseudomonas*, indicate the presence of that genus in multiple samples from both sequencing methods and in larger quantities. *Pseudomonas* and *Staphylococcus* genera are easily identified in dark green and yellow, because they have higher relative abundance compared to other genera.

Overall, 16S sequencing results revealed between 7 and 518 genera identified from the healthy cohort chip and swab samples, and WGS sequencing results revealed between 38 and 192 genera identified from the healthy cohort chip and swab samples. Two different sequencing companies were used to obtain these results, and Ilumina 16S data from the UAGC was uploaded to the Cosmos ID database to compare to the WGS results on the same platform. There is no indication why more genera were identified from the 16S compared to WGS in some samples, but this could be due to the specific region identified and sequenced, compared to the whole genome set. WGS was only performed on the 'healthy cohort' chip and swab samples, not the diabetic foot samples or the AMP test samples, so we can only compare 16S and WGS differences in the healthy cohort chip and swab samples. Another benefit of the WGS method was identification of antibiotic resistance genes in our samples. Sequencing information highlights the difference between two types of data collection methods: 16S identifies rRNA genes from a specific region of the genome, while WGS identified all genomes from the whole genome. 16S provides enough information to taxonomically identify a sample, usually down to the genus level, but does not provide complete genomic information that WGS does. 16S can help identify samples, while WGS can help understand microbial communities, which can be helpful for understanding and planning wound care and treatment.

Comparison of 16S and WGS on Chip and Swab Sampling Methods

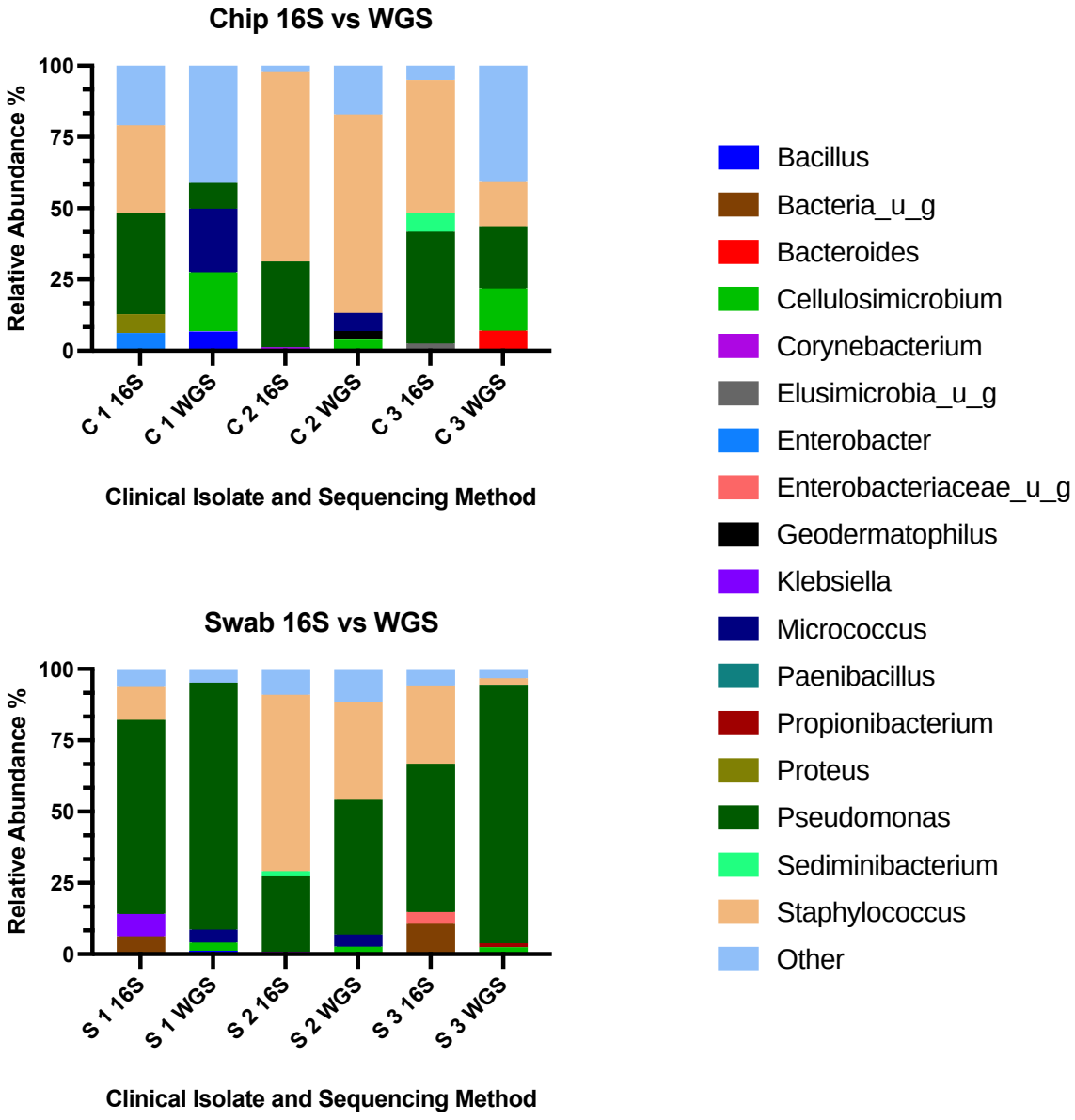

Figure 5a Chip (top) and 5b Swab (bottom):

Top five bacterial genus identified from five 'healthy cohort' individuals, comparing two different sequencing methods, whole genome sequencing (WGS) and 16S sequencing. WGS was performed by Cosmos ID and 16S sequencing was performed by the UA Genetics Core. The figure shows each individual, ie. 1, and their chip 16S and WGS data next to their swab 16S and WGS data. Chip is indicated by 'C' and swab is indicated by 'S'. This human subject's study was the first test of this hydrogel 'chip' on a human cohort and was approved by the University of Arizona (IRB # 2012309920).

There were similar results from the two sequencing methods, 16S and WGS, for some subjects, namely subject 2 where *Staphylococcus* was the topmost abundant genera for both 16S and WGS and from the chip and swab collection material, but for other subjects, like subjects 1 and 3, 16S and WGS data revealed different topmost abundant genera, and differences between the swab and chip material collection. The swab and chip materials are inherently different, the former a dry fabric and the latter a moist, stickier material, which could have altered which bacteria were picked up more or easily by the swab or chip, based on bacteria genera characteristics like charge, cell membrane, or extracellular receptors. This may explain 16S and WGS differences, however, those methods are different and provide different information. Differences in data between sequencing method and collection material may be problematic for individuals with aggressive, chronic wounds that are unresponsive to antibiotics, where complete genetic information from WGS may be beneficial over 16S sequencing, and more than one sampling method is warranted, both a dry swab and a hydrogel chip, or sampling from debridement, to expand potential material and bacteria collected for better sequencing.

Antibiotic Resistance in Healthy Subjects Microbiome

Subject		Class C beta-lactamases Cephalexin cefadroxil gene	Macrolide 137 1805 Branch	Macrolide 41 1826 Branch	Tetracycline 41 2477 Branch
1	Chip	X			
	Swab	X			
2	Chip		X		
	Swab		X		
3	Chip			X	
	Swab	X			
4	Chip		X		
	Swab		X		
5	Chip				X
	Swab	X			

Figure 6:

Presence of Antibiotic Resistance in Healthy Subjects Microbiome. Most prevalent antibiotic resistance from each subject 1-5 whole genome sequencing (WGS) in Cosmos ID, and for each sample, chip or swab. Subjects 1, 2 and 4 had the same antibiotic resistance in both chip and swab, while subjects 3 and 5 had different resistance.

Figure 6 (above) shows the antibiotic resistance genes identified from WGS sequencing in our 'healthy skin' cohort. Cosmos ID compared our sequenced samples to their antibiotic resistance database, which revealed dozens of genes present in our healthy cohort, many of which were the same in different subjects and from different collection materials. Each sample from both the chip and swab materials revealed several antibiotic resistance genes. Figure 6 illustrates that the top antibiotic resistance genes were present among different patients, so for example, the 'Class C beta-lactamases Cephalexin cefadroxil gene' that appeared in swabs 1, 3, and 5 and chip 1, means that those individuals have bacteria present on their skin which have an enzyme that break down cephalexin antibiotics (40). This means that bacteria colonizing skin in an otherwise healthy individual, may have antibiotic resistance genes that can become problematic if an infection or skin break occurs. This information is relevant because it shows the extent and prevalence of antibiotic resistance, which is a growing concern and main indicator

for the need for AMP treatments for antibiotic resistant infections, and for detailed information from sequencing to characterize infections for best treatment options, avoiding medications where bacteria already have resistance.

Microbiome Freshly Isolated from DFI Patients- Arm 2

Diabetic patients presenting to the SALSA Clinic at the Keck School of Medicine of USC with a new foot wound were recruited to participate in Arm 2. Diabetic patients were screened and only patients not currently taking antibiotic therapy were enrolled. Two swabs were taken after sampling from diabetic foot wounds, one for culturing and one for direct 16S genome sequence analysis. A total of 10 completely de-identified swabs were received from 10 subjects who volunteered to participate in Arm 2. Wound swabs were cultured, characterized, and samples were frozen back for further analysis. Of the 10 donors, 3 (USC 1, USC 2, and USC 7) with the most genetic material extracted from cultured samples given to the UA Genetics Core, were used to test our AMP treatment. See Appendix for Consent Form. Study Title: Towards a Hydrogel Therapeutic for Diabetic Foot Wounds: IRB Human Subjects Study: Protocol 2012309920 Approved by Univ. of Arizona IRB (Closed Jan 2022).

AMP Treatments

To understand the impact of AMP treatments on the growth of clinical DFU isolates, initial tests were performed on laboratory isolates to establish a standard workflow, visualize expected data, and establish controls for comparison. Initial AMP treatment tests were performed on two laboratory isolates, *S. aureus, P. aeruginosa,* and a co-culture of both *S. aureus* and *P. aeruginosa*. Figure 7 (below) shows growth curves of control or laboratory isolates and clinical DFU isolates, with and without AMP treatments, free, untethered LL37 at 50 uM and ELP

tethered S-Pex at 12.5 mg/ml. Growth curves provide visual guidance for assessing changes in growth, and quantitative data based on the area under the curve or integral.

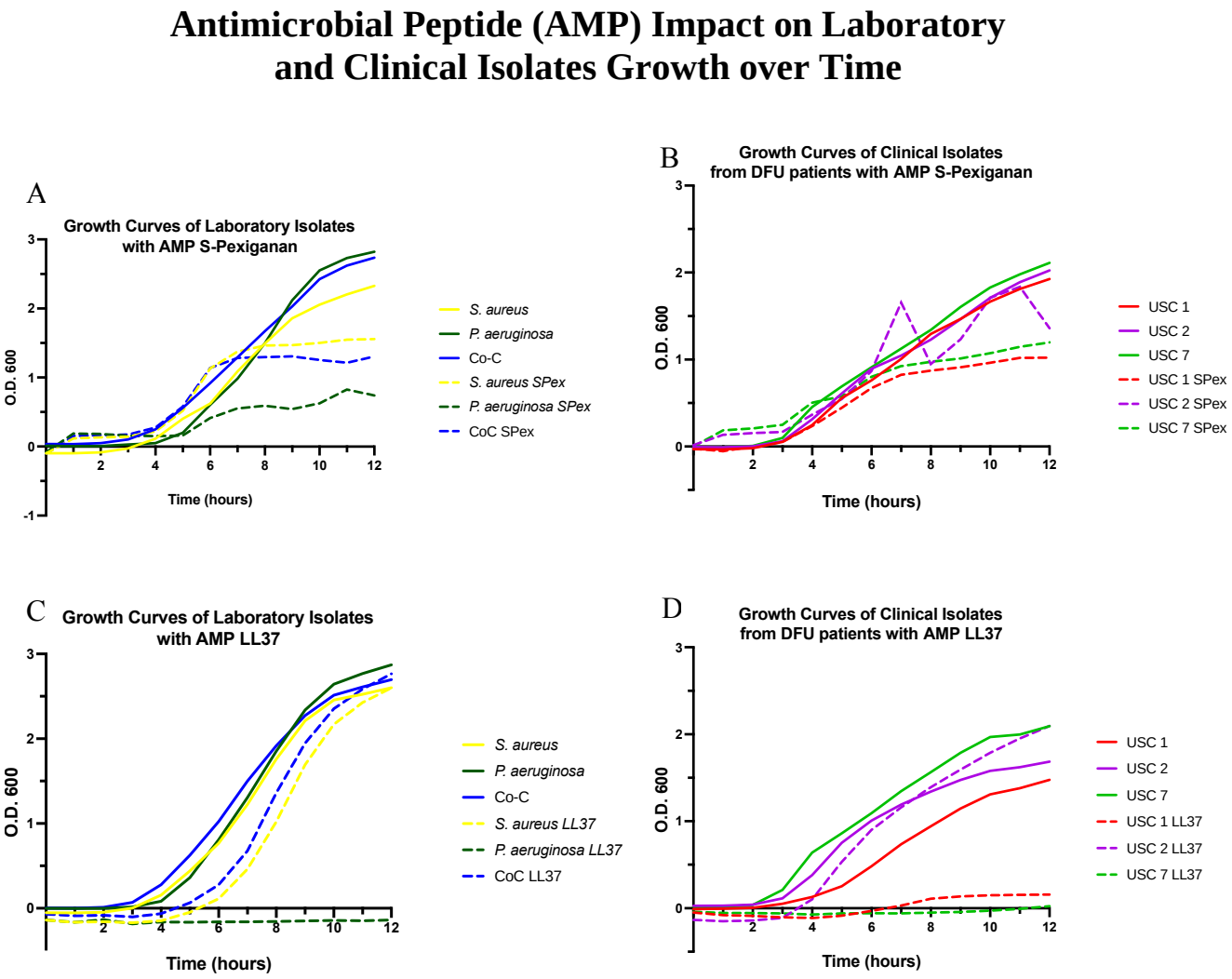

Figure 7:

Growth curves calculated from O.D. 600 measurement read every 10 minutes, plotted over 12 hours. Controls include laboratory isolates (*S. aureus*, *P. aeruginosa*, and a co-culture of *S. aureus* and *P. aeruginosa*) and clinical isolates (USC 1, USC 2, and USC 7) and are represented by solid-colored lines, and AMP treatments include S-Pexiganan (SPex) and commercial free LL37 (LL37) and are represented by dotted colored lines.

Growth Curves and Plating

Overnight cultures from control bacteria, *S. aureus* and *P. aeruginosa,* and three clinical samples USC 1, 2, and 7 were set up 16-18 hours prior, by adding a 1 ul loopful of bacteria from frozen glycerol cultures to 3 ml trypticase soy broth in a 10 ml sterile conical tube. The sterile loop was swirled in the broth to deliver total contents of the loopful, and then tubes were capped and vortexed to mix well. Then tube caps were loosened to allow for oxygen exchange, and tubes were placed in an incubator at 37^{o}C, with constant, medium shaking for 16-18 hours. 200 ul sterile water was added to the perimeter of the 96 well plate to humidify the plate and reduce evaporation from the test and control wells. For the control wells, 75 ul Mueller Hinton broth, and 75 ul of overnight culture diluted down to an O.D. 600 of 0.005 was added to each control well. Overnight cultures of each *S. aureus, P. aeruginosa,* co-culture, USC 1, USC 2, and USC 7 were added to their respective wells in duplicate.

ELP-AMP materials were transformed and then expressed in *E. coli* for high yield amplification of the protein via overnight culture in nutrient rich media, centrifuged, lysed via sonification, purified, lyophilized, and then resuspended in phosphate buffer (PB) by the Kim laboratory (9). AMP materials were resuspended in PB because it is a standard, neutral buffer that mimics human body conditions. Resuspended materials were received from the Kim laboratory, then sterile filtered and then the concentration was measured and recalculated in mg/ml. Depending on the initial concentration of the AMP treatment materials resuspended in PB, a percentage of PB was added to each control well including *S. aureus, P. aeruginosa,* a co-culture of the two, and patient cultures USC 1, 2, and 7, to mimic the same growing conditions of the test wells with AMP and PB in the control wells. The higher the concentration of the AMP treatment materials, the less material was needed, and therefore the lower the percentage PB in

the well. Bacteria grew best in 100% MH broth, and grew worse with increasing amounts of PB, so a higher starting concentration of materials was preferable. The percentage of PB was considered in each assay, so control wells of each clinical sample had the same percentage of PB to match control and treatment growth conditions.

Once the 96-well plate was set up, it was added to a BioTek Synergy 2 plate reader for continuous plate reading. The plate reader captured the O.D. 600 measurement of each well every 10 minutes, over a period of 12 hours. Growth curves were then plotted with the O.D. 600 value on the Y-axis, and time on the X-axis. Resulting growth curves can be seen in figure 7 above. The growth curves plot control bacteria, both laboratory and clinical isolates, and the control bacteria plus AMP treatment. Growth curves in figure 7 indicate growth reduction of all laboratory isolates in the presence of S-Pex (dotted lines) compared to controls (solid lines). The dotted lines are below the solid lines, which indicates a lower O.D. 600 measured.

The O.D. 600 measurement captures optical density of a well, and measures bacterial growth based on density. A higher O.D. 600 means less light is passing through the well, and there are more particles in the well, which translates to more bacteria in the well. This indicates that S-Pex suppresses the growth of those bacterial species, *P. aeruginosa* the most, *S. aureus* somewhat, and the co-culture in between. The AMP treatments used, S-Pex and LL37, did have opacity when used in a well plate, but this value was captured in uninoculated wells as our 'background subtraction'. For every well with an AMP treatment, we subtract the O.D. 600 values of the well with the treatment and media, equal to our inoculated wells, to plot the values of the clinical or laboratory isolates alone, without the O.D. 600 values of the treatment. This way, we can assess if the AMP treatment is affecting the growth, without the density of another substance interfering. With the AMP LL37 treatments, we do not see the same growth

suppression as with S-Pex. Figure 7C in the lower left, the controls (solid lines) are closely following the treatments (dotted lines) which means the AMP treatment of LL37 did not alter or suppress the growth for our clinical isolates.

Interestingly, both S-Pex and LL37 had some growth suppressing effects for our clinical isolates USC 1, USC 2, and USC 7, in figures 7B and 7B. Figure 7B the treatment lines (dotted) are mostly below the control growth lines, which indicates some growth reduction. USC 2 with S-Pex treatment, the purple dotted line in 7B, shows several jagged spikes, which could indicate noise or possible contamination. USC 1 and USC 2 treatment lines in dotted red and green are clearly seen below the control growth lines in solid red and green, which means the treatment suppressed growth for those two isolates. Free LL37 was the most effective in growth suppression in figure 7D, with the dotted green and red lines for USC 1 and USC 7 measuring O.D. 600 values of near 0, meaning close to no bacterial growth, over the 12-hour period. The effectiveness of the treatment is further supported by the standard growth of the controls with no treatment, in solid red and green, which show typical growth phases.

After growth curve completion, data analysis was performed and included calculation of the integral based on the area under the curve. Figure 8a (below, left) shows integral results from initial growth curve tests of 12.5 mg/ml ELP in green (control polymer material), ELP-LL37 in blue, ELP-Pexiganan in orange, and the control bacteria *S. aureus* in red, and the % growth based on the area under the curve. The two AMP treatments, ELP-LL37 and ELP-Pex (short for Pexiganan) in blue and orange show a significant reduction of growth by 50% compared to control *S. aureus* in red. Figure 8b (below, right) shows a comparison of two different concentrations of ELP-Pexiganan tested, 6.25 mg/ml and 25 mg/ml in orange, and the control bacteria *S. aureus* in red. We expected an increase in treatment effectiveness with an increase in

concentration, and the CFU/ml data shows a log reduction in growth between the higher concentration treatment 25 mg/ml and the lower concentration treatment 6.25 mg/ml, indicating that the higher concentration is more effective at reducing *S. aureus* colony numbers. Reduction of colony count is a treatment goal, so higher concentrations should be used for more effective treatment. The concentration must be balanced with toxicity tests, to address if the treatment material has any adverse effects and is planned for future research. Our initial growth curves tested 6.25, 12.5, and 25 mg/ml of control material tethered to treatment (ELP-LL37 and ELP-Pex), and/or control material (ELP alone).

ELP-AMP Treatment Effect on Control and Clinical Samples

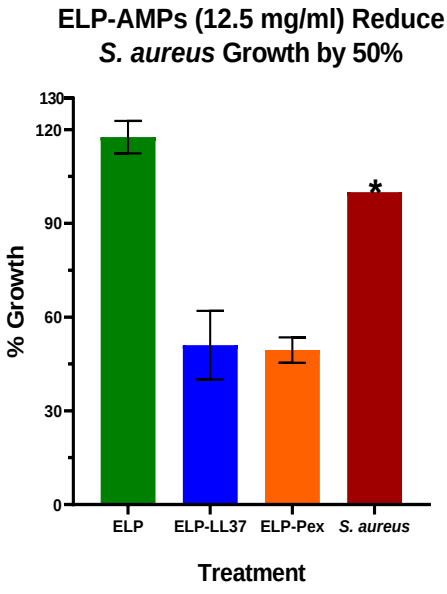

Figure 8a:
Integral from 0 to 48 hours of LL37 and Pex AMP treatments compared to control ELP and control *S. aureus*. * Error bars at +/- 0

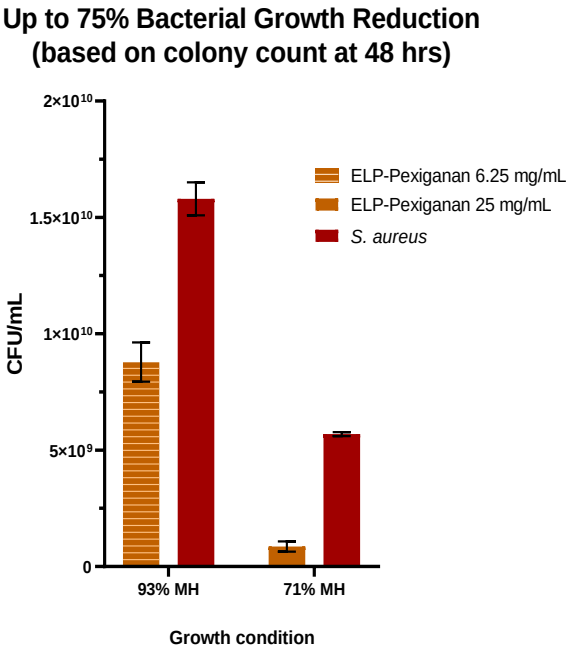

Figure 8b:
Bacterial cultures in Mueller Hinton (MH) broth and PB were plated after 48 hours of growth and CFU (colony forming units) were calculated. Log reduction in growth between treatment and control *S. aureus*.

These concentrations were chosen based on previous work by Li, 2019, based on a literature search of the minimum inhibitory concentration (MIC) of LL37 for *Staphylococcus aureus*. In figure 8b, the high (25 mg/ml) and low (6.25 mg/ml) concentrations were tested, to address if there was a concentration gradient for growth inhibition.

After the integral calculation workflow was established, we expanded to test and quantify additional AMP treatments and tether configurations (specified as 'A' and 'S') and the growth effects on laboratory isolates. Figure 9 below shows the percent growth of control bacteria, *S. aureus* (left), *P. aeruginosa* (middle), and a co-culture of both *S. aureus* and *P. aeruginosa* (right) in the presence of three different AMP treatments. The black bar on the left in each graph is the respective control bacteria, the pink bar is free LL37 treatment, free meaning no tether, at 50 uM, the blue-green bar indicates a tethered form of LL37 treatment, A-LL37, at 12.5 mg/ml, and the purple bar is another tether configuration with Pexiganan treatment, S-Pexiganan, at 12.5 mg/ml. 'A' and 'S' stand for the first letter of the amino acid chain, with both being different and changing the shape and function of the tether.

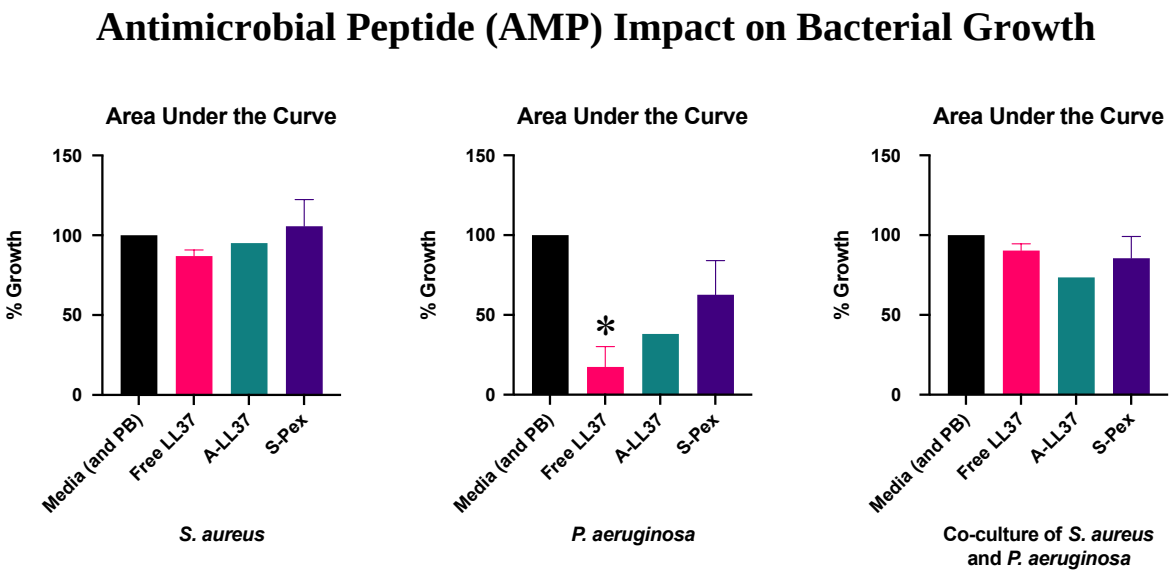

Figure 9:

Area under the curve calculated from 12-hour growth curves, measuring the O.D. 600 value every 10 minutes, plotted over time. Control experimental conditions include 'Media (and PB)' and treatment conditions include 'Free LL37', 'A-LL37', and 'S-Pex' which were added to assess growth alterations (% growth).

***** Significance determined via Kruskal-Wallis non-parametric test in Graph Pad prism.

Tethers like ‘A’ and ‘S’ are important to stabilize the AMP treatment, so it does not degrade as rapidly. We wanted to test both ‘A’ and ‘S’ to see if there were differences in the tethers based on growth inhibition, and if both materials were able to function with a tether.

Results in figures 8a and 9 are based on the area under the curve obtained from calculating the integral in Gen5 software. In figure 9, compared to the control (black bar), free LL37 and A-LL37 appear to inhibit the growth of bacteria at varying levels. S-Pexiganan (purple) did not appear to inhibit the growth of *S. aureus* or the co-culture but did so with *P. aeruginosa.* This indicates that S-Pexiganan may be more effective against gram-negative organisms, but additional organisms would need to be tested. Growth inhibition differences between figures 8a and 9 may be due to variability of ELP-AMP batches, as many batches of material were expressed and processed over several years. Sequencing data could be used to compare the presence and amounts of gram-negative bacterial species and/or genera after LL37 treatment and see if these integral results match the sequencing data. Overall, we found that S-Pexiganan treatment reduced growth of *P. aeruginosa*, free LL37 treatment reduced growth of *S. aureus* and *P.* aeruginosa, and A-LL37 treatment reduced growth of *P. aeruginosa* and a co-culture of both *S. aureus* and *P. aeruginosa*, With the above results showing growth inhibition of laboratory bacterial isolates with various AMP treatments, our next steps were to expand our studies with the clinical, polymicrobial DFU isolates to assess AMP treatment effects on overall microbiome composition.

In figure 9, the only statistically significant value comparing treatment and control bacteria was with free LL37 treatment on *P. aeruginosa* (Figure 9, middle graph asterisk). Statistical significance was determined using the Kruskal-Wallis non-parametric test in Graph Pad prism. This test measures if there is a difference between two groups based on a dependent

variable (% growth), so for our groups, control bacteria and bacteria with LL37 treatment, or with A-LL37 treatment or with S-Pex treatment. While we can see some visual differences between control growth (black bars) and treatment growth (colored bars), those differences were not different enough to be considered different, or significant, which renders them the same. We do see growth differences, they are minor, so additional tests (changing treatment concentration or using different tethers) are needed to determine if our AMP treatments cause a true difference in growth across treatments and bacterial species.

The Kruskal-Wallis test was not performed on each treatment condition, because there were not enough experimental replicates to assess for a difference. Typically, a sample size of 5 or more is needed to perform this test, and our sample sizes were generally smaller than 5 due to limited treatment availability and high cost of AMPs. Within a growth curve experiment, each test was run in duplicate, and some experiments were repeated, We tested free LL37 in several experiments, so there were enough data to perform the Kruskal-Wallis test on free LL37 treatment and each bacteria, *S, aureus, P. aeruginosa,* and a co-culture of the two, but of the three bacteria, only *P. aeruginosa* was statistically significant. A-LL37 was only tested in one experiment and S-Pex in two experiments because we had limited amounts of those AMPs as they are expensive, so we could not perform repeat experiments. So, with two replicates of A-LL37 and four of S-Pex, we were unable to perform statistical analysis. The results in figure 9 can then be considered qualitative results and we can discuss the information from a qualitative perspective, as we were unable to perform quantitative analysis due to lack of available data and cannot make quantitative conclusions.

DFU Clinical Isolate Sequencing and AMP Treatment

16S sequencing of the DFU clinical isolates was performed by the UA Genetics Core and resulting data were uploaded to Cosmos ID for comparison to the 'healthy' skin cohort WGS data. Figure 10 below shows the top 5 genera identified from DFU clinical isolates: USC 1 (top graph), USC 2 (middle graph), and USC 7 (bottom graph). In each graph, the top 5 genera are identified from 5 different experimental conditions. On the far left of each graph, the direct ex vivo column is the clinical swab sample collected from the DFU patient's wounds. Two experimental controls, one each for LL37 and S-Pex experiments, are the same clinical sample after overnight culture on different days, and then the same clinical sample on different days with treatment added: 50 uM LL37 experiment and 12.5 mg/ml S-Pex experiment. These sequencing data allows us to visualize which genera are present in the initial wound swab sample, which are present after culturing, and which are present after different AMP treatments. This can help identify patterns of susceptibility for different genera or types of bacteria, for example based on cell wall type.

The direct ex vivo bar on the left shows the top 5 genera after sequencing from the wound swab. USC 1 top species include *Prevotella, Fusobacterium, Enterobacter,* and *Corynebacterium.* USC 2 top species from direct ex vivo sequencing include *Proteus, Anaerococcus,* and *Peptonophilus.* USC 7 top species from direct ex vivo include *Finegoldia, Staphylococcus,* and *Streptococcus.* Most of the top species identified were diverse from the other samples, which indicates that each wound environment is unique, and the polymicrobial classification may vary in magnitude. Interestingly, after overnight culturing, USC 1 top genus shifted to include a majority of *Klebsiella* at approximately 70% for both experiments, when that genus was not initially present in the top 5 genera form direct ex vivo sequencing. After USC 1

treatment with 50 uM LL37, *Klebsiella* was no longer in the top 5 genus identified, and *Enterococcus* was identified at 95% relative abundance. USC 1 treatment with S-Pex revealed similar results as the overnight culturing, with *Klebsiella* at around 75% abundance. These results indicate that *Klebsiella*, a gram-negative genus, may be sensitive to AMP LL37, but not to S-Pexiganan.

Antimicrobial Peptide (AMP) Impact on DFU Microbiome Diversity

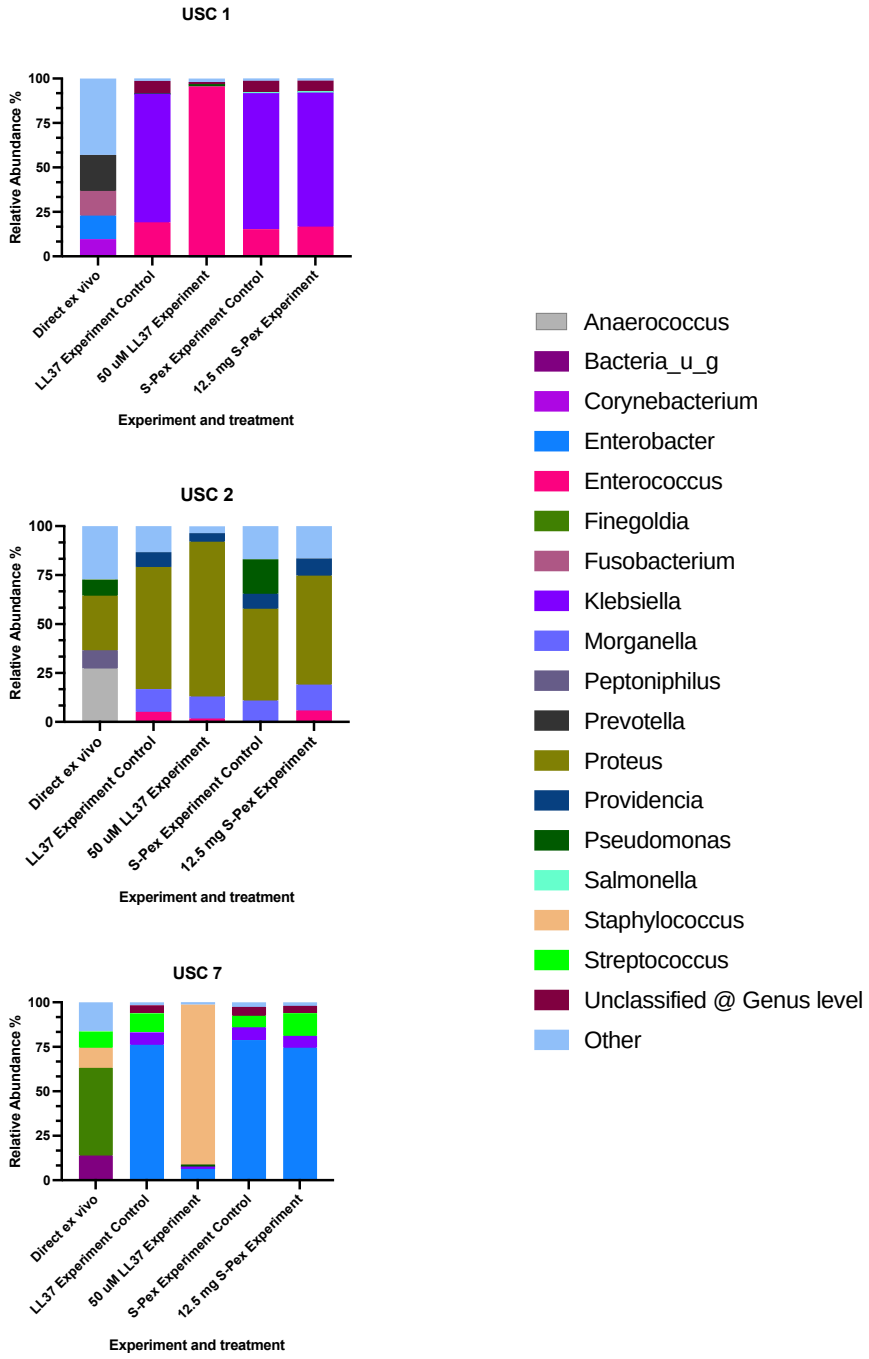

Figure 10:

The relative abundance percentage of bacterial genus from 16S sequencing of three DFU subjects direct ex vivo; the same clinical sample after overnight culture (experiment control) on different days; and the same clinical sample with AMP treatments added: 50 uM LL37 and 12.5 mg S-Pex. The clinical samples 'experiment' tests were grown up to an O.D. 600 of 1 after an overnight culture, and then diluted down to O.D. 600 of 0.0025 to inoculate in a 96 well plate for a 12-hour growth curve.

USC 2 results shifted from top genus *Anaerococcus* and *Proteus* at approximately 25% relative abundance, to a higher % relative abundance of *Proteus* in both experimental controls after overnight culturing. With LL37 treatment, USC 2 top genera were *Proteus* at approximately 60% relative abundance and *Morganella* at approximately 11%. With S-Pex treatment, USC 2 genera included approximately 55% *Proteus* and 13% *Morganella.* These results indicate that after overnight culturing, there was more *Proteus* identified in USC 2, and neither treatment appeared to be particularly effective in altering the growth of those top two identified genera. LL37 and S-Pex may have inhibited the growth of other genera, allowing greater proliferation of *Proteus* and *Morganella,* both gram-negative and motile genera.

USC 7 genera identified from direct ex vivo include *Finegoldia* at approximately 50%, but after overnight culturing, that genus disappears from the top 5 most abundant genera, and *Enterobacter* is the most relatively abundant genus at approximately 75%. With LL37 treatment, *Enterobacter* is greatly reduced in relative abundance, down to approximately 6% and *Staphylococcus* is the most abundant genus at approximately 90%. This result indicates that *Enterobacter,* another gram-negative bacterium, may be sensitive to the AMP LL37, which could be useful for individuals with abundant *Enterobacter* identified in DFU or other wounds. Additionally, *Staphylococcus* emerged as the topmost abundant genus after LL37 treatment, which may indicate that AMP is not effective against *Staphylococcus,* a gram-positive bacterium, and may suppress the growth of other gram-negative bacteria, allowing *Staphylococcus* and other non-susceptible bacteria to proliferate with reduced pressure from other, potentially competing genera and species.

Overall results in figure 10 indicate that culturing techniques affect the bacteria identified from different methods, and different AMP treatments may target different bacteria and have

different interactions, which can alter the composition of a polymicrobial sample. All our experiments were performed under aerobic conditions, which potentially limited our results, as many chronic wounds contain a considerable number of anaerobic bacteria, as wound environments often contain anaerobic regions. Anaerobic bacteria are not always cultured in clinical settings, which is another reason genetic sequencing is indicated, for more accurate results and wound characterization (27). Testing for and including anaerobic bacteria is something we hope to include in our future research and is indicated in future clinical wound characterization (27, 32).

After taxonomic identification of clinical isolates with and without treatment, we aimed to quantify AMP treatment effects by calculating the integral based on the area under the curve from a 12-hour growth curve experiment. Figure 11 below shows the area under the curve of clinical control samples, USC 1, USC 2, and USC 7, in black, and treatment conditions LL37 in pink, a tethered form of LL37, A-LL37 in blue-green, S-Pex in dark and light purple, and light blue, differentiated by experiment dates.

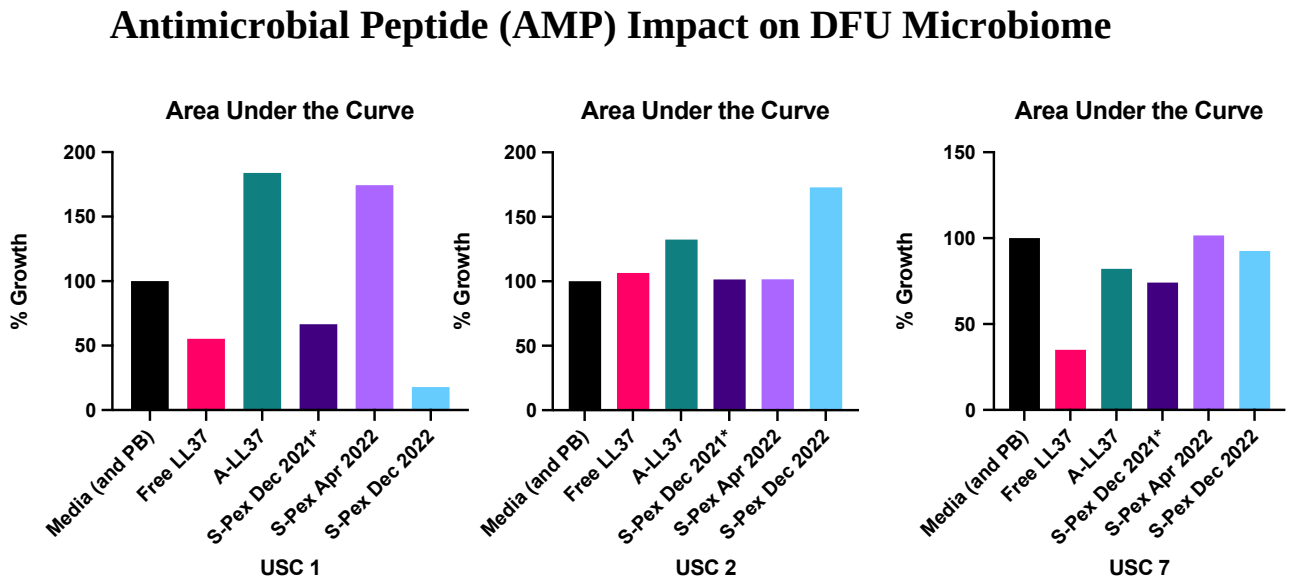

Figure 11:
Area under the curve calculated from 12-hour growth curves, measuring the O.D. 600 value every 10 minutes, plotted over time. Control experimental conditions include 'Media (and PB)' and treatment conditions include 'Free LL37', 'A-LL37', and 'S-Pex' which were added to assess growth alterations (% growth).

Overall results from figure 11 indicate AMP treatments had different effects on clinical isolates from DFU patients. We were unable to perform statistical analysis on data in figure 11, due to a lack of sufficient replicates needed for non-parametric tests. The AMP treatments were limited and costly, so we tested the treatments on the clinical samples to gather qualitative information. Within a graph, the colored bars vary in growth, some above the control bacteria growth (black bars) and some below, but without multiple experiments and sufficient replicates, we cannot conclude that the control bacteria and bacteria with treatment are different. These data above indicate that AMP treatments may be changing growth, but additional tests are needed to determine if the growth with and without treatment varies enough to be considered different, or statistically significant.

Though we do not have sufficient replicates for quantitative data analysis, we can make qualitative observations about the data. Compared to controls, free LL37 appeared to reduce USC 1 and USC 7 growth but grew more than the control in USC 2. S-Pex had varying effects on growth for all three samples and was inconsistent across experiments on different days. The flora cultured from clinical isolate USC 1 and USC 7 appear susceptible to free LL37, growing at around 50% compared to control, which indicates an estimated 50% growth reduction compared to control. USC 1 and USC 2 were not sensitive to AMP A-LL37 shown in teal, as the integral value was greater than 100% and grew more than the clinical isolate in broth, however USC 7 was somewhat susceptible to A-LL37 based on reduced growth percentage compared to control. The AMP S-Pexiganan (S-Pex) was tested on several different dates, and from different batches of the AMP. USC 1, 2, and 7 showed varying growth inhibition in the presence of S-Pex. USC 1 was susceptible to S-Pex in the December 2021 experiment (around 40% growth inhibition) and in the December 2022 experiment (around 70% growth inhibition), but not the April 2022

experiment where USC 1 in the presence of S-Pex grew more than control USC 1 in media. Our results show there was not consistent reduction of bacterial growth with increasing concentrations of free LL37 treatment. Integrals are one method to visually assess bacterial growth in the presence of AMPs, and integral data can be compared to sequencing data for further insight. Based on sequencing data, LL37 altered the topmost abundant genera identified in USC 1 and 7 but not USC 2, so LL37 appears to affect clinical isolates and species differently, which we can suspect from our sequencing data and further confirm with our integral data.

Figure 11 illustrates quantification of the growth curves 7B and 7D in Figure 7. The growth curves in 7B show clinical samples USC 1, 2, and 7 controls and with S-Pex treatment. Of note, USC 1 and USC 7 with S-Pex treatment, indicated with the red and green dotted lines reach an O.D. 600 of around 1.0, compared to the USC 1 and USC 7 control growth curves with no treatment, represented by the solid red and green lines, which reach an O.D. 600 of around 2.0. The S-Pex treatment in USC 1 and USC 7 appear to reduce the growth curve by about half, which translates to reduced growth overall. The visualization of this growth curve is then quantified by calculating the area under those curves, illustrated in figure 11. The calculated area under the curve for USC 1 with S-Pex treatment was around 40%, and for USC 7 with S-Pex treatment was around 30%, as stated above. Comparing the two tests is helpful to clarify visual estimates of growth inhibition with a numerical estimate. S-Pex appeared to reduce USC 7 growth curve by about half or close to 50%, but with the area under the curve data, that value was closer to 30% which is quite different from a visual estimate. Because the AMP treatments seemed to affect clinical isolates in different ways and target different bacterial genera, we next wanted to identify gram-positive or gram-negative patterns with AMP treatment.

Figures 12a and 12b below indicate overall more gram-negative bacteria (pink) than gram-positive bacteria (purple). With AMP treatments, LL37 appears to affect the gram-negative bacteria in USC 1, because there is an increase in the number of gram-positive bacteria after LL37 treatment, as seen in figure 12a with the increase in purple between the top two pies. Most other pies in figures 12a and 12b have minimal changes between the number of gram-positive and gram-negative bacteria, before and after treatment. Previous research found LL37 to have broad spectrum activity against both gram-positive and gram-negative species. Our results did not show the same activity against gram-positive species, as *S. aureus* growth appeared relatively unchanged with LL37 treatment. This may be due to different bacterial strains used, environmental pressure based on the freeze and thaw method each time bacteria is cultured, or differences in commercial or synthesized LL37.

AMP and ELP-AMP Differentially Kill Gram Positive and Negative Clinical Isolates

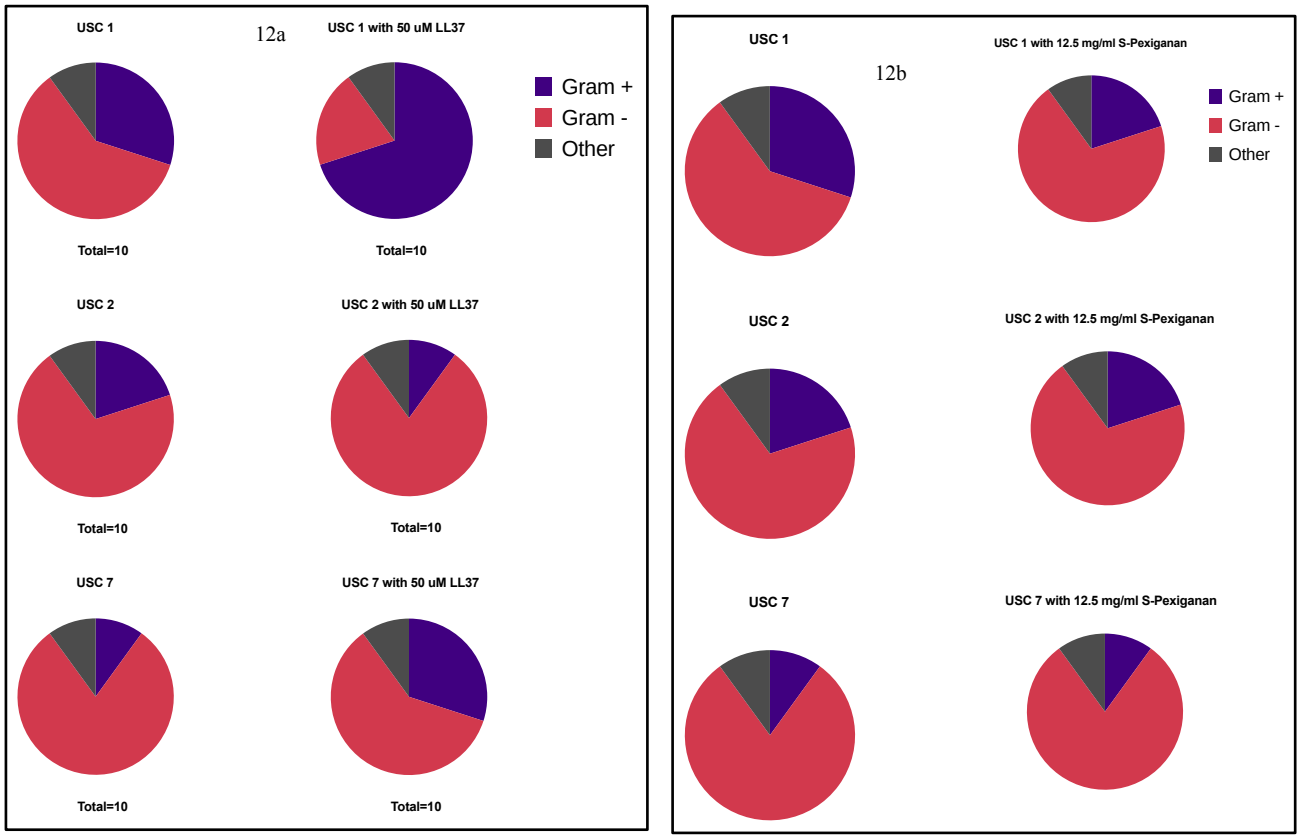

Figure 12a and 12b:

Classification of the top most abundant genus above 1% relative abundance from clinical isolates USC 1, 2, and 7 comparing gram-negative and gram-positive cell wall (12a) and after treatment with 50 uM LL37 AMP (12b).

Gram-positive versus gram-negative genera and species that are susceptible to different AMP treatments may be useful in identifying specific bacteria in clinical samples USC 1 sensitive to LL37, and combinations of bacteria that may exhibit different relationships and competition. The AMP treatments appear to slightly increase the number of gram-positive bacteria from the majority gram-negative bacteria, but the exact mechanisms and interactions resulting in these changes are unclear.

DISCUSSION

This study provides evidence for the expansion of DFU characterization techniques and DFU treatment options. Infections are becoming increasingly difficult to treat, and some antibiotics are no longer effective against certain bacteria; like methicillin resistant *Staphylococcus aureus* (MRSA) (15). Therefore, this two-fold problem requires more precise and expanded identification of infectious organisms. This will better inform treatments targeting these organisms, with consideration of antibiotic resistant genes.

Bacterial Sequencing for Precision DFU Treatment

The current standard sample collection for DFU wounds is a swab which we tested as a collection method in both our 'healthy' skin and diabetic foot ulcer cohorts, yet an increasing amount of research shows that this collection method is inaccurate and provides an inadequate sample of a wound. We noted differences in the most abundant genera identified by sequencing data between direct ex vivo and overnight culturing of the same sample. This may be due to a variety of changes imposed on the microorganisms from collection to re-culturing. Some of the changes could be due to the altered growth conditions, namely a change in oxygen levels, in nutrients, and exchange of the complex, multi-cellular and metabolically active human skin wound environment for a simplified, sterile liquid media environment. Other effects could stem from a stress period of growth from the sample collection on a dry swab or hydrogel chip, and temperature changes from human body temperature at 37C to ambient, fridge, and freezer temperatures of 25, 4, -80C. Therefore, alternative sampling methods are indicated for improved data collection. Tissue samples from a biopsy, in addition to swab collection and culturing, are becoming more standard for DFI characterization. A biopsy collects more of the wound

microbiome than what adheres to a swab, it samples at greater depths, and includes host tissue that yields greater DNA sample collection and preservation.

AMP Treatment Effects on DFU and Healthy Skin Samples

Based on the AMP concentrations we tested we saw a moderate amount of growth inhibition (%) based on our qualitative observations, but we would like to determine a dose for more significant growth inhibition, for more effective treatment of bacterial infection. A moderate amount of growth inhibition indicates the AMP treatments are altering the polymicrobial sample and are promising therapeutics, but to be considered effective and a treatment, these often need to meet bactericidal or bacteriostatic standards (27). Though there is still some debate around exact qualifications for bactericidal and bacteriostatic agent status, these classifications are often determined by the minimum inhibitory concentration (MIC) test results, and for antimicrobials, the target value is <10 ng/ml. The MIC is the minimum amount of an antibiotic or antimicrobial or other treatment that is needed to inhibit the growth of the bacteria or bacterium, so if a treatment can reduce the bacteria amount to less than 10 nanograms per milliliter, a very small amount. In the future, we plan to perform MIC tests after treatment with our AMP and ELP-AMPs to help us identify the minimum amount of AMP LL37 and ELP-AMP Pexiganan required to reduce our clinical and laboratory bacteria samples to <10 ng/ml (27). To do so, we would set up serial dilutions of AMP treatments in a 96 well plate and test different bacteria to visually determine at what concentration the bacteria growth is inhibited. In a 96 well plate, a well with bacteria has a ‘button’ of sediment that forms at the bottom, which is bacteria, and with different concentrations of treatment, there is often a breaking point where the button disappears, which reveals the concentration at which bacterial growth is inhibited. This test can be confirmed by then plating from those wells to determine if there is additional growth on agar

plates, and how much, to determine if the value is the minimum inhibitory concentration for that bacteria.

Increasing concentrations of AMP treatments would need to be balanced with toxicity tests, for safety of topical use on human skin. In these experiments, we tested AMP treatments, LL37 and Pexiganan separately, due to limited resources, but recent literature by Duong et al. showed that combining multiple AMPs has synergistic effects, so combining multiple AMPs that target different microorganisms or types of bacteria on DFI wound samples may have improved therapeutic effects and provide further guidance for clinical applications. Our initial tests of LL37 and Pexiganan on clinical DFU isolates provide evidence that these AMPs alter polymicrobial samples from human DFU (Figures 7, 10, 11, and 12), and further investigation into the capabilities of these AMPs and other alternative antimicrobials is necessary, to combat growing antibiotic resistance. Our 'healthy' skin cohort revealed a surprising amount of antibiotic resistance genes – in subjects 1, 2, and 3; 39, 38, and 42 different antibiotic resistant genes were identified from the chip sample, respectively, and in subjects 1, 2, and 3; 20, 38, and 27 different antibiotic resistant genes were identified from the swab sample, respectively. In subject 1, between 20 and 38 antibiotic resistant genes identified can translate to multiple antibiotics being less ineffective. The amount of antibiotic resistant genes identified is another indication of the need for additional research into antimicrobials because antibiotic resistance genes are widespread and colonize 'healthy' as well as wound environments.

Hydrogel Chip Platform Safety and Effects on Healthy Skin

The initial tests of our hydrogel chip on humans were successful, and established safety of the novel device on human skin. We sampled the skin via swab before application of the gel chip, to see if the chip and the swab picked up similar bacteria. In 'healthy' skin subject 2, the

16S sequencing data revealed the chip picked up *Staphylococcus* at 66.37% relative abundance, *Pseudomonas* at 30.14%, and *Corynebacterium* at 1.03%, compared to subject 2 swab which picked up *Staphylococcus* at 61.91% relative abundance, *Pseudomonas* at 26.39%, *Sediminibacterium* at 1.83%, and *Corynebacterium* at 0.89%. In this example, the swab picked up one different genus than the chip did, with a relative abundance of 1.83% which is small compared to the top two most abundant genera. The other genera identified were the same in both sampling methods and had relative abundance scores within 15% of one another – 66.37% *Staphylococcus* from the chip and 61.91% *Staphylococcus* from the swab, which indicates that similar amounts of the same genera were picked up in both sampling methods. However, we did not sample the skin after gel chip removal, and this information will be important to determine if the gel chip alters 'healthy' skin flora.

Our target population, namely diabetic patients, also indicates a potential need for changing testing temperatures. The experiments performed in this study were all at 37C, but testing lower temperatures is important for this target population because DFU on the foot, ankle, and lower leg are often lower in temperature due to reduced blood flow and neuropathy. In diabetic patients experiencing neuropathy, lower leg extremity temperatures on average read 27C (Schmidt et al., 2020), which is considerably lower than normal human body temperature. Therefore, we should consider AMP behavior at 27C, as well as AMP and ELP tether behavior at 27C, and a diabetic foot epithelium tolerance of topical application at 27C. This will also help us assess whether the different temperature promotes or inhibits wound healing and bacterial growth, and if there are temperature dependent interactions with the hydrogel chip.

Overall, similar numbers and types of bacterial species were identified by our hydrogel chip and by our swab, with 281 species identified by WGS and 490 species identified by 16S in

our ‘healthy’ skin cohort ‘chip’ 2 sample, compared to 311 species identified by WGS and 689 species identified by 16S in our ‘healthy’ skin cohort ‘swab’ 2 sample. Sequencing data from the swab sample collection method provided detailed and valuable information about our DFU clinical samples, including 485 different species identified by 16S in USC 1, 364 species in USC 2, and 356 species in USC 7. With this large number of species identified from our ‘healthy’ skin cohort, and in three of our clinical samples, we can confirm that these sequencing methods provide more information compared to culturing and selective media testing methods. With several hundred species identified, our clinical wound samples are intrinsically diverse, two of the subjects had similar profiles yet the third subject was markedly unique. This amount of information cannot be obtained from traditional methods like gram stain, blood agar, catalase tests, MacConkey agar, or morphologic features. Additionally, antibiotics typically target groups of bacteria, and based on our sequencing data and classification of gram positive and negative status, one antibiotic, even broad-spectrum, will not likely kill all the microorganisms identified in our clinical wounds.

Compared to culturing methods, sequencing identified 3-5 times more species and with greater confirmation methods and confidence based on Cosmos-ID and UAGC internal controls and confidence reports. There were minor differences noted between the 16S and WGS data, but both sequencing techniques provided a library of specific, genetic information that we could not obtain from traditional culturing techniques and differential media. WGS provides more detailed taxonomic information compared to 16S sequencing, including capturing entire genomes rather than a specific region of a genome which reveals a greater number of reads and includes subspecies and strain level identification, which 16S does not identify, and the presence of antibiotic resistance genes. WGS also provides identification of non-bacterial microorganisms

such as fungi, which may be advantageous in cases where infections include fungus species, multiple treatments and antibiotics have not worked, and infections are rapidly progressing. Due to limited funding, two different companies were used for sequencing, Cosmos ID for WGS and the UAGC for 16S. Thus, to compare data between platforms, we looked at the genus taxonomic level rather than the species level due to variations in data reporting of subspecies and strains. Overall, the genomic data obtained from the two sequencing methods and companies yielded similar results from our samples and provided much more information compared to our culturing results. This specific information from sequencing is necessary to reframe standard treatment protocols and prescription selections, to incorporate precision medicine techniques for targeted treatments, specific to individuals and even infections. Figure 6 shows the top antibiotic resistance genes found among our healthy skin cohort, which further supports the need for targeted treatments. Every healthy skin sample collection included a variety of resistance genes, with most subjects sharing at least one of the top resistance genes detected. Even among our healthy skin cohort, bacteria with antibiotic resistance genes were found in both samples from our hydrogel chip and swab. These resistance genes were similarly found in our clinical DFU samples. Therefore, targeted treatments, like AMPs are needed to treat a polymicrobial infection properly and effectively with varying degrees of antibiotic resistance, whether alone or in combination with other AMPs, antibiotics, or other therapeutics and antimicrobials.

This project showed that the presence of AMPs alters bacterial samples, whether laboratory or commercially produced, but did not appear to affect control bacterial samples differently from our clinical DFU samples. We successfully tested our hydrogel chip on a healthy human skin cohort, and determined the gel chip is safe and non-irritating to skin for 8 +/- 2 hours. Therefore, there is potential use for the hydrogel chip as a therapeutic and stabilizer for

AMP treatments for DFUs. Additionally, this project showed that the hydrogel chip was able to collect genetic information from healthy, clean, dry, and intact skin. Thus, the hydrogel chip may support the topical function of a wound dressing, by sticking to bacteria, and helping remove them from the wound environment.

Data from this project supports three changes to consider for improved DFU treatment: shifting medications from antibiotics to incorporate AMPs, detection methods from culturing to sequencing, and drug delivery systems from oral to topical. There is potential to harness the power of the innate immune system but utilizing naturally occurring AMP proteins to target and kill or inactivate microorganisms, rather than relying on creation or discovery of new antibiotics, as more bacteria become resistance to more antibiotics. Recent articles (1, 14, 21, 26, 27, 35, 36, 37, 44) mention the need for exploration of AMPs in clinical settings and applications, so our initial work on a limited number of clinical samples provides support for future testing with expanded clinical samples to determine optimal AMP treatment concentrations, configurations, and treatment combinations, of both AMPs and antibiotics.

FUTURE RESEARCH

Next steps for this project include testing different concentrations of AMPs to determine optimal levels, testing different ELP platforms that tether the AMPs to determine the best configuration and combination for stability and activity of each AMP, testing combinations of different AMPs to assess synergistic effects, and finally sampling a healthy human cohort for sequencing before and after gel chip application, to measure hydrogel chip effects on healthy skin bacteria. Based on the target patient population, future tests should also include testing materials on clinical samples and controls at temperatures lower than average human body temperature of 37C, as Schmidt et al., 2020 noted on average 27C in this population. Lower

temperatures are often seen in diabetic patients and in patients experiencing neuropathy, particularly in lower extremities where there is reduced blood flow. Reduced temperature tests should consider AMP behavior at 27C, AMP and platform tether behavior at 27C, and foot epithelium tolerance of the hydrogel chip application at 27C. With these future tests, we hope to better understand the role AMPs have on clinical wound samples, and in different concentrations, configurations, combinations, and temperatures.

CONCLUSION

Diabetes and DFUs are becoming increasingly difficult to treat - chronic conditions which affect diverse communities and individuals around the world. Treating infection and supporting wound healing are two clinical priorities that our research aims to support and investigate. We sought to characterize normal foot flora in healthy subjects, characterize DFU flora in diabetic subjects, compare sample collection methods between standard cotton swabs and an artificial polypeptide-based gel chip matrix, address the issue of antibiotic resistance and non-healing wounds in DFU infections by testing alternative treatments of AMPs on control and clinical bacterial samples, and investigate the safety and tolerance of our gel chip matrix and whether or not it alters normal foot flora. AMPs are indicated for infection treatment in the literature, and our results show activity against certain bacteria. These results can be considered in the future when considering dual treatments of both LL37 and Pexiganan for synergistic effects.

APPENDIX

Antimicrobial Properties of Artificial Polypeptide-Based Materials. Melcher LG, Doole FD, Kim M and Wertheimer AM (2020).

- Poster and presentation at the American Society for Microbiology 'Microbe 2020' Conference.

"Figure 1. Common Pathway of Diabetic Foot Ulcer Occurrence and Recurrence" Armstrong et al., 2017. **Reproduced with permission from Armstrong et al., 2017, Copyright Massachusetts Medical Society**.

Sequencing sample labels for Cosmos ID and UA Genetics Core and their shorthand names used in the methods, results, and discussion.

#	Name in Illumina	Shorthand
1	USC001	USC 1
2	USC002	USC 2
3	USC007	USC 7
4	22-S-PexplusUSC-1-12-28-21	USC 1 w Spex
5	9-S-PlexplusUSC-2-12-28-21	USC 2 w Spex
6	23-S-PexplusUSC-t-12-28-21	USC 7 w Spex
7	24-USC-1-10-LL37-12-30-21	USC 1 w 10 LL37
8	10-USC-2-10-LL37-12-30-21	USC 2 w 10 LL37
9	25-USC-7-10-LL37-12-30-21	USC 7 w 10 LL37
10	27-USC-1-50-LL37-12-30-21	USC 1 w 50 LL37
11	13-USC-2-50-LL37-12-30-21	USC 2 w 50 LL37
12	28-USC-7-50-LL37-12-30-21	USC 7 w 50 LL37
13	2-Co-Culture-12-28-21	CoC control Spex
14	8-S-PexplusCo-culture-12-28-21	CoC w Spex
15	20-Co-C-12-30-21	CoC control LL37
16	12-CoC-10-LL37-12-30-21	CoC w 10 LL37

17	14-CoC-50-LL37-12-30-21	CoC w 50 LL37

Consent to Participate in Research

Study Title: Towards a Hydrogel Therapeutic for Diabetic Foot Wounds:

Arm 1: The non-diabetic skin flora and response to hydrogel exposure

Principal Investigator: Anne Wertheimer PhD

Summary of the research

This is a consent form for participation in a research study. Your participation in this research study is voluntary. It contains important information about this study and what to expect if you decide to participate. Please consider the information carefully. Feel free to ask questions before making your decision whether or not to participate.

You are being asked to participate in the product development phase of our material. We are conducting a study about the safety of our new gel chip material and we want to determine if they remove any bacteria normally found on- the foot. This study will take at most up to 10 hours where your foot will be swabbed, have a gel chip or two about the size of a dime adhered to it with a Band-Aid. The chip(s) will stay in place for an hour, have it checked and then remain adhered for 8 +/- 2 hours. The chip(s) will be removed and the area where the chip had been placed will be swabbed again for bacteria. There is some risk of irritation to the skin around the gel chip. There is no direct benefit to you.

Why is this study being done?

The purpose of this study is to determine if our gel chips cause any irritation, and if they remove any of the bacteria normally present on your foot. Eventually we want to use similar chips but ones that have been modified to contain an antimicrobial compound in them, as a way of treating bacterial infections of the skin. The gel chips in this product development stage can be inherently sticky and some research has noted the chips can be capable of removing bacteria in lab settings. This purpose of this study to see if the gel chips are safe, do not cause any irritation and to see if they change the normal skin in anyway particularly altering the bacteria normally found on a person's skin. Our long term goal is to modify the gel chips to contain various compounds, thus developing them into treatments for infected skin wounds.

What will happen if I take part in this study?

Before you arrive, you will be asked to have not bathed that day leading up to your visit. When you arrive for your participation, the bottom of your foot will be swabbed using a FLOQ swab (like a Q-tip). The other foot will have the gel chip(s) adhered to it with a stretchy gauze material (Coban). After one hour the foot with the gel chip will be checked to ensure that there are no skin irritation, redness or itching. If no issues are seen, then the gauze will be rewrapped

around, and the gel chip will be left on for a total of 8 +/- 2 hours. You will be expected to not interfere or disturb the gel chip once it is adhered. You will return after 8 hours and the chip will be removed. The area that the gel chip(s) was will be swabbed and you will be done with your participation in the study. The gel chip(s) will then be taken to the laboratory for additional experiments. A portion of the chip (or an entire chip) will be placed in microbiological growth media to enrich for the bacteria that may have become attached to it. Another portion of the chip (or the second chip) will be examined directly by extracting the DNA to determine what bacteria are present. Photographs of your feet may be taken. You maybe contacted within 48hrs of your participation for additional follow-up to verify the was no delayed reaction to the hydrogel application.

How long will I be in this study?

You are expected to participate at a maximum of two days for this study as the gel chip(s) will remain on you for a maximum of 10 hours.

How many people will take part in this study?

We anticipate no more than 10 individuals but will recruit up to 25 in the event we see some people experiencing skin irritation after 1hour.

What benefits can I expect from being in this study?

There is no intended clinical benefit for you in this study. You will be informed of your skins microbiome but there is no clinical gain that you should expect.

What risks, side effects or discomforts can I expect from being in the study?

You may experience some irritation and reddening around the gel chip site. There are no other foreseeable risks associated, however if there are significant new finding that may impact your participation you will be informed of such.

What other choices do I have if I do not take part in this study?

Your participation is voluntary. If you decide to take part in the study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your usual benefits. Your decision will not affect your future relationship with the University of Arizona. If you are a student or employee at the University of Arizona, your decision will not affect your grades or employment status.

When may participation in the study be stopped?

If you start to have irritation due to the gel chip you may stop your participation in the study to prevent further irritation to the area the gel chip was exposed to. There will be no penalties or loss of benefits to you if you are forced to have your participation halted. We would appreciate you returning to the clinic if you experience irritation at the site.

What happens if I am injured because I took part in this study?
The University of Arizona have no funds set aside for the payment of treatment expenses for this study.

What are the costs of taking part in this study?
The gel chips and services performed for research only will be provided at no charge to you or your insurance company. Routine medical care performed while participating in study will be billed to you and / or your insurance company. This will include but is not limited to administration of medications, and the treatment of side effects.

Will I be paid for taking part in this study?
You will receive $10 compensation for taking part in the study.

Will my data or specimens be stored for future research?
Identifiers might be removed from the private information or biospecimens, and that after such removal, the information or biospecimens (the bacteria that we culture or identify through sequencing the genetic material from the swab or the gel chip) may be used for future research studies without additional informed consent for other studies investigating the human microbiome. You may be contacted in the future for additional studies related to your participation in this study.

Will my specimens be sold for commercial profits?
Your specimens will not be sold for commercial profit even if identifiers have been removed. You will not collect any commercial profit from the sale of your specimens.

Will I hear back on any results that directly impact me?
You will be informed of any determined bacteria that are found on your foot, please provide your contact information.

Will Whole Genome Sequencing be done with my specimen?
No only the bacterial sequencing will be performed.

Will my study-related information be shared, disclosed, and kept confidential?
It is anticipated that there will be circumstances where your study related information and Protected Health Information (PHI) will be released to persons and organizations described in this form. If you sign this form, you give permission to the research team to use and/or disclose your PHI for this study. Your information may be shared or disclosed with others to conduct the study, for regulatory purposes, and to help ensure that the study has been done correctly. These other groups may include:

- Office for Human Research Protections, Food and Drug Administration, or other federal, state, or international regulatory agencies
- The University of Arizona (UA) and the UA Institutional Review Board
- The sponsor and/or funder supporting the study, their agents or study monitors
- Your primary care physician or a specialist taking care of your health.

Who can answer my questions about this study?

If at any time you feel you have had a research-related injury, or for questions, concerns, or complaints about the study you may contact **Dr. Wertheimer at 503-956-0147**.

For questions about your rights as a participant in this study or to discuss other study-related concerns or complaints with someone who is not part of the research team, you may contact the Human Subjects Protection Program at 520-626-6721 or online at http://rgw.arizona.edu/compliance/human-subjects-protection-program.

If you are injured as a result of participating in this study or for questions about a study-related injury, you may contact **Dr. Wertheimer at 503-956-0147.**

To cancel your authorization for access to PHI you must notify the *Principal Investigator* and/or *Research Team* in writing at the following address:

awerth@email.arizona.edu

Signing the consent form

I have read (or someone has read to me) this form, and I am aware that I am being asked to participate in a research study. I have had the opportunity to ask questions and have had them answered to my satisfaction. I voluntarily agree to participate in this study and I authorize the use and/or disclosure of my PHI. I am not giving up any legal rights by signing this form. I will be given a signed copy of this form**.**

Printed name of subject	**Signature of subject**	**Date**

HSPP Use Only:
Consent form Medical v2019-07

Consent to Participate in Research

Study Title: Towards a Hydrogel Therapeutic for Diabetic Foot Wounds:

Arm 2: Skin Microbiome of Foot Wounds

Principal Investigator: David G. Armstrong, DPM, MD, PhD

Summary of the research

This is a consent form for participation in a research study. Your participation in this research study is voluntary. It contains important information about this study and what to expect if you decide to participate. Please consider the information carefully. Feel free to ask questions before making your decision whether or not to participate.

You are being asked to participate in a study about the treatment capabilities for human patients to treat/prevent bacterial infections of the foot/ankle. This study will take place during your routine office visit. Your wound will be swabbed as part of your standard of care and a second swab will be conducted and shipped for research purposes to further analyze the bacteria that may be present. There is no direct benefit to you.

Why is this study being done?

The purpose of this study is to 1) determine if the bacteria present in the wound respond to our new treatment, and 2) identify the types of bacteria present on the swab. Our long-term goal is to adapt the treatment that we are developing to heal infected skin wounds or prevent infection.

What will happen if I take part in this study?

You will be asked if you are interested in participating in the study, either before you arrive for your appointment or at your appointment. If the medical evaluation of your wound reveals the need for a culture swab for diagnosis, and you agree to participate in the study the wound will be swabbed twice: once for your clinical laboratory follow up and once for our research program. The swab we receive for research will be placed in microbiological growth media to enrich for the bacteria that may have become attached to it. These bacteria will then be tested 1) to determine if our new treatment kills the bacteria and then 2) a sample of the bacteria will be sent to our research facility to determine the identity of the bacteria.

How long will I be in this study?

You are expected to participate for the time it takes to consent you and obtain the second swab approximately no more than 15 minutes.

How many people will take part in this study?
We anticipate no more than 10 individuals but will recruit up to 25 in the event we fail to identify any bacteria from the swab.

What benefits can I expect from being in this study?
There is no intended clinical benefit for you in this study. You will be informed of your skins microbiome but there is no clinical gain that you should expect.

What risks, side effects or discomforts can I expect from being in the study?
You may experience some irritation and reddening around the swab site no different than the standard of care swab.

What other choices do I have if I do not take part in this study?
Your participation is voluntary. If you decide to take part in the study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your usual benefits. Your decision will not affect your future relationship with The *University of Southern California or the Keck Medical Center of USC* If you are a student or employee at the *University of Southern California* your decision will not affect your grades or employment status.

When may participation in the study be stopped?
If you start to have irritation at the wound site contact your physician (Dr. Armstrong (323-865-1544)). There will be no penalties or loss of benefits to you if you are forced to have your participation halted. We would appreciate you returning to the clinic if you experience irritation at the site.

What happens if I am injured because I took part in this study?
The University of Arizona have no funds set aside for the payment of treatment expenses for this study.

What are the costs of taking part in this study?
The services performed for research only will be provided at no charge to you or your insurance company.

Will I be paid for taking part in this study?
You will receive $10 compensation for taking part in the study.

Will my data or specimens be stored for future research?
Identifiers might be removed from the private information or biospecimens, and that after such removal, the information or biospecimens (the bacteria that we culture or identify through

sequencing the genetic material from the swab or the gel chip) may be used for future research studies without additional informed consent for other studies investigating the human microbiome. You may be contacted in the future for additional studies related to your participation in this study.

Will my specimens be sold for commercial profits?

Your specimens will not be sold for commercial profit even if identifiers have been removed. You will not collect any commercial profit from the sale of your specimens.

Will I hear back on any results that directly impact me?

You will be informed of any determined bacteria that are removed from your body through the treatment process.

Will Whole Genome Sequencing be done with my specimen?

No only the bacterial sequencing will be performed.

Will my study-related information be shared, disclosed, and kept confidential?

It is anticipated that there will be circumstances where your study related information and Protected Health Information (PHI) will be released to persons and organizations described in this form. If you sign this form, you give permission to the research team to use and/or disclose your PHI for this study. Your information may be shared or disclosed with others to conduct the study, for regulatory purposes, and to help ensure that the study has been done correctly. These other groups may include:

- Office for Human Research Protections, Food and Drug Administration, or other federal, state, or international regulatory agencies
- The University of Arizona (UA) and the UA Institutional Review Board
- The University of Southern California Institutional Review Board and Human Subjects Protection Program
- The sponsor and/or funder supporting the study, their agents or study monitors
- Your primary care physician or a specialist taking care of your health.

Your PHI may no longer be protected under the HIPAA privacy rule once it is disclosed by the research team.

Who can answer my questions about this study?

If at any time you feel you have had a research-related injury, or for questions, concerns, or complaints about the study you may contact D**r.** Armstrong at 323-865-1544.
For questions about your rights as a participant in this study or to discuss other study-related concerns or complaints with someone who is not part of the research team, you may contact:
USC IRB

Telephone: 323-442-0114
Fax: 323-224-8389
Email: irb@usc.edu

If you are injured as a result of participating in this study or for questions about a study-related injury, you may contact **Dr. Wertheimer at 520-626-5850 or Dr. Armstrong at 323-865-1544.**

To cancel your authorization for access to PHI you must notify the *Principal Investigator* and/or *Research Team* in writing at the following address:

Insert address for Investigator: awerth@email.arizona.edu

Signing the consent form

I have read (or someone has read to me) this form, and I am aware that I am being asked to participate in a research study. I have had the opportunity to ask questions and have had them answered to my satisfaction. I voluntarily agree to participate in this study and I authorize the use and/or disclosure of my PHI. I am not giving up any legal rights by signing this form. I will be given a signed copy of this form**.**

Printed name of subject	**Signature of subject**	**Date**
Printed name of person authorized to obtain consent	**Signature of subject**	**Date**

HSPP Use Only:
Consent form Medical v2019-07

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