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High Levels of Oxidative Stress and the Skin Microbiota Contribute Towards Initiation
and Development of Chronic Wounds in Diabetic Mice

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular and Developmental Biology

by

Jane Hannah Kim

March 2022

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Chavez; graduate students, Frantzeska Giginis, Benjamin Yang and Terrence Lin; and our undergraduate students, Kelly Chen, Elyson Gavin Lebig, Amanda Tedesco, Alaa'I Ahamed, Shayan Saeed, Anthony Castro, James Thongkham, and Jade Ramirez.

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Dedication

I dedicate this dissertation to my mother and father, Carol and Charles Kim. Thank you for all your love and support. I greatly appreciate your commitment to my education over the years. I also dedicate my work to my siblings, Heidi, Charlie, and Walter Kim, who over the course of my research career have blossomed into amazing individuals with dreams and aspirations. Lastly, I dedicate this dissertation to my best friends Emelline Chen, Danielle Dayto, and Jason Estep, who are always there for me, watching me succeed and become the best person I can be.

"Discovery consists of seeing what everybody has seen

and thinking what nobody has thought."

-Albert von Szent-Györgyi

ABSTRACT OF THE DISSERTATION

High Levels of Oxidative Stress and the Skin Microbiota Contribute Towards Initiation
and Development of Chronic Wounds in Diabetic Mice

by

Jane Hannah Kim

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, March 2022
Dr. Manuela Martins-Green, Chairperson

The skin is the largest organ of the human body. It is barrier that defends and
protects the body against environmental assaults and has the important role in
maintaining physiological homeostasis, functioning as a highly regulated interface
between the outside and inside of the body. Residing on the skin is a microbial
community known as the skin microbiota which consists of bacteria, fungi, and viruses.
These microbes residing in specific niches of the skin interact with each other and
potentially impact the health of the skin through crucial skin-microbe interactions.
Research on the resident skin microbiota is ongoing; however, current research suggests
that beneficial microbes, commensal microbes, and pathogens all have a role in
maintaining healthy skin or causing infection and disease in the skin.

When the skin is injured, the human body activates the cutaneous wound healing
process to close the wound as quickly and thoroughly as possible to re-establish the
protective and defensive roles of the skin. Skin of healthy individuals will heal quickly;
however, skin that is afflicted with oxidative stress or inflammation may not heal in a
timely manner. When healing is delayed for any number of reasons, the wound may
develop into a chronic wound. Chronic wounds are defined as wounds with prolonged

healing time greater than four weeks and have very high risk of chronic infections which in turn can further increase healing time. Infected chronic wounds are colonized by bacteria and fungi which can form biofilm, polymeric structures that protect the microbes from the immune system and prolong the infection of the pathogenic microbes.

Chronic wounds are difficult to heal as the biofilm present on the wounds are recalcitrant to conventional antimicrobial therapies including antibiotics. To understand how chronic wounds are initiated and what role bacteria play on the skin, I employed a novel chronic wound model in obese, diabetic mice previously developed in our lab. Using this model, I studied how levels of oxidative stress is crucial for the initiation and development of chronic wounds. The level of oxidative stress stimulates bacteria in the wound to produce biofilm soon after injury. I also used to model to study the community of the bacteria that reside on healing and chronic wounds over time. I show that the diversity of the bacteria in healing wounds is very diverse while chronic wounds which also contain biofilms are not. I identified bacterial species associated with either healing wounds or wounds. Lastly, I provide a review on the importance of bacteria in healthy skin, ailing skin and chronic wounds. Taken together, my finding show that oxidative stress is crucial for the initiation and development for chronic wounds and the biofilm that infect them. Further research on the wound-microbe interaction is required to understand the underlying mechanisms that lead to the development of chronic wounds. This knowledge will lead to the discovery and development of new therapies that can heal and prevent chronic wounds.

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Introduction

The skin is the first line of defense and protection against environmental assaults, functioning as an interface between the outside and inside of the body. It has evolved to act as a physical, chemical, and biological barrier through careful and precise management of the cellular composition and structure. The skin is populated with a microbiota which consists of bacteria, fungi, and viruses that can interact with and impact the health of the skin. Beneficial microbes can contribute to healthy skin management by stimulating the adaptive and innate immune system. In contrast, pathogens can cause infection and disease if their population is not controlled. Commensal microbes, while mostly benign, can function in aiding beneficial microbes or participate in infection and disease by promoting the colonization of pathogens.

When the integrity of the skin is compromised, essential and critical functions of the skin are impaired and disrupted, allowing the microbes that populate the skin to enter the body and potentially cause systemic infection and death. In response to injury, four major stages of cutaneous wound healing are activated. These include homeostasis, inflammatory phase, proliferative phase, and remodeling phase and result in reinforcing the protection and defense abilities of the skin. Minor cutaneous injuries including scrapes, abrasions and cuts will heal quickly in healthy individuals. However, large surgical incisions and serious trauma such as lacerations and burns will take a longer time to heal and are at risk of developing infection. Despite of the different types of wounds, when cutaneous injuries achieve closure in a timely fashion, it is referred to acute wound healing. In contrast, severe, non-healing wounds with prolonged healing time greater than

four weeks are referred to as chronic wounds and are at even greater risk of developing chronic infections which in turn can further increase healing time.

Chronic wounds are a significant and increasing burden on the health care system. More than 6.5 million people are affected by chronic wounds, and billions of dollars are spent annually in the US alone. Managing chronic wounds is a costly burden especially to the elderly and diabetic patients who are at a significantly increased risk of developing chronic wounds compared to the general population. Patients that are immobile, have cancer or co-morbidities, such as obesity or cardiovascular diseases, which result in poor blood flow such peripheral artery disease and venous insufficiency, are at highest risk of developing chronic wound. Patients with chronic wounds also have decreased quality of life compounded by chronic or intense pain, long hospitalization admittance and reduced mobility which can affect their ability to function.

Severe injuries that develop into non-healing wounds fall under several major categories: pressure ulcers, venous leg ulcers, and diabetic foot ulcers. They are commonly characterized by a sustained activation of inflammation. Many factors such as oxidative stress can activate inflammation, but the mechanism of activation is still not completely understood. Regardless, delaying treatment to manage inflammation and oxidative stress significantly increases risk of infection. If the wound ultimately cannot be healed, physicians may resort to amputations in order to contain spread of infection and prevent risk of developing sepsis. Amputation significantly increases the risk of mortality to elderly and diabetic patients.

Research into chronic wounds has led to evidence that suggests that host-microbial interactions, more specifically the interaction between the skin and its microbiota, is an important driver of skin ailments, including wound chronicity. The colonization of microbes including bacteria and fungi in wounds, as a community, may impact wound healing processes and preventing healing. Several skin microbiota studies have shown that chronic wounds are colonized by a diverse wound microbiome, and that the composition and structure of the wound microbiome plays an important role in dysregulating normal wound healing processes including the activation of the innate immune system and inflammation. What is not understood is why certain species are observed in wound infections and whether certain species are contributing towards the cellular and molecular mechanisms that lead to chronic wound development. These types of questions are pushing scientists and healthcare professionals to research how dysregulated host-microbe interactions affect normal cutaneous wound healing.

As the global population continues to age and experience increasing rates of chronic diseases such as diabetes, there is a need to understand the pathophysiology of chronic wounds and the wound microbiota that colonizes them to develop effective therapies that can reduce or remove microbial bioburden, repair tissue damage, and restore healing. Because the skin is crucial to overall health and wellbeing, countless therapeutics for wound treatment, including traditional and more novel, advanced methods, are available to assist in rapid healing and increased wound healing quality. Research into acute cutaneous wound healing is ongoing to discover or develop more efficient and cost-effective approaches. While some of these practices can be applied to

chronic wounds, due to the complexity of the wounds and the microbiota, current and future studies need to focus on understanding how the interaction between host and microbe impacts delayed healing and why wounds become chronic. Options for convenient, effective therapies need be explored and quickly developed.

While significant efforts have been made to understand how chronic wounds develop in humans, results that can lead to the development of efficient treatment strategies for chronic wounds has been slow. This is because effective chronic wound research requires a stable, reliable, and reproducible model to understand how these wounds initiate and develop. There was a lack of animal models that recapitulate the complexity of human chronic wounds, and experiments to create chronic wounds simply cannot be conducted on humans. Instead, wound healing specialists researched chronic wounds in patients that have already been chronic for weeks and months. Another approach has been to use animal models with impaired wounds that ultimately heal rather than becoming chronic wounds. To overcome these shortcomings, we developed a chronic wound model in mice. This model is a powerful tool to study chronic wounds because it shares many of the same features observed in human diabetic chronic wounds. Most importantly, it is characterized with prolonged inflammation by increasing oxidative stress and with natural biofilm formation derived from the skin microbiota. In our lab, we use this model to study how chronic wounds initiate and develop. For my dissertation, using our novel model of chronic wounds, I have studied host-microbe interactions that occur during the development of chronic wounds-and how those interactions contribute to chronicity.

In the first paper, I studied the effect of oxidative stress, in a dose-dependent manner, on the initiation and development of chronic wounds. Not only is the severity of

the wound and quality of healing affected, the level of oxidative stress is also observed to impact the diversity of bacteria that colonizes the wound. In the second paper, I collected and sequenced the wound microbiome through the progression of acute wound healing in control wounds and through the initiation and progression of chronic wound development. In these studies, I identified specific species of bacteria to be putative probiotics that may contribute to wound healing as well as commensal species that may assist pathogenic species in colonizing and infecting wounds. The third paper reviews the importance of the skin and the skin microbiota in health and disease. I discuss how the anatomy and function of the skin interacts with the complex skin microbiota to support healthy skin and normal wound healing. The review also discusses how microbial dysbiosis, perturbations to the skin microbiota, may result in the development of human skin diseases and ailments, including the development of chronic wounds. Skin ailments such as atopic dermatitis, acne vulgaris, rosacea, and psoriasis have skin microbiota different from normal skin. Similarly, the community of biofilm-forming microbes differs with some uniqueness across different etiology of chronic wounds such as venous ulcers, pressure ulcers, and diabetic foot ulcers. The review also addresses a new generation of therapeutics where the skin microbiota is manipulated through probiotics, prebiotics and skin microbiota transplants in order to prevent and treat skin ailments and chronic wounds.

Chapter 1

High Levels of Oxidative Stress and Skin Microbiome are Critical for Initiation and

Development of Chronic Wounds in Diabetic Mice

Abstract

A balanced redox state is critical for proper healing. Although human chronic wounds are characterized by high levels of oxidative stress (OS), whether OS levels are critical for chronic wound development is not known. For these studies, we used our chronic wound model in diabetic mice that has similar characteristics as human chronic wounds, including naturally developed biofilm. We *hypothesize* that OS levels in wound tissues are critical for chronic wound initiation and development. We show that increased OS levels in the wound correlate with increased chronicity. Moreover, without increased OS levels, biofilm taken from chronic wounds and placed in new excision wounds do not create chronic wounds. Similarly, high OS levels in the wound tissue in the absence of the skin microbiome do not lead to chronic wounds. These findings show that both high OS levels and bacteria are needed for chronic wound initiation and development. In conclusion, OS levels in the wound at time of injury are critical for biofilm formation and chronic wound development and may be a good predictor of the degree of wound chronicity. Treating such wounds might be accomplished by managing OS levels with antioxidants combined with manipulation of the skin microbiome after debridement.

Introduction

Cutaneous wound healing requires a concerted effort of cellular and molecular processes to heal the skin that has been injured[1–3]. It involves four highly regulated overlapping phases: homeostasis, inflammation, granulation tissue formation, and tissue remodeling[1,3–5]. After injury, homeostasis needs to be achieved to prevent blood loss and block the influx of microorganisms from the environment into the blood stream; blood vessels constrict to decrease blood flow and to allow platelets to stick to the injured site. Fibrinogen is activated to form fibrin fibers that associate with platelets and red blood cells to form a clot. Once homeostasis is achieved, blood vessels dilate to allow immune cells, antibodies, nutrients, and oxygen to flow into the wound tissue. Molecules, such as growth factors and cytokines released from the platelets during clotting, attract neutrophils to the injury site. These leukocytes secrete proteolytic enzymes and release reactive oxygen species into the wound bed to combat invading bacteria. Monocytes, also recruited to the wound, differentiate into pro-inflammatory macrophages to aid in the inflammatory process by clearing dead neutrophils, cellular debris and bacteria through phagocytosis[5]. They also secrete new growth factors and cytokines that are important for the next phase of healing – proliferative phase.

As inflammation resolves, the proliferative phase begins and rebuilding of new tissue initiates[6,7]. Keratinocytes proliferate and migrate over the wound to create a new epidermis through re-epithelialization. To begin reconstruction of the dermis, fibroblasts and endothelial cells migrate and proliferate beneath the newly formed epithelium, generating new dermal tissue called granulation tissue[2,4,5]. Fibroblasts proliferate and

differentiate into myofibroblasts, which aid in healing through contraction of the wound and secretion of extracellular matrix (ECM) proteins such as collagen, a component of the scar tissue. Angiogenesis, a process by which new blood vessels develop by proliferation and migration of endothelial cells, brings nutrients and oxygen to the wound site to support the reconstruction of the granulation tissue[2,5,7].

Remodeling, which is the last stage of wound healing, begins after re-epithelization and granulation tissue formation. The excess cells are eliminated from the tissue by undergoing apoptosis and being removed by resident macrophages and histiocytes[5]. The tensile strength of the tissue is dependent on various components of the ECM and on fibroblasts in the scar tissue so that the newly formed tissue becomes strong[4]. Finally, the skin barrier is re-established to protect the newly formed tissue from the environment[8].

Chronic wounds develop when one or more of the processes described above are disrupted or occur out of sequence. Patients with underlying pathological conditions, such as metabolic diseases, are particularly susceptible to developing chronic wounds[9,10]. For example, patients with Type II diabetes have significantly higher probabilities of developing chronic wounds, particularly in the feet, called diabetic foot ulcers (DFUs). Of the ~30 million Americans who have diabetes, approximately 25% will experience a foot ulcer during their lifetime[4,8,9]. DFUs are commonly infected with microbial biofilms that prevent healing since they can be resistant to antimicrobial therapies. When the wound is unable to heal, amputation of the lower limbs may be necessary to contain the spread of infection. DFUs wounds cost the US up to $13 billion a year[11].

Many chronic wounds persist despite antibiotic therapy, wound debridement, and applications of wound dressings[12,13]. It is known that chronic wounds in humans contain elevated levels of oxidative stress (OS)[5]. OS occurs when an imbalance of redox chemicals exists in the tissues being affected. Reactive species are separated into two major groups, reactive nitrogen species (RNS) and reactive oxygen species (ROS)[14,15]. When oxidizing radicals build up within a tissue, it is usually due to inhibition or insufficiency of detoxifying antioxidant enzymes. Therefore, improper regulation and response to OS results in significant damage to DNA, proteins and lipids[16,17]. Oxidative damage to DNA can lead to oxidized nucleotides and single strand or double-strand breaks. Oxidative damage to amino acid residues can change protein structure, ultimately altering their function in the cell[18]. Given that chronic wounds are known to contain elevated levels of OS[2,19], providing the wound bed with additional antioxidants has been shown to support healing[20].

We have previously created chronic wounds in *db/db*^{-/-} mice by inhibiting catalase and glutathione peroxidase (GPx), immediately after wounding[20]. These two enzymes are critical antioxidant molecules during wound healing. When 7 mm diameter full thickness cutaneous wounds in *db/db*^{-/-} mice are treated with phosphate buffer saline (PBS) at pH 7.4, the wound typically heals around 20-25 days whereas the same size wound made in C57BL/6 mice closes between 11-12 days. Upon treatment with inhibitors for catalase and GPx at time of injury, changes in the wound tissue lead the biofilm-forming bacteria present in the skin to create biofilm within 3-5 days. In our diabetic mouse model, biofilm-forming bacteria are not artificially introduced after wounding; they are present

in the skin microbiota prior to wounding and take advantage of the wound

microenvironment created by the high levels of OS to form biofilm. 10-15 days after

wounding, the amount of the biofilm greatly increases, and the wounds remain open and

infected with biofilm for weeks if the mice survive[20].

Based on these findings, *we hypothesized* that OS is critical for chronic wound

initiation and development and that chronicity is directly proportional to the levels of OS

in the wound tissue. To test this hypothesis, we treated wounds in *db/db*^{-/-} mice at the time

of injury with increasing doses of inhibitors to the antioxidant enzymes, catalase and

GPx, to create increasing levels of OS in the wound tissue. We show that OS is critical

for the initiation of chronicity and that higher the levels of OS induced at wounding, the

more severe the chronic wounds become. We also show that, although OS is necessary

for development of chronicity, it is not sufficient; the presence of bacteria on the skin is

also necessary. We conclude that in order for the wounds to become chronic, we need

both increased levels of OS and the bacteria present in the skin.

Materials and Methods

Reagents: 3-Amino-1, 2, 4-triazole (ATZ) from Tokyo Chemical Industry Co., Ltd.

(Portland, OR). Mercaptosuccinic acid (MSA) from Sigma-Aldrich (St. Louis, MO).

Buprenorphine (buprenex) from Henry Schein (Dublin, OH). Isoflurane from Henry

Schein (Dublin, OH). Tegaderm Film 1624W from 3M (Maplewood, MN). 10%

Povidone-Iodine Solution from Equate (Bentonville, AR). Bovine serum albumin (BSA)

from Fisher Scientific (Hampton, NH). Paraformaldehyde (PFA) from Fisher Scientific

(Hampton, NH). Goat serum from Sigma-Aldrich (St. Louis, MO). Vectashield from

Vector Laboratories, Inc. (Burlingame, CA). Power Block from BioGenex (Fremont, CA). Krystalon Mounting Medium from EMD Millipore Sigma (Burlington, MA).

Antibodies: The following primary antibodies were used: anti-collagen IV (col IV) ab6586 from Abcam (Cambridge, UK), EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80) MCA497 from Bio-Rad, formally from AbD Secrotech, (Hercules, CA). The following secondary antibodies were used: goat anti-rabbit antibody conjugated with Alexa Fluor 594 A11012, and goat anti-rat antibody conjugated with Fluorescein A10527 from Invitrogen (Carlsbad, CA).

Chronic wound model: All experiments were completed in accordance and compliance with federal regulations and the University of California policy and procedures approved by the UCR IACUC (Institutional Animal Care and Use Committee). The description of how to obtain chronic wounds in *db/db*^{-/-} mice have been published in detail by us previously[20–22]. Briefly, *db/db*^{-/-} mice are bred in our conventional vivarium from B6.BKS(D)-*Lepr*[db]/J heterozygotes obtained from the Jackson Laboratories (Stock no. 000697). Only *db/db*^{-/-} that are 5-6 months old and weigh at least 50 g are used to create chronic wounds. By this age, the mice have had high blood glucose for a long period of time and are fully diabetic and obese, something that does not occur when the mice are 2 months old, the most common age at which db/db^{-/-} mice are used for experiments. To remove the hair, the back of each mouse is shaved, and Nair applied to expose the skin. To create the chronic wound, one 7 mm full thickness skin excision wound is made under isoflurane anesthesia and then covered with Tegaderm. OS in the wound tissue is increased by using specific inhibitors for catalase and GPx, 3-amino-1, 2, 4-triazole

(ATZ) and mercaptosuccinic acid (MSA), respectively. ATZ is injected intraperitoneally at 1 g ATZ/kg of mouse weight in sterile PBS approximately 20 min before surgery. MSA is administered topically onto the wound site under the Tegaderm at 150 mg MSA/kg of mouse weight in sterile PBS within 10min after surgery. All mice are treated with buprenex, a pain reliever, injected intraperitoneally at 0.05 mg buprenex/kg of mouse weight in sterile PBS before surgery and 6 h after surgery. After wounding, the mice are housed individually in our conventional vivarium to prevent biting and scratching from other mice.

Creating microbe-depleted wounds: The *db/db*$^{-/-}$ mice are normally housed in a conventional vivarium, but for experiments where wounds cannot have bacteria on the skin, we housed the mice separately and away from the normal *db/db*$^{-/-}$ colony in special cages supplied with autoclaved water, irradiated bedding and irradiated vivarium chow. The skin of the mouse was wiped and disinfected with iodine and 70% ethanol immediately prior to surgery in order to remove as much of the natural skin microbiota as possible. As soon as the wounds were made, they were sealed with sterile Tegaderm which provides a barrier to external contaminants and environmental bacteria from the cage and bedding. The mice were given the dose of the inhibitors for high levels of OS at wounding.

Wound Area: The area of each wound was measured over time with inSight by eKare Inc. (Fairfax, VA). Briefly, the device was held parallel to the wound and always at the same distance from the wound. The device was calibrated to measure the wound area accurately after manual delineation of the wound margin.

Histology and histological staining: Wound tissues were collected and fixed in 4%

paraformaldehyde (PFA) in PBS for 4-6 h at room temperature. The tissues were washed

three times in PBS for 15 min to remove excess PFA; non-crosslinked PFA was

quenched by incubation in 0.1 M glycine in PBS for 30 min. The tissues were washed

with PBS and then incubated first in 15% sucrose in PBS for 4-6 h at room temperature,

and then 30% sucrose in PBS overnight at 4 °C. The tissues were then embedded in

optimal cutting temperature compound (OCT), sectioned (8-10 µm thick) in a cryostat,

and stained with hematoxylin & eosin (H&E) and Masson's trichrome (MT) as we have

previously described[20]. Staining with picrosirius red (PSR) (Polysciences Inc) was

performed as previously described[23]. Briefly, wound tissues embedded in OCT were

sectioned and fixed in 4% PFA for 10 min. The sections were rinsed with DI water before

stained with PSR (Solution B) for 90 min, rinsed two times with 0.1 N HCl, pH 4.0

(Solution C) for 1 min before rinsing again in DI water. The sections were dehydrated in

70% ethanol for 30 s and allowed to dry before mounting in Krystalon. Stained sections

were visualized using a Nikon Microphot-FXA microscope (Nikon Instruments Inc.,

Melville, NY) and photographed between cross polarizers.

Immunolabeling: Frozen sections were fixed in 4% PFA in PBS for 20 min. After

washing with PBS, excess PFA was quenched with 0.1 M glycine in PBS for 20 min.

Tissue sections were incubated in 0.3% Triton X-100 for 30 min when probing for

intracellular proteins. Sections were blocked with Power Block for 4 min and then

incubated with the primary antibodies in 1% BSA/PBS for 4 h at room temperature,

washed three times with 0.1% BSA in PBS and then incubated with secondary antibodies

for 1 h at room temperature. After washing with PBS, slides were mounted in Vectashield

containing DAPI (4′,6-diamidino-2-phenylindole). Immunofluorescence was visualized

and imaged using a Nikon Microphot-FXA fluorescence microscope with a Nikon DS-

Fi1 digital camera and NIS-Elements software (Nikon Instruments Inc., Melville, NY).

Bacterial collection: Bacteria were collected with sterile swabs using the Levine

method[24]. Briefly, a sterile swab was rolled around the wound area, approximately 1 cm^2

area. Bacterial samples were taken from the skin after injury, and from days 1, 3, 5, 10,

15, and 20 following surgery. For sequencing, the swabs were stored at -80 °C without

freezing media.

DNA extraction: DNA extractions were performed on thawed swabs using the MOBio

PowerSoil DNA Isolation Kit (which became the Qiagen PowerSoil DNA Isolation Kit)

as described by the manufacturer with a 90 s bead-beating step.

Bacterial rRNA internal transcribed spacer (ITS) analysis: Illumina bacterial rRNA

ITS libraries were constructed as follows: PCRs were performed in an MJ Research PTC-

200 thermal cycler (Bio-Rad Inc., Hercules, CA) as 25 µl reactions containing: 50 mM

Tris (pH 8.3), bovine serum albumin (BSA) at 500 µg/ml, 2.5 mM MgCl$_2$, 250 µM of

each deoxynucleotide triphosphate (dNTP), 400 nM of the forward PCR primer, 200 nM

of each reverse PCR primer, 2.5 µl of DNA template, and 0.625 U JumpStart Taq DNA

polymerase (Sigma-Aldrich, St. Louis, MO). PCR primers targeted a portion of the small-

subunit (ITS-1507F, GGTGAAGTCGTAACAAGGTA) and large-subunit (ITS-23SR,

GGGTTBCCCCATTCRG) rRNA genes and the hypervariable ITS region[25], with the

reverse primers including a 12 bp barcode and both primers including the sequences

needed for Illumina cluster formation; primer binding sites are the reverse and complement of the commonly used small-subunit rRNA gene primer 1492R[26] and the large-subunit rRNA gene primer 129F[27]. PCR primers were only frozen and thawed once. Thermal cycling parameters were 94 °C for 5 min; 35 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 40 s; followed by 72 °C for 10 min. PCR products were purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

DNA sequencing (single-end 150 base) was performed using an Illumina MiSeq (Illumina, Inc., San Diego, CA). Clusters were created using template concentrations 2.5 pM and phi X at 107 K/mm^2. Data processing was performed with USEARCH v10.0[28]. We used the UPARSE pipeline for de-multiplexing, length trimming, quality filtering and operational taxonomic unit (OTU) picking using default parameters or recommended guidelines that were initially described[29] and have been updated at https://www.drive5.com/usearch/manual10/uparse_pipeline.html. Briefly, after demultiplexing and using the recommended 1.0 expected error threshold, sequences were trimmed to a uniform length of 149 bp and then dereplicated. Dereplicated sequences were subjected to error-correction (denoised) and chimera filtering to generate zero-radius operational taxonomic units (ZOTUs) using UNOISE3[30]. An OTU table was then generated using the otutab command. ZOTUs having non-bacterial DNA were identified and enumerated by performing a local BLAST search[31] of their seed sequences against the nucleotide database. ZOTUs were removed if any of their highest scoring BLAST hits contained taxonomic IDs within the rodent family, Fungal or Viridiplantae kingdoms, or

PhiX. Taxonomic assignments to bacterial ZOTUs were made by finding the lowest

common taxonomic level of the highest BLAST hits excluding unclassified designations.

Data were normalized within each sample by dividing the number of reads in each OTU

by the total number of reads in that sample.

Bioinformatics and statistics: One-way ANOVA was used to calculate the significance

of the wound area between different doses, followed by Bonferroni's multiple-

comparison test to determine significant differences between groups. One-way ANOVA

was also used to calculate the significance of the number of blood vessels and

macrophages between different doses, followed by Dunnett's test to determine significant

differences between groups compared to basal level of OS as control. ggplot2 (2.2.1) in R

(3.4.0) was used to graph the mean% of the microbiome. Alpha diversity among the

treatment groups was analyzed with a statistical model that uses a first-order

autoregression covariance structure, assuming heterogenous variances and heterogenous

correlations between time and the dose given to each mouse. The least square means

were used to perform F-tests and calculate significance.

Results

Dose-dependent effect of OS on development of chronic wounds

The *db/db*^{-/-} mice have delayed and impaired wound healing but they do not

develop chronic wounds. The healing takes approximately twice as long when compared

to that of normal C57BL/6J mice. However, when catalase and GPx are inhibited to

increase OS to high levels in the wound microenvironment, the wounds become fully

chronic within 20 days[20]. These wounds become larger than the original wound because

the tissue damaged due to the increased levels of OS disintegrates and contributes to the formation of the biofilm. To determine the importance of the levels of OS in wound chronicity, we performed dose-dependent experiments (**Fig. 1**). Mouse wounds treated at wounding with only PBS as vehicle are denoted as having *basal level* of OS, representing the natural level of OS induced by the injury. Mouse wounds treated with the inhibitors of catalase and GPx at high doses, 1.0 g ATZ/kg and 150 mg MSA/kg of mouse weight in sterile PBS, are denoted as having *high levels* of OS and go on to develop *fully chronic wounds*. To create wounds with *low levels* of OS, inhibitors at a dose of 0.125 g ATZ/kg and 17.9 mg MSA/kg of mouse weight were administered. For wounds with *moderate levels* of OS, a dose of inhibitors at 0.25 g ATZ/kg and 35.7 mg MSA/kg of mouse weight were administered. For wounds with *moderate/high levels* of OS a dose of inhibitors at 0.5 g ATZ/kg and 75 mg MSA/kg of mouse weight were administered (**Fig. 1**).

After one 7mm diameter full thickness wound was made, wounds with basal levels of OS closed in approximately 20 days and those with low levels of OS took a few more days (**Fig. 2**). Wounds treated with moderate levels of inhibitors showed considerable amounts of tissue damage and took several more days than 20 to close. Increasing the levels of OS further to moderate/high or high levels resulted in extensive damage to the wound tissue with formation of bacterial biofilm. Biofilm formation in wounds with moderate/high OS levels began around days 10-12 days after wounding whereas wounds with high OS levels began much earlier, around days 3-5 after wounding (**Fig. 2**). Wounds with high OS levels significantly expand after day 10 because the

bacteria in the wound break down the dead host skin tissue after it has been damaged by OS. The broken-down tissue was used as nutrients for the bacteria and as building material for the biofilm they build in the chronic wounds. Calculation of percent wound closure showed significant differences in area of the wounds between day 0 and day 20 (**Fig. 3**).

To determine how the levels of OS affect wound healing quality, for each treatment, wound tissues were collected after closure (the higher the OS levels the longer the time to closure) and various stains were performed (**Fig. 4**). H&E staining was performed to visualize overall structure of the wound tissue after closure (**Fig. 4A-D**). Staining showed that wounds containing basal to moderate levels of OS were able to close with an intact epidermis connected to the newly formed granulation tissue underneath (**Fig. 4A-C**). The wounds with moderate/high levels of OS were able to form an epidermis; however, the connection between the epidermis and the granulation tissue was poor, indicating poor healing (**Fig. 4D**). MT staining was used to show whether the wound tissue contained interstitial collagen, which is important for remodeling and maturation of the wound tissue after wound closure (**Fig. 4E-H**). Compared to wounds with basal levels of OS, the dermis of the wounds with low and moderate levels of OS shows more intense collagen staining in the granulation tissue (**Fig. 4F-G**). However, this could be due to wound compression during sectioning of the sample for histology. Wounds containing moderate/high levels of OS showed that the granulation tissue had a sparse structure and less compact interstitial collagen (**Fig. 4H**).

The closed wounds were also stained with PSR in order to distinguish between and visualize the distribution of collagen I (col I) and collagen III (col III) in the granulation tissue (**Fig. 4I-L**). Under cross-polarizing light, the PSR staining showed col I as red and col III as green. When they colocalized, the staining becomes yellowish/orange. In wounds with basal levels of OS, col I appeared as thicker fibers that are evenly distributed in the granulation tissue. Col III fibers were also found distributed in the tissue, with some overlap with col I deeper in the granulation tissue (**Fig. 4I**). In contrast, wounds with increasing levels of OS showed that after closure, the amount of col I was lower than that of col III and lower than that in the wounds with low OS levels, suggesting that the collagen present in the granulation tissue was primarily col III **(Fig. 4J-K).** This pattern was accentuated in the wounds with moderate/high levels of OS (**Fig. 4L**).

In order to further examine the quality of the wound tissue with increased OS levels, we immunostained the tissues for col IV which localizes to the basement membrane under the epidermis and in the basement membrane around the microvessels of the granulation tissue. For these studies, we used an antibody specific for col IV (**Fig. 4M-T**). The basal lamina was well formed under the epidermis of wounds with basal to moderate levels of OS (**Fig. 4M-O**). However, in the wounds with moderate/high levels of OS, the basement membrane under the epidermis was not well formed (**Fig. 4P**). The col IV staining in the basement membrane of the blood vessels allowed us to examine the microvasculature in the granulation tissue of the wounds (**Fig. 4Q-T**). We observed that the blood vessels present in the granulation tissue of wounds with basal, low and

moderate OS levels were similar in morphology and abundance (**Fig. 4Q-S**). The granulation tissue of the moderate/high levels of OS contained significantly fewer blood vessels (**Fig. 4T**).

The data described above showed the quality of wound healing in the presence of increased levels of OS once the wounds have closed. This occurred at different times, with moderate and moderate to high levels of OS taking several days more than wounds with basal and low levels of OS. To observe the effect of OS on the wound tissue structure as the wounds were healing, wound tissues were collected 17 days into the healing process (**Fig. 5**). H&E staining (**Fig. 5A-D**) showed that an epidermis has already formed in the wounds with basal and low OS levels (**Fig. 5A-B**). Wounds with moderate and moderate/high OS levels completely lacked an epidermis, indicating that the wounds were still open at 17 days into healing (**Fig. 5C-D**). MT staining (**Fig. 5E-H**) of the wounds with low OS levels showed that collagen deposition was delayed comparing to wounds with basal OS levels at the same time post-wounding (**Fig. 5E-F**). Both moderate and moderate/high levels of OS resulted in virtually no interstitial collagen deposition at this stage of healing (**Fig. 5G-H**). The structure of the granulation tissue of wounds with moderate/high levels of OS was also very weak as the tissue was disrupted (**Fig. 5H**). This pattern was also seen, but to a lesser degree, in wounds with moderate OS levels (**Fig. 5G**). PSR staining of these wounds showed that as the OS levels increased, the wounds have less deposition of both col I and col III as shown with the MT staining (**Fig. 5I-L**). Immunofluorescence for col IV showed that the pattern of blood vessels in wounds with basal to low levels of OS (**Fig. 5M-N**) was similar to that of closed wounds with

basal and low levels of OS (**Fig. 4Q-R**). Wounds with moderate OS levels were open at

day 17 but they contained significant number s of blood vessels (**Fig. 5O**). In wounds

with moderate/high OS levels, the number of new blood vessels in the granulation tissue

was low, showing that angiogenesis was delayed (**Fig. 5P**). Quantitation of the

microvessels shows significant decrease as the levels of OS increase (**Fig. 5R**).

Immunostaining for macrophages with F4/80 (**Fig. 5Q**) to examine the level of

inflammation in these wounds showed that, at 17 days into the healing process, the

macrophages were no longer present in the wounds with basal, low and moderate levels

of OS but were still very much present in the wounds with moderate/high levels of OS

(**Fig. 5R**).

Wound bacterial microbiome is affected by oxidative stress levels

Because we observed correlation between the levels of OS and the degree of

wound chronicity, we investigated whether the levels of OS also affected the degree of

biofilm that developed as the wounds became chronic. Analysis of the wound

microbiome was conducted by sequencing the bacterial rRNA ITS, a hypervariable

region between the 16S and 23S rRNA genes. Variable regions of 16S rRNA gene are

more commonly used to obtain descriptions of bacterial communities. However, we used

the rRNA ITS region because analysis of this region often enabled identification of

bacterial OTUs at species level [25]. From the dose dependent study (**Fig. 1**), 243

bacterial samples were collected on individually wrapped sterile swabs via the Levine

method and stored frozen in -80 ℃ before DNA extraction, library construction and

sequencing.

To determine whether a correlation between the diversity of bacteria and wound healing outcomes existed in our chronic wound model, we calculated the alpha diversity of bacteria in the wounds using the Shannon diversity index and performed statistics accounting for repeated measures (**Fig. 6A**). Shannon diversity index is a measure of both species' richness (number of taxa) and evenness (a measure of the relative abundances) of each of the species. Low indices indicated lower diversity, found typically in infections (e.g., one microorganism dominates and causes disease). High indices indicated higher diversity, found typically in stable, healthy communities. In the following analysis we discovered that the level of OS significantly contributed to a difference in Shannon diversity (p-value < 0.0001). The diversity across time was also significant (p-value < 0.0198). The greatest difference in diversity is between wounds with basal levels of OS and wounds with low, moderate, moderate/high, and high levels of OS (p-values $= 0.0007$, $= 0.0006$, < 0.0001, and < 0.0001, respectively). Wounds with closer levels of OS had similar diversity in their bacteria: low and moderate levels (p-value $= 0.9996$), moderate and moderate/high (p-value $= 0.9324$), and moderate/high and high (p-value $= 0.2455$). Wounds with low and moderate OS levels had a Shannon diversity index that was very different from wounds with high OS levels (p-value $= 0.0523$ and 0.0860, respectively).

Wounds with basal levels of OS had a bacterial microbiome profile that was dynamic and diverse. Diversity was maintained as the wound healed. Several species were present in the wound throughout healing, with the percent composition changing in the wound over time. The dynamics of the microbiome kept the diversity high throughout

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the healing (**Fig. 6B**). Interestingly, the presence of biofilm-forming bacteria such as

Enterobacter cloacae, *Streptococcus thermophilus*, *Propionibacterium acnes*,

Staphylococcus xylosus, *Pseudomonas aeruginosa*, *Corynebacterium sp.*, *Acinetobacter*

johnsonii and, *Achromobacter xylosoxidans,* was observed in these wounds but never

with biofilm formation. Wounds treated to create low levels of OS were populated with a

number of bacterial species found in the wounds with basal OS levels but in different

proportions; only a few bacteria dominated the wound: *E. coli*, *E. cloacae*, *S xylosus* and

P. aeruginosa (**Fig. 6C**). In days 1-3, a majority of the wound was colonized by *E. coli*

and *S. xylosus*. At day 5, *E. cloacae* and *P. aeruginosa* began to colonize the wound until

day 10 when *S. xylosus* appeared to be outcompeted by these two bacterial species. The

relative abundances among *E. cloacae*, *E. coli*, and *P. aeruginosa* were stabilized by day

10 and there was no biofilm formation; the wound healed similarly to basal OS level

wounds (**Fig. 2**). This observation showed that these bacteria do not form biofilm and

colonize the wound in a pathogenic manner unless the levels of OS reached a specific

threshold (**Fig. 6B**). In contrast, wounds with moderate levels of OS, which had a similar

profile as wounds with low levels of OS, contained some biofilm (**Fig. 6D**). With the

presence of damaged tissue caused by the MSA, the microenvironment began to provide

conditions that are conducive to biofilm development. In these wounds, *S. xylosus* did not

comprise a large percentage of the microbiome; its presence was observed on day 1 at

15% of the population and in the subsequent days less than 5%. *P. aeruginosa*, a known

biofilm-forming species of human chronic wounds, very quickly colonized the wound

after wound initiation; at day 3, it composed more than 25% of the wound, increasing to

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more than 40% by day 5. Other bacteria such as *E. coli* and *E. cloacae* competed with *P. aeruginosa* for dominance in these wounds. Wounds treated with moderate/high levels of OS in the wound were colonized by the same biofilm-forming bacteria found in the wounds with low and moderate levels of OS (**Fig. 6E**). Wounds with high levels of OS were dominated by the most aggressive biofilm forming bacteria such as *P. aeruginosa* and *A. johnsonii* which began their colonization at 24 hours after wounding and quickly expanded their populations, outcompeted all other bacteria in the wound and formed strong biofilm (**Fig. 6F**).

While the average percentage of bacteria in the wound showed multiple bacteria present in a wound in a single day, individual mice with high levels of OS typically showed a single or a couple of bacteria dominating the wound over the course of several days. For example, a mouse wound containing high level of OS had a microbiome entirely composed of *P. aeruginosa* starting from day 3, while another was entirely composed of *A. johnsonii* (data not shown). We observed that when wounds contain low levels of OS, the diversity of bacteria during healing was high. Contrarily, when wounds contain higher levels of OS, the wounds were open and have lower bacterial diversity. These wounds were ultimately colonized by a few or a single bacterial species.

Oxidative Stress and the microbiome are each necessary but not sufficient for chronic wound initiation

The wound profiles showed that OS had a significant impact on wound healing initiation development and progression. We determined whether OS alone was necessary and sufficient to create chronic wounds without the presence of bacteria in the wound.

For that, we removed the bacteria from the skin before wounding and then created high levels of OS in the wound tissue by inhibiting catalase and GPx at wounding (**Fig. 7**). Compared to wounds with high levels of OS, in which the bacteria were not removed and go on to become chronic, the wounds in the absence of bacteria but with high levels of OS, did not become chronic. Cell death by necrosis in the tissue around the wound was clearly visible but biofilm did not develop, and the wounds did not become chronic; bacterial infection and biofilm were not seen at 20 days post-wounding when the wounds would normally become fully chronic in the presence of both high levels of OS and bacteria **(Fig, 7, column 3)**.

To determine whether bacteria alone were able to make biofilm in the wounds without an environment with high levels of OS, we transplanted biofilm from fully chronic wounds to new wounds without increasing the OS levels in the wound (**Fig. 7, column 4**). These wounds did not become chronic and went on to heal, much like non-chronic wounds, despite the presence of biofilm obtained from other chronic wounds (**Fig. 7, column 4**). Wound areas were determined in all four conditions. We showed that wounds with high OS levels had an area significantly increased after day 10 when compared to the areas of the other groups (**Fig. 8**). These results show that OS and the microbiome in the skin were each necessary but not sufficient to achieve chronicity.

Discussion

OS has many important roles in regulation of wound healing[5,32]. At basal levels, it creates an environment conducive to initiation of the healing process involving all four phases of healing that we described in the introduction. Increasing OS after wounding

will derail these processes. Excessive levels of ROS and RNS, which are highly reactive, can damage DNA, proteins, metabolites and lipids in the wound. Damage sustained by DNA in the form of oxidized nucleotides or single/double stranded breaks initiates various response to DNA damage pathways in order to repair oxidative damages and also regulates fundamental cell cycle processes. However, if the damage cannot be corrected or reversed, this signals to cells and tissues to undergo unregulated cell death in the form of necrosis. Damaged metabolites or oxidized lipids can disturb the cellular redox balance and lead to mitochondrial dysfunction, which can also lead to cell death. Inflammation, whose initiation and termination are strictly controlled in order to facilitate healing, is highly sensitive to the redox state of the wound and can lead to a stronger inflammatory response or prolong the process beyond normal regulation. Damage to regulatory chemicals such as chemokines, cytokines, and growth factors will prevent proper regulation of leukocyte chemotaxis and function in response to injury. Regulation of angiogenesis (microvessel development) is also disturbed by high levels of hydrogen peroxide and nitric oxide leading to inhibition of microvessel development.

The studies presented here showed that the development of chronic wounds in diabetic and obese mice was directly proportional to the levels of OS in the wound tissue (**Fig. 9A**). When wounds were treated with inhibitors of catalase and GPx to increase OS levels in the wound, wounds treated to cause low levels of OS closed just slightly delayed when compared with wounds with basal levels of OS (*db/db*$^{-/-}$ non-chronic wounds). The quality of healing and the % of bacteria present in the wounds were similar to non-chronic wounds and neither form biofilm. Wounds containing moderate levels of OS

were further delayed in closure; they remained open for a longer period of time, but the wound underwent re-epithelialization, the basal lamina under the epidermal cells was also developed and the quality of wound healing after wound closure was similar to that of wounds with low levels of OS. Wounds with moderate/high to high levels of OS had a higher degree of tissue disorganization, had considerably larger area compared to the original wound and developed strong biofilm around 10 days after injury. Wounds with high levels of OS did not close and began to develop biofilm with 2-3 days after wounding. The importance of levels of OS is emphasized when observing its effect on the bacteria that colonized the wound. Wounds with lower levels of OS generally showed more dynamic shifts in population percentages over time, whereas wounds with higher levels of OS showed decreased dynamic shifts with fewer biofilm-forming bacterial species colonizing the wounds. Therefore, the levels of OS were important in the microbiome that developed in the wound; increased OS levels decreased the diversity of the wound microbiome by providing a microenvironment conducive to biofilm-forming bacteria to thrive and form biofilm in the wound. Therefore, the levels of OS in a wound at the time of injury are critical for biofilm formation and chronic wound development and may be a good predictor of the degree of wound chronicity. We also found that, when mature bacterial biofilm was transplanted onto wounds after injury without increasing OS levels, wounds were able to heal in a timely manner. Similarly, wounds treated to eradicate existing skin microbiome and administered inhibitors for antioxidant enzymes to increase OS levels, underwent moderate tissue damage without the development of a chronic wound (**Fig. 9B**).

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We also show that the quality of healing after closure of wounds treated with moderate levels of OS can be similar to wounds with low or basal levels of OS and they do not develop biofilm. However, in some cases wound healing is delayed with biofilm formation that is not as strong as that found in wounds with moderate/high OS levels. Because damage to the tissue is not extensive, this results in the formation of mild chronic wounds. These results suggest that in the wounds with moderate levels of OS, other factors such as the types of bacteria present in the skin of the mouse, have strong effects on the healing outcome. Circumstances, such as bacterial interactions in the wound, may prevent strong biofilm-forming bacteria from colonizing the wound and forming biofilm. If this is the case, wound healing will not be very delayed and the wound closes even if the levels of OS in the wound are moderate. However, if a strong biofilm-forming bacteria is abundant in the skin to start with, moderate levels of OS might create a microenvironment conducive for biofilm formation preventing healing.

Wounds with moderate to high levels of OS are able to undergo re-epithelialization, but the connection between the epidermis and the granulation tissue underneath is weak. The basement membrane lacks col IV, indicating that the wounds have no basal lamina. These wounds also had a higher number of macrophages, indicating that inflammation has continued days past when inflammation in healing wounds should have resolved. We conclude that wounds with excessive levels of OS are at increased risk of having impaired healing, biofilm formation and developing chronic wound. Studies to determine whether inducing high levels of OS as the healing is

progressing results in derailing of the healing process and causes chronicity will be under way soon.

In humans, the skin microbiome has important implications in cutaneous wound healing[32–34]. Culture-independent methods using 16S rRNA and/or shotgun metagenomic sequencing, surveyed complex wound microbiomes across domains with dynamic interactions between microbes over time corresponding to healing and impaired wounds[33,35–37]. Host-microbe interactions studies in skin further stressed the complex relationship between wound healing processes and the skin-residing microbes[38,39]. Of the bacteria found in human chronic wounds, the following were found in our chronic wound model: *Acinetobacter sp.*[40–45], *Bacillus sp.*[42,44,46,47], *Enterobacter sp.*[40,41,43–45,48], *Escherichia sp.*[40,41,44,45,47,49], *Propionibacterium sp.*[43,46,47], *Enterococcus sp.*[40,42,44,46,48,49], *Pseudomonas sp.*[40–43,46–49], and *Staphylococcus sp.*[40,43,44,46–48]. In our *db/db*^{-/-} mice, much like in humans, the bacterial profile can diverge from litter to litter and even in cage mates so that each mouse followed over time shows an individualized profile in the % of the bacterial species colonizing the respective wound in response to OS levels. These finding indicate that our mouse has the ability to form mature biofilm in the chronic wounds from the skin microbiome that parallels that of human chronic wounds.

High OS in human chronic wounds can be a result of a hypoxic wound microenvironment or ischemia, which significantly impairs wound healing in human wounds[50]. When tissues have poor vascularization and blood flow, the wound tissue fails to have sufficient oxygen pressure and nutrients to support healing and instead lead to the breakdown of the tissue or development of ulcers and infection. This effect is worsened

especially in lower extremities of diabetic patients because they also suffer from chronic hyperglycemia, neuropathy, arterial insufficiency, and dysfunctional leukocytes. Angiogenesis, which we observed to be delayed in wounds with high OS levels, is also found to be improperly regulated and delayed in human chronic wounds. We observed that re-epithelization is delayed as wounds were subjected to higher levels of OS. Human chronic wounds are also difficult to heal because re-epithelialization of the wound is either delayed or does not occur. Histology of human chronic wounds shows that at the wound edge, the epidermis is thick and hyperproliferative, the epithelial tongue does not form, and epithelial migration does not occur. This suggests that important keratinocyte functions, such as activation, differentiation and migration, are aberrant or disturbed[51].

In conclusion, the degree of chronicity of the wounds depends on the levels of OS when the wound is first created. Wounds with lower levels of OS heal and are colonized by a diverse bacterial community. These wounds may have biofilm-forming bacteria present in the wound, but these bacteria do not form biofilm. Wounds with higher levels of OS are colonized by pathogenic bacterial species that form biofilm and do not heal normally. Furthermore, the levels of OS present in the wound after injury greatly impacts wound healing progression and prognosis and also it affects the type of bacteria that colonize the wound, acquiring either pathogenic or commensal/non-pathogenic roles. Both OS and bacteria in the skin are crucial for the development of chronic wounds; high levels of OS are necessary but not sufficient for chronic wound development, and mature bacterial biofilm in the absence of high OS levels does not lead to development of chronic wounds. These studies provide a platform to further study the

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effects of OS in both the progression of wound healing and the dynamics of the

microbiome over time, potentially leading to new strategies to treat these recalcitrant

wounds.

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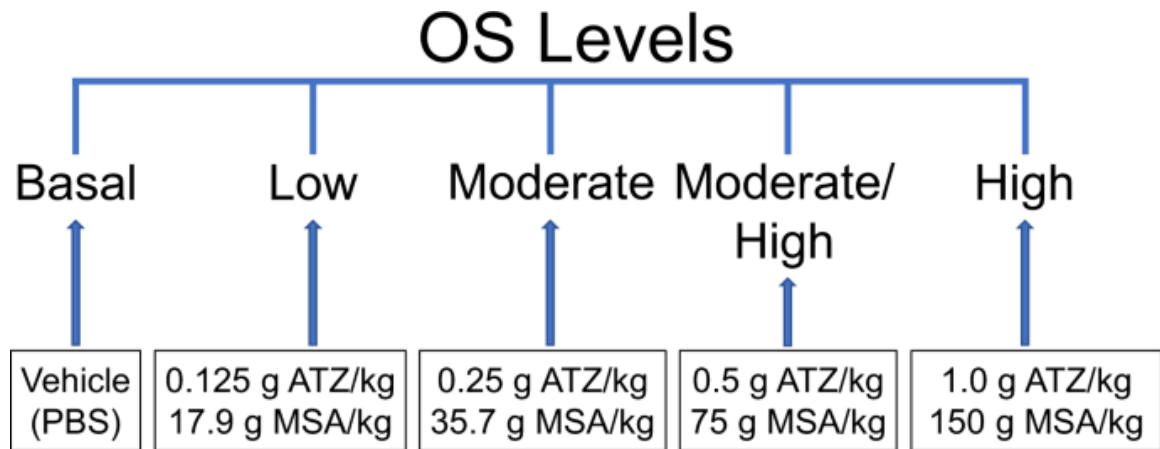
Figure 1. Treatment of the wounds to cause different levels of OS. Different doses of ATZ and MSA, which inhibit catalase and GPx, respectively, were used to induce different levels of OS in the wound. Basal OS levels represents the natural level of OS induced by wound healing in the diabetic mice model *db/db$^{-/-}$*.
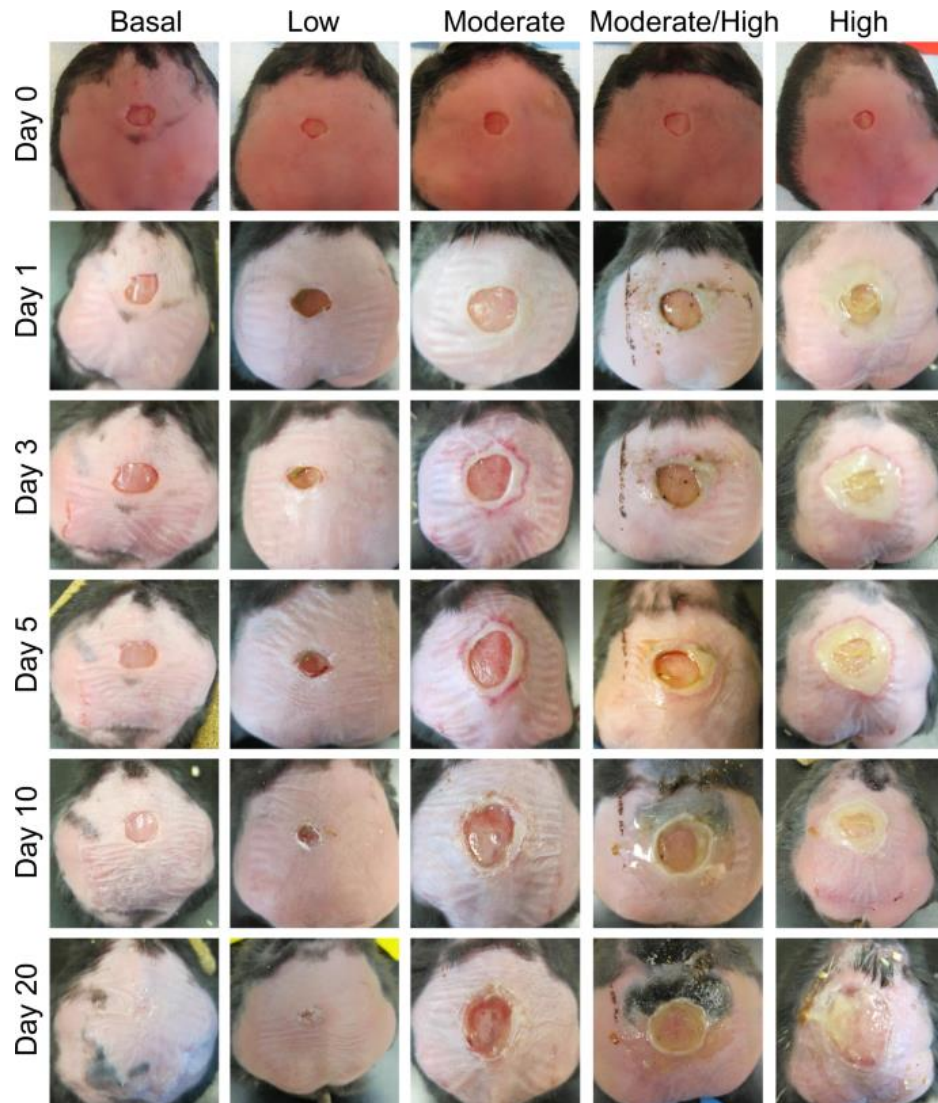
Figure 2. Dose-dependent effects of OS on levels of wound chronicity. Wound healing in *db/db*$^{-/-}$ mice is delayed and impaired as OS levels are increased. To create chronic wounds, a full thickness wound is made with a 7 mm biopsy punch and the inhibitors of antioxidant enzyme catalase and GPx applied at the time of wounding. We studied the effects of various levels of OS in the wound tissue: *Basal levels of OS*: Wounds were treated only with vehicle; the wounds were able to close within 20 days and have no biofilm formation. *Low levels of OS*: Wounds were treated with low dose of inhibitors; wound healing is slightly delayed but still occurs normally. *Moderate levels of OS*: Wounds were treated with moderate dose of inhibitors; wound closure is delayed with tissue margins damaged by OS, but re-epithelialization occurs, and the granulations tissue forms and biofilm can form. *Moderate to high levels of OS*: Wounds were treated with higher doses of inhibitors: Significant damage to the tissue is sustained, wound healing is delayed, and wounds are colonized by bacteria that form aggressive biofilm in the wound.
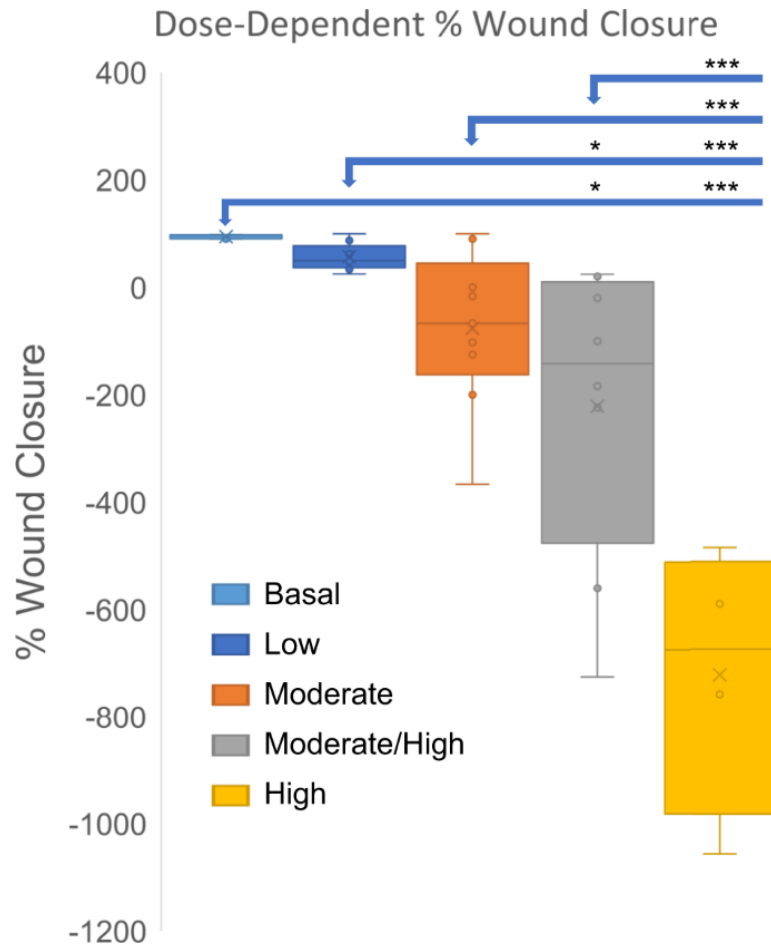
Figure 3. Wound closure is delayed in wounds with higher OS levels. Percent wound closure was calculated by measuring the area at wounding and 20 days after wounding. The wounds with lower levels of OS healed significantly faster than wounds with higher levels of OS. Chronic wounds with the full dose of inhibitors did not heal and had abundant biofilm formation. Wound area results were compared and analyzed using one way ANOVA, followed by Bonferroni's multiple-comparison test to determine significant differences between groups. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. Basal OS, n=6, low OS, n= 9, moderate OS, n= 8, moderate/high, OS n= 9, high OS, n=5.

	Basal	Low	Moderate	Moderate/High
H&E	A	B	C	D
MT	E	F	G	H
PSR	I	J	K	L
Col IV	M	N	O	P
Col IV	Q	R	S	T
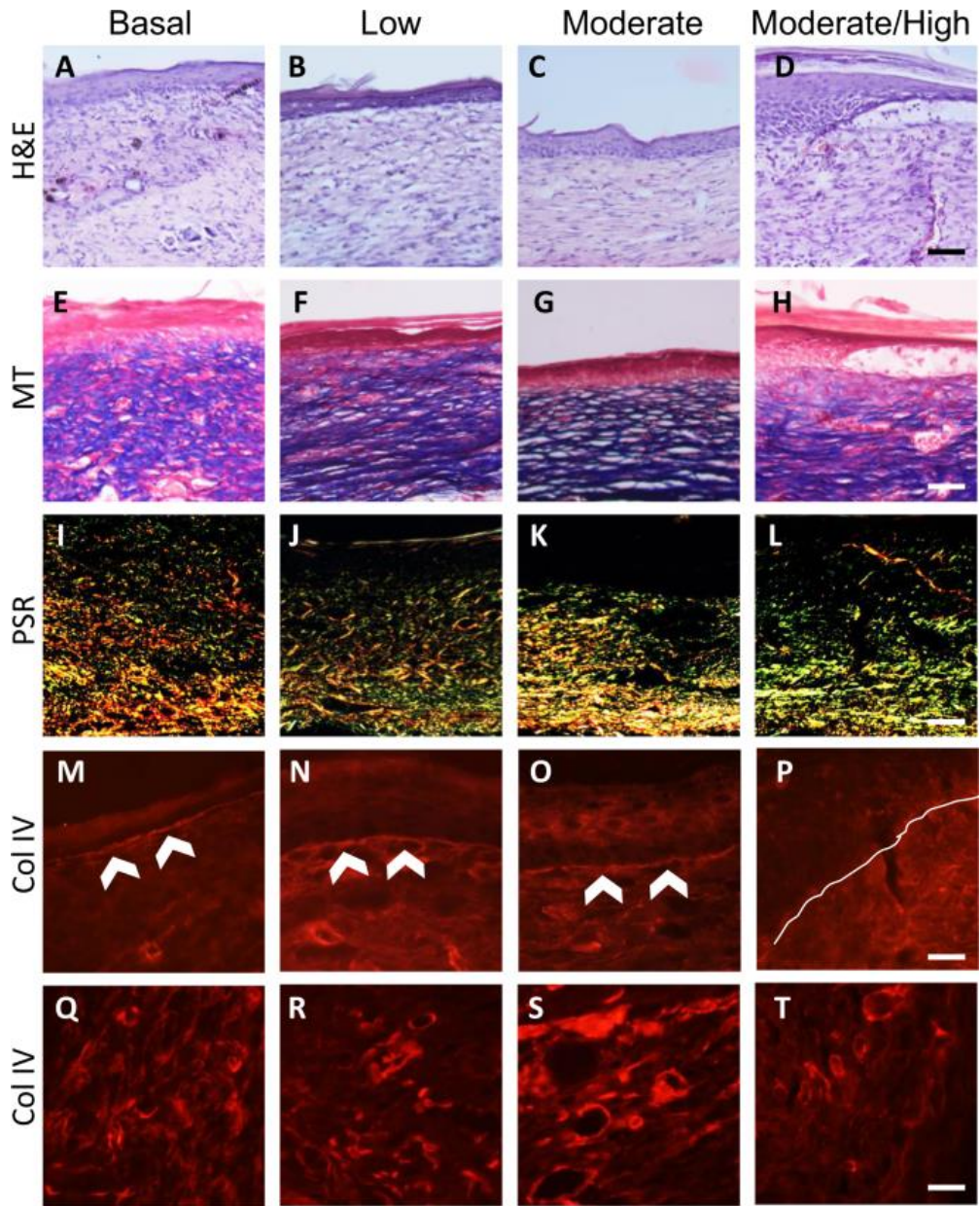
Figure 4. Dose-dependent effects of OS on the quality of wound tissue after wound closure. Cryosections of the skin were taken from wound tissue at the time of complete closure in each basal, low, moderate, and moderate/high levels of OS in the wound tissue. Wounds with high OS levels do not heal so histology was not performed at wound closure. (**A-D**) H&E staining of sections of the wound after closure shows formation of an epidermis and quality of the granulation tissue decreasing as levels of OS increased. Scale bar = 100 μm. (**E-H**) MT staining of wounds show collagen deposition in the granulation tissue after closure. Scale bar = 100 μm. (**I-L**) Picrosirius red staining of wounds show the different composition and location of col I (red) and col III (green) under cross polarizing light. Areas with both col I and col III are shown in orange-yellow. Scale bar = 100 μm. (**M-P**) Immunofluorescent staining for col IV shows col IV presence in the basal lamina underneath the epidermis. White arrows indicate the basal lamina formed below the basal cells of the epidermis. The white line in (**P**) A is drawn under where the basal lamina should have formed in wounds with moderate/high levels of OS but is lacking even though the wound has undergone re-epithelialization. Scale bar = 20 μm. (**Q-T**) Images show staining for col IV around the microvessels in the granulation tissue. The density of blood vessels is significantly lower in the wounds with moderate to high level of OS. Scale bar = 20 μm.
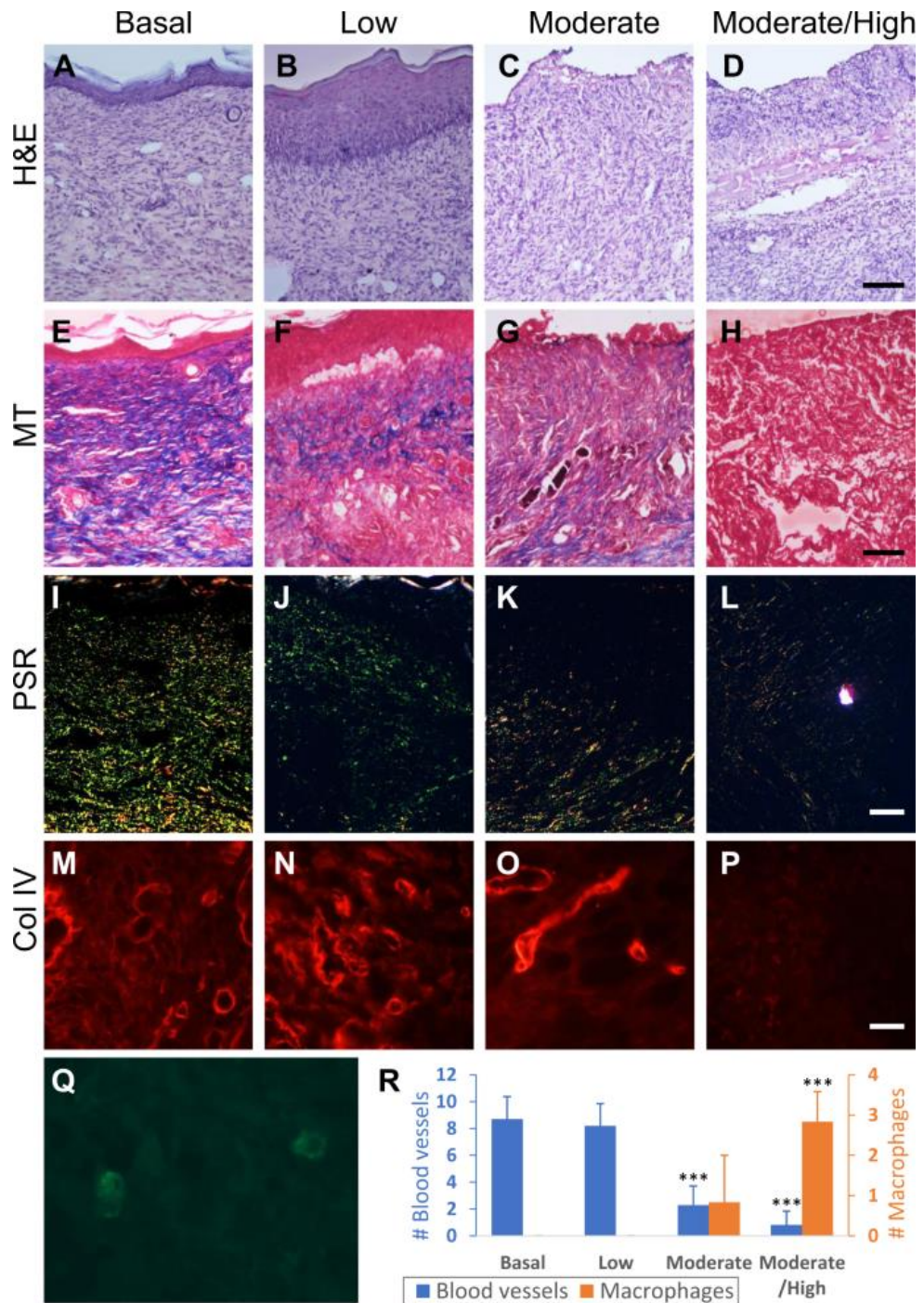
Figure 5. Dose-dependent effects of OS on the quality of wound tissue when the wounds are still opened. Cryosections of the skin were taken from basal, low, moderate, and moderate/high levels of OS wound tissue, 17 days after wounding. (**A-D**) H&E staining of sections of the wound at day 17 shows development of an epidermis in wounds with basal and low OS levels. Higher levels of OS in the wound tissues resulted in the wounds being still opened. Scale bar = 100 μm. (**E-H**) MT staining of wounds show collagen deposition in the granulation tissue. Dark blue collagen fibers are present in wounds with basal levels of OS. Wounds with low levels of OS showed that the dermal/epidermal junction was weak and the interstitial collagen very sparse. When the wound tissues contained more than moderate levels of OS, interstitial collagen in the wound was absent and the wounds were still opened. Scale bar = 100 μm. (**I-L**) Picrosirius red staining of wounds showed col I (red) and col III (green) under cross polarizing light. Areas with both col I and col III are shown in orange-yellow. Wounds with basal OS levels have both col I and col III in the wound tissue whereas wounds with higher OS levels have very little of each col I and col III. Scale bar = 20 μm. (**M-P**) Immunofluorescent staining for col IV shows the presence of blood vessels in the granulation tissue. No blood vessels can be found in wounds with moderate/high levels of OS. Scale bar = 20 μm. (**Q**) Staining for F4/80 showed macrophages in the granulation tissue of wounds with higher levels of OS. (**R**) The number of blood vessels and macrophages were counted in 10 and 6 frames (area = 0.02 mm^2), respectively, of the granulation tissue. Blood vessel and macrophage results were compared and analyzed to basal level OS wounds using one way ANOVA, followed by Dunnett's test. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.
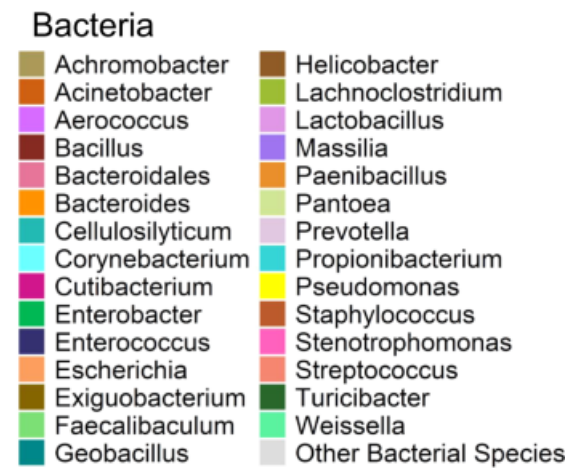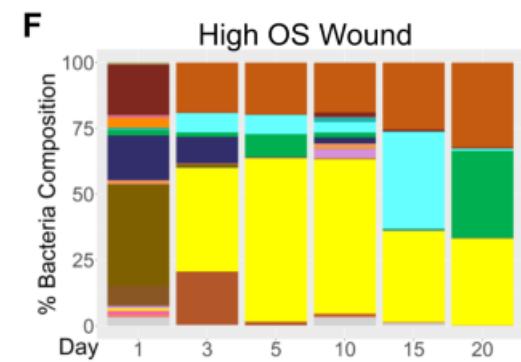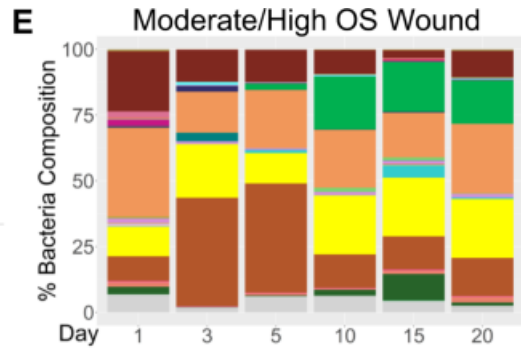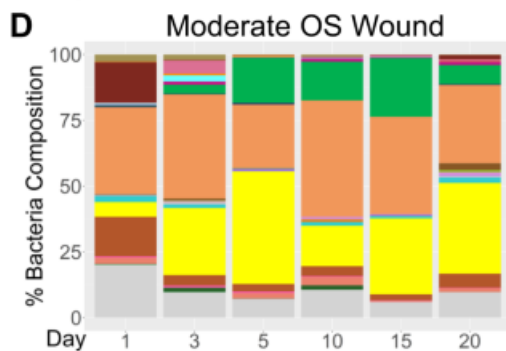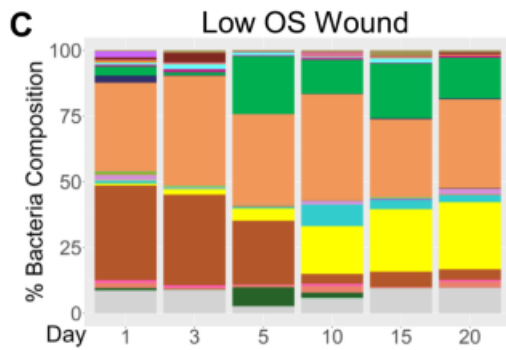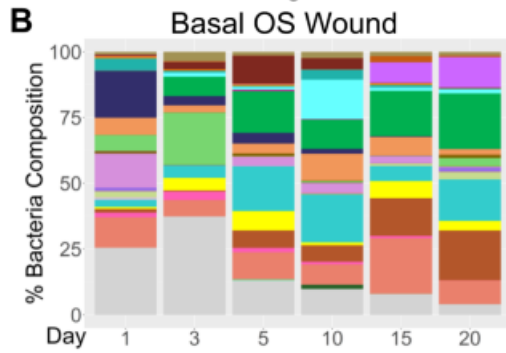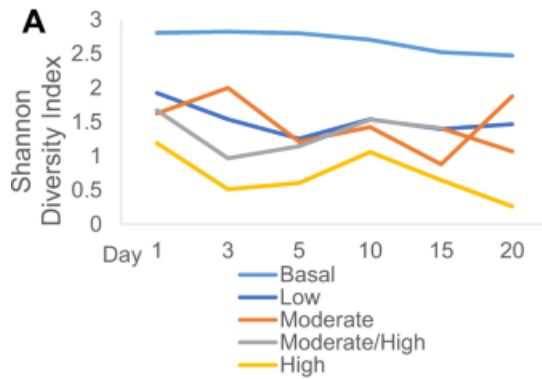
Figure 6. Effects of increasing levels of OS on wound bacteria over time. This analysis was conducted by sequencing the rRNA ITS of DNA isolated from wound swabs. Over 1400 unique OTUs were identified; only the top 30 genus level OTUs were plotted as mean % compositions for each treatment group; the remaining OTUs were combined under "Other Bacterial Species" category. (**A**) Diversity of bacterial microbiome in the wound is affected by OS in a dose dependent manner. Diversity considers species diversity and abundances, not just species richness. Alpha diversity measured with Shannon's index shows that diversity among doses is significant ($p < 0.0001$). (**B**) Wounds with basal levels of OS show a diverse bacterial profile throughout wound healing. The same bacterial species are found at almost every timepoint, but their percent composition of the wound is dynamic. (**C**) Wounds with low levels of OS are populated with similar bacteria throughout the profile which includes *E. coli*, *P. aeruginosa, S. xylosus* and *E. cloacae*. (**D**) Wounds with moderate levels of OS have wound profiles similar to wounds with low levels of OS; however, the population of *S. xylosus* is lower and *P. aeruginosa* can be found present in higher proportions earlier in wound development. (**E**) Wounds with moderate/high levels of OS have microbiomes colonized by the same biofilm-forming bacteria as in B and C but with more *S. xylosus* present in the earlier times. (**F**) Wounds with high level of OS can be dominated by more aggressive biofilm-forming bacteria, such as *P. aeruginosa* and *A. johnsonii*. Their colonization begins early and quickly expand their population to outcompete other bacteria in the wound. Basal OS, n=6, low OS, n= 9, moderate OS, n= 8, moderate/high, OS n= 9, high OS, n=5.
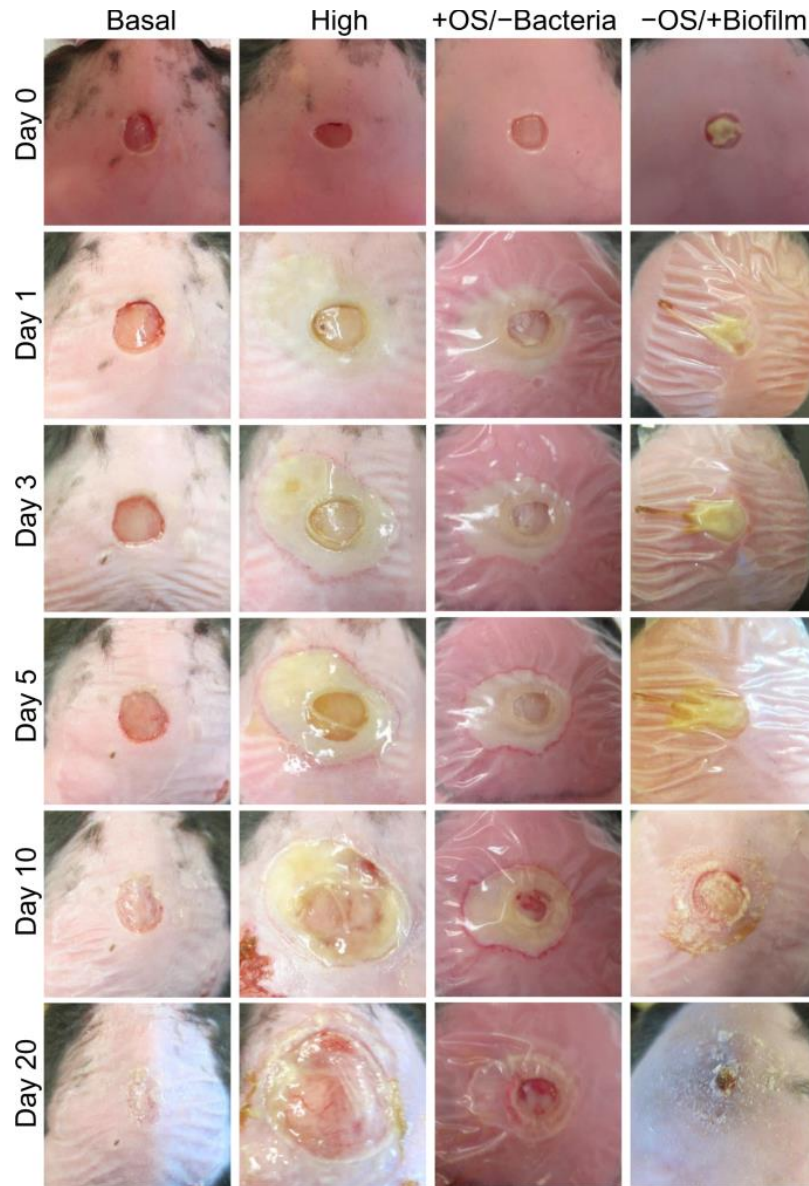
Figure 7. Both high levels of OS and biofilm-forming bacteria are needed to create chronic wounds. To test whether OS and bacteria are critical for chronic wound formation, wounds were either cleaned to remove skin microbiome or transplanted with chronic wound biofilm in the presence or absence of OS. *Basal levels of OS*: wounds heal within 20 days and do not form biofilm. *High levels of OS*: wounds develop into chronic wounds with biofilm formation. *+OS/-Bacteria*: When high levels of OS were induced with skin microbiome removed, the tissue sustained damage from OS, but the wound did not develop into a chronic wound. *-OS/+Biofilm*: When wound tissues were transplanted with mature biofilm from other chronic wounds in the absence of additional OS, the biofilm breaks down and the wound heals much like wounds with basal OS levels.
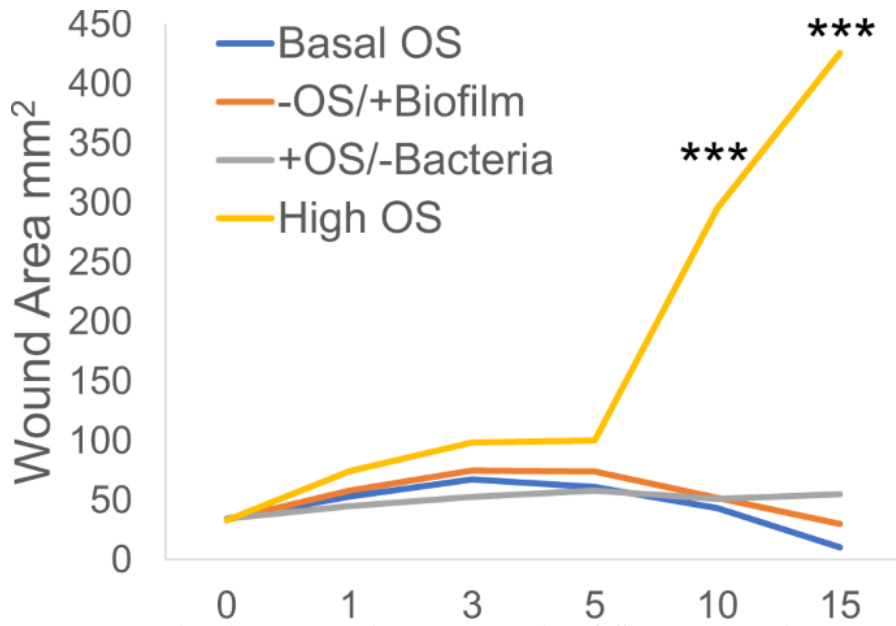
Figure 8. Wound healing is delayed in wounds with OS and bacteria. Measurements of the wound area shows that the area of wound with either OS or biofilm do not increase significantly like wounds with high levels of OS**.** Wound area results were compared and analyzed using one way ANOVA, followed by Dunnett's test to determine significant differences between wound areas to basal OS levels. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. Basal OS, n=5, +OS/-Bacteria, n=4, -OS/+Biofilm, n=7, high OS, n=4.
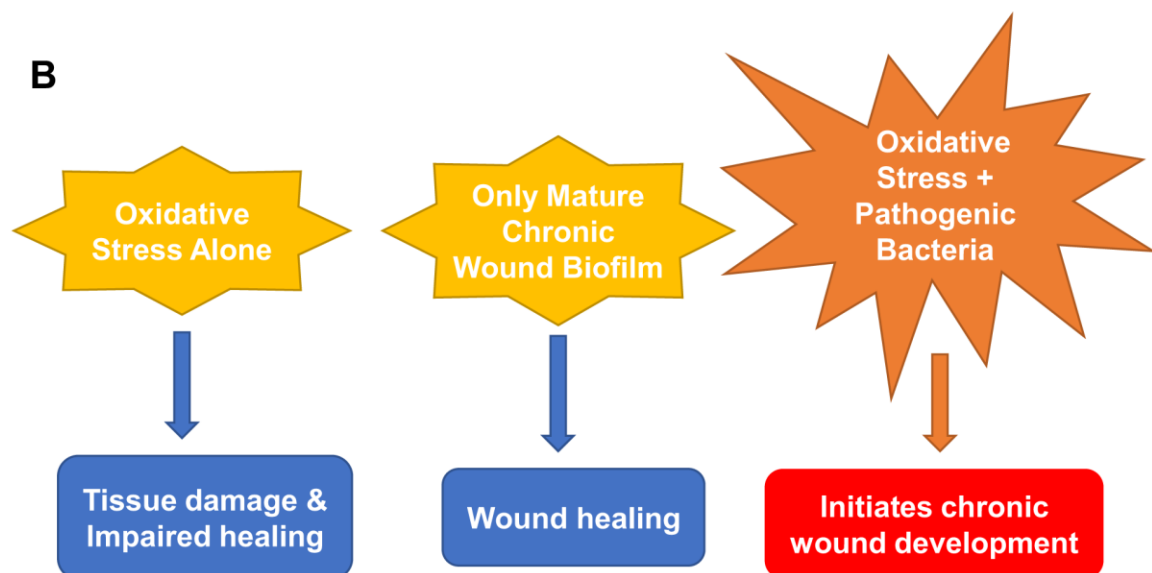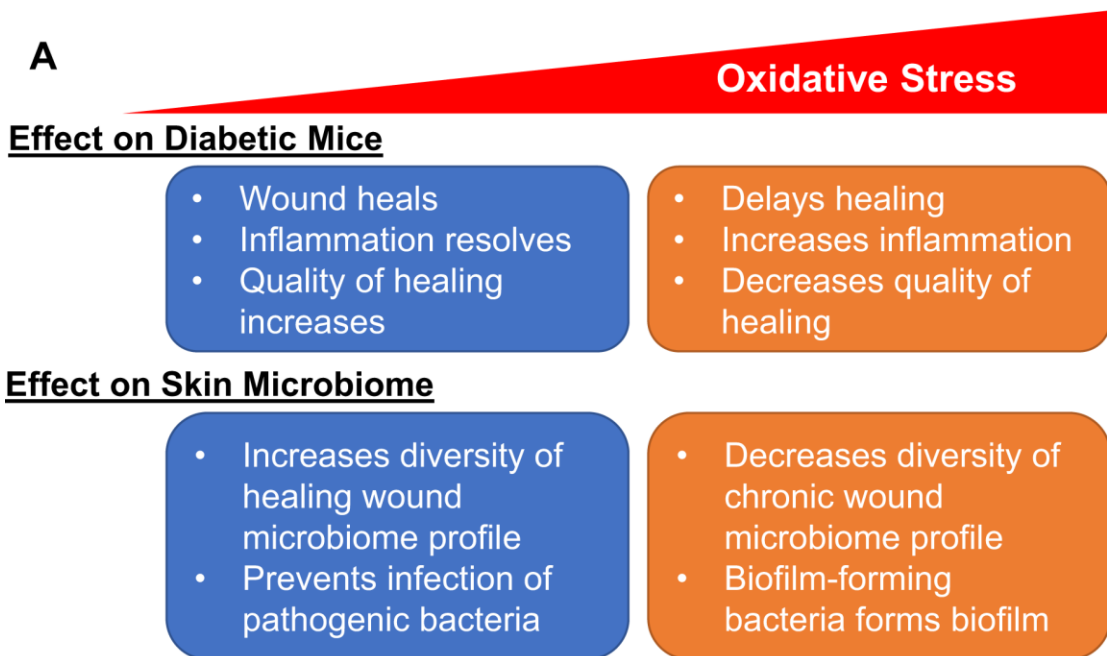
Figure 9. Schematic summary of the effects of OS on wound healing and the bacterial microbiome. (**A**) OS has a significant effect on both the diabetic mouse and the skin bacteria that lead to the development of chronic wounds in the mouse in a dose dependent manner. (**B**) OS and chronic wound biofilm alone are not sufficient to create chronic wounds. Only when both OS and skin bacteria are present does a chronic wound development.

Chapter 2

High Levels of Oxidative Stress Create a Microenvironment that Significantly

Decreases the Diversity of the Microbiota in Diabetic Chronic Wounds and

Promotes Biofilm Formation

Abstract

Diabetics chronic wounds are characterized by high levels of oxidative stress (OS) and are often colonized by biofilm-forming bacteria that severely compromise healing and can result in amputation. However, little is known about the role of skin microbiota in wound healing and chronic wound development. *We hypothesized* that high OS levels lead to chronic wound development by promoting the colonization of biofilm-forming bacteria over commensal/beneficial bacteria. To test this hypothesis, we used our *db/db^{-/-}* mouse model for chronic wounds where pathogenic biofilms develop naturally after induction of high OS immediately after wounding. We sequenced the bacterial rRNA internal transcribed spacer (ITS) gene of the wound microbiota from wound initiation to fully developed chronic wounds. Indicator species analysis, which considers a species' fidelity and specificity, was used to determine which bacterial species were strongly associated with healing wounds or chronic wounds. We found that healing wounds were colonized by a diverse and dynamic bacterial microbiome that never developed biofilms even though biofilm-forming bacteria were present. Several clinically relevant species that are present in human chronic wounds, such as *Cutibacterium acnes*, *Achromobacter* sp., *Delftia* sp.*,* and *Escherichia coli*, were highly associated with healing wounds. These bacteria may serve as bioindicators of healing and may actively participate in the processes of wound healing and preventing pathogenic bacteria from colonizing the wound. In contrast, chronic wounds, which had high levels of OS, had low bacterial diversity and were colonized by several clinically relevant, biofilm-forming bacteria such as *Pseudomonas aeruginosa*, *Enterobacter cloacae, Corynebacterium*

frankenforstense, and *Acinetobacter* sp. We observed unique population trends: for example, *P. aeruginosa* associated with aggressive biofilm development, whereas *Staphylococcus xylosus* was only present early after injury. These findings show that high levels of OS in the wound significantly altered the bacterial wound microbiome, decreasing diversity and promoting the colonization of bacteria from the skin microbiota to form biofilm. ***In conclusion***, bacteria associated with non-chronic or chronic wounds could function as bioindicators of healing or non-healing (chronicity), respectively. Moreover, a better understanding of bacterial interactions between pathogenic and beneficial bacteria within an evolving chronic wound microbiota may lead to *better solutions for chronic wound management.*

Introduction

The human skin is an important organ that functions as an interface between the human body and the environment. The skin is inhabited by a complex and diverse community of microbes whose impact on health and disease is still under investigation[1–3]. The skin microbiota consists of both benign commensal bacterial species and opportunistic pathogens that are able to cause infection and disease if not controlled[3]. Commensal bacteria have recently been shown to stimulate the adaptive and innate immune system and directly or indirectly prevent pathogens from causing infections[3–6].

Long-term bacterial infections are a characteristic of chronic wounds. These wounds develop when regulation of wound healing processes is defective or does not occur. Chronic wounds are characterized by high levels of oxidative stress (OS) and chronic inflammation that cause extensive damage to the host tissue. This is due to a continuous influx of inflammatory cells that release cytotoxic enzymes, increasing free oxygen radicals and resulting in cell death[7–10]. The healing process for chronic wounds is complicated by pathogenic bacteria which take advantage of host nutrients that are leeched in the destructive inflammatory microenvironment and contribute to the damaging of the host tissue when they form biofilm[9,11–16]. These biofilms are recalcitrant to conventional antibiotic therapies because the structure of the biofilm decreases the efficacy of antibiotic therapy by significantly decreasing their diffusion rate. The biofilm extracellular polymeric substance (EPS) also helps the bacteria in the biofilm evade the host innate and adaptive immune system[17–19].

Bacterial biofilms are composed of a matrix of EPS, extracellular DNA, proteins/peptides, and lipids surrounding bacterial cells that are attached to abiotic and biotic surfaces[12,16,20–22]. *In vivo*, biofilms are composed of a multitude of aerobic and anaerobic bacterial species across the phylogenetic tree that aggregate into sessile microcolonies[23]. Bacteria within the biofilm differ significantly from their planktonic counterparts in their morphology, mode of communication, and metabolism[24,25]. The biofilm provides a unique environment to facilitate bacterial cell-to-cell signaling by the production of and detection of quorum-sensing molecules, which promote collective behavior such as optimizing nutrient acquisition and regulation of virulence, leading to sustained pathogenicity in the wound[7,16]. At the same time, stochastic processes and nonuniform gene expression can lead to the appearance of subpopulations of bacteria with different phenotypes and environmental responses[7].

Interactions between different bacterial species are very complex in both biofilm and free-living states. In the presence of biofilm-forming bacteria, commensal bacteria can be out-competed for resources and eradicated[26]. Similarly, benign colonizers of normal skin microbiota can participate in multi-species biofilm production and sustain a high burden of infection[27]. Biofilms in chronic wounds are complex, capable of harboring many species of bacteria, each with very different nutritional demands and roles[28,29]. Common wound-associated bacteria such as *Staphylococcus*, *Streptococcus*, and *Pseudomonas* can produce exotoxins that cause broad damage to host tissue, destruction of host cells, and disruption of normal cellular metabolism leading to further tissue

necrosis[27]. An increased burden of infection can increase the risk of amputation needed to stop the spread the infections in patients with chronic wound[30,31].

Chronic wounds in humans can develop when any process of the wound healing program, which includes homeostasis, inflammation, proliferation, and remodeling, is disrupted or impaired. Patients with one or more underlying pathological conditions, such as metabolic diseases, are particularly at risk for developing chronic wounds[31]. For example, injuries in Type II diabetes patients can develop into chronic wounds; in the foot, these are called diabetic foot ulcers (DFUs)[32]. With over ~30 million Americans with diabetes, DFUs are a major health concern and cost the US health care industry $13 billion a year, since approximately 25% will experience a foot ulcer during their lifetime[19,33,34]. DFUs must be treated frequently and diligently because they are commonly infected with bacterial biofilms that prevent healing. Amputation of the lower extremities may be necessary if the wound is unable to heal and the infection and biofilm cannot be controlled. These patients have a 50% mortality risk within 5 years of amputation, attributed to the pathophysiology of diabetes and other co-morbidities such as obesity[35].

To investigate how bacteria participate in chronic wound development, we used our murine *db/db*^{-/-} diabetic chronic wound model[18,36]. This model is characterized by obesity, diabetes, chronic inflammation and lack of angiogenesis that result in impaired healing. To create the chronic wounds, we treated the mice once, immediately after wounding, with inhibitors of the antioxidant enzymes catalase and glutathione peroxidase. The inhibition of these two enzymes results in high levels of OS. In the

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db/db^{-/-} chronic wounds, biofilm starts to form around three days after wounding and induction of OS in the wound tissue. The wounds became fully chronic within 20 days and contained mature biofilm, comprised of bacteria and extracellular polymeric substance[18,37], also found in human diabetic chronic wounds.

We have previously shown that OS levels correlate inversely with the levels of diversity of the microbiota in the wound using the Shannon diversity index. This index measures both the species richness (number of taxa) and evenness (relative abundances) of each of the species. Low indices indicate lower diversity, found typically in infections (e.g., one microorganism dominates and causes disease). High indices indicate higher diversity, found typically in stable, healthy communities. We found that the level of OS significantly contributed to a difference in Shannon diversity. The greatest difference in diversity was found between wounds with basal to low levels of OS and wounds with high levels of OS. The former showed high diversity and the latter low diversity[36].

Based on these findings, *we hypothesized* that high levels of OS create a microenvironment that significantly decreases the diversity of the microbiota in diabetic chronic wounds and stimulates biofilm-forming bacteria to grow and form biofilm. We show here that in non-chronic wounds, the microbiota of the wound is as diverse as that of the skin and the bacteria do not form biofilm even though biofilm-forming bacteria are present in the wound during healing. In contrast, chronic wounds have much less bacterial diversity in comparison with the microbiota of the skin and are strongly colonized by biofilm-forming bacteria. These findings suggest that bacteria found in non-

chronic wounds may benefit or assist in wound healing, and/or participate in the exclusion of pathogenic biofilm-producers.

Materials and Methods

Reagents: 3-Amino-1,2,4-triazole (ATZ) from Tokyo Chemical Industry Co., Ltd. (Portland, OR). Mercaptosuccinic acid (MSA) from Sigma-Aldrich (St. Louis, MO). Buprenorphine from Henry Schein (Dublin, OH). Isoflurane from Henry Schein (Dublin, OH). Tegaderm Film 1624W from 3M (Maplewood, MN).

Chronic Wound Model: All experiments were completed in accordance and compliance with federal regulations and University of California policy and all procedures have been approved by the University of California, Riverside Institutional Animal Care and Use Committee (IACUC). The detailed description of how to obtain chronic wounds in *db/db*^{-/-} mice has been previously published by us[18,38]. Briefly, *db/db*^{-/-} mice were bred in house from B6.BKS(D)-*Lepr*[db]/J heterozygotes catalog #000697 from the Jackson Laboratories. The mice were housed in a conventional non-barrier vivarium and after wounding they were housed in separate cages. Control and experimental groups were age-matched and both males and females were used in the study indiscriminately because both genders form chronic wounds similarly[39,40]. A total of 77 mice were used for this experiment. Only 5–6-month-old *db/db*^{-/-} that weighed at least 50g were used. The hair was cut short with an electric shaver and then removed with a chemical depilatory 24 hr prior to wounding**.** A wound was created on the back of *db/db*^{-/-} mice by first gently wiping the skin with 70% ethanol and then excising one 7-mm full-thickness skin biopsy punch under isoflurane anesthesia. Buprenorphine, an analgesic, was administered by

intraperitoneal injection at 0.05mg buprenorphine/kg of mouse weight in sterile

phosphate buffer solution (PBS), given 20 mins before surgery and 6 hr after the surgery.

Tegaderm, which is a gas permeable bandage but cannot be penetrated by bacteria, was

applied firmly to prevent contamination of the wound site. A non-chronic wound was

created by treating the wound with the vehicle PBS. To create chronic wounds, OS in the

wound tissue was increased by using specific inhibitors for catalase, ATZ, and

glutathione peroxidase, MSA. ATZ was injected intraperitoneally at 1g ATZ/kg of mouse

weight in sterile PBS approximately 20 min before surgery. MSA was administered

topically onto the wound between the Tegaderm and the wound site at 150mg MSA/kg of

mouse weight in sterile PBS 10 min after surgery.

Bacterial Sampling and DNA Extraction: Bacterial samples were collected from

wounds with a sterile cotton swab via the Levine method[41]. Briefly, the Tegaderm was

removed, and a sterile cotton swab was rolled around $1cm^2$ in the center of the wound for

10 seconds. A new piece of Tegaderm was placed securely on the wound and skin after

sample collection. Bacterial swabs were collected at 0, 1, 2, 3, 5, 10, 15, and 20 days after

wounding (D0, D1, D2, etc., respectively). The swabs were stored dry, without freezing

media, in sterile microcentrifuge tubes at -80°C until DNA extraction. DNA extractions

were performed on thawed swabs using the MOBio PowerSoil DNA Isolation Kit (which

became the Qiagen PowerSoil DNA Isolation Kit) as described by the manufacturer, with

a 90 s bead-beating step.

Bacterial rRNA Internal Transcribed Spacer (ITS) Analysis: Illumina bacterial rRNA ITS gene libraries were constructed as follows: PCRs were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA) as 25μl reactions containing, 50 mM Tris (pH 8.3), bovine serum albumin (BSA) at 500μg/ml, 2.5mM $MgCl_2$, 250μM of each deoxynucleotide triphosphate (dNTP), 400nM of the forward PCR primer, 200nM of each reverse PCR primer, 2.5μl of DNA template and 0.625 units JumpStart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO). PCR primers targeted a portion of the small-subunit (ITS-1507F, GGTGAAGTCGTAACAAGGTA) and large-subunit (ITS-23SR, GGGTTBCCCCATTCRG) rRNA genes and the hypervariable ITS region[42], with the reverse primers including a 12-bp barcode and both primers including the sequences needed for Illumina cluster formation; primer binding sites were the reverse and complement of the commonly used small-subunit rRNA gene primer 1492R[43] and the large-subunit rRNA gene primer 129F[44]. PCR primers were only frozen and thawed once. Thermal cycling parameters were 94°C for 5 min; 35 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 40 s; followed by 72°C for 10 min. PCR products were purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Bacterial ITS Sequencing and Bioinformatics: DNA sequencing (single-end 150 base) was performed using an Illumina MiSeq (Illumina, Inc., San Diego, CA). Clusters were created using template concentrations 2.5 pM and PhiX at 107 K/mm^2. Data processing was performed with USEARCH v10.0[45]. The UPARSE pipeline for de-multiplexing, length trimming, quality filtering and operational taxonomic unit (OTU) picking using

default parameters or recommended guidelines that were initially described[46] and which

have been updated at https://www.drive5.com/usearch/manual10/uparse_pipeline.html.

Briefly, after demultiplexing and using the recommended 1.0 expected error threshold,

sequences were trimmed to a uniform length of 149 bp and then dereplicated.

Dereplicated sequences were subjected to error-correction (denoised) and chimera

filtering to generate zero-radius operational taxonomic units (ZOTUs) using UNOISE3[47].

An OTU table was then generated using the otutab command. ZOTUs having non-

bacterial DNA were identified and enumerated by performing a local BLAST search[48] of

their seed sequences against the nucleotide database. ZOTUs were removed if any of

their highest scoring BLAST hits contained taxonomic IDs within the Rodent family,

Fungal or Viridiplantae kingdoms, or PhiX. Taxonomic assignments to bacterial ZOTUs

were made by finding the lowest common taxonomic level of the highest BLAST hits

excluding unclassified designations. Data were normalized within each sample by

dividing the number of reads in each OTU by the total number of reads in that sample.

The bacterial rRNA ITS sequences have been deposited in the National Center for

Biotechnology Information (NCBI)'s Sequence Read Archive (SRA) under the

BioProject Accession Number PRJNA623025.

Statistics: Alpha diversity using the Shannon index was calculated with Qiime (version

1.9.1). One-way ANOVA in R (version 3.6.1) was used to calculate the significance of

the bacterial alpha diversity in chronic and non-chronic wounds. Indicator species

analysis[49] was used to classify bacterial species that were highly associated with either

non-chronic wounds or chronic wounds. Calculations for the indicator values were done

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with indicspecies (version 1.7.6)[50]. Statistical significance of the indicator values was tested with permutation tests using permute (version 0.9-5).

Results

Bacterial colonization of non-chronic and chronic wounds

We sampled the microbiome from 77 mice with either non-chronic or chronic wounds from wounding to 20 days post-wounding (**Fig. 1**). The non-chronic wound cohort consisted of 40 mice and the chronic wound cohort consisted of 37 mice. The analysis of the wound microbiome was conducted by sequencing the ITS rRNA of the bacterial genome, a region of nucleotides that was flanked by the 16S and 23S rRNA genes. After 20 days, mice treated with placebo (PBS) undergo re-epithelization and heal. During the healing of non-chronic wounds, we observed a clear exudate, indicating the lack of biofilm formation and the analyses of the microbiota in the wound over time showed a diverse microbiome very similar to that of undamaged skin (data not shown). Conversely, wounds in mice treated with inhibitors for antioxidant enzymes (chronic wounds) showed yellowish fluid and thick biofilm on the wound and exhibited reduced microbiome diversity. Indeed, the microbiota was composed of a few bacteria species which are known to form strong biofilm.

Over 50 million counts were categorized, representing ~8000 OTUs with ~1470 uniquely identified species. Three major phyla, *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, comprised a large majority of the bacteria in non-chronic and chronic wounds (**Fig. 2**). The average proportion of bacteria in each phylum remained relatively the same in non-chronic wounds; however, the proportion of the phyla changed in

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chronic wounds. The *Proteobacteria* population of bacteria in chronic wounds began to increase starting from D2 as the population of bacteria from *Actinobacteria* and *Firmicutes* decreased. Major contributors to the *Proteobacteria* populations were *Pseudomonas aeruginosa* and *Enterobacter cloacae*. They were found in approximately 23 non-chronic wounds and 18 chronic wounds and approximately 39 non-chronic wounds and 34 chronic wounds, respectively. *Achromobacter*, also belonging in the *Proteobacteria* group, was found in approximately 21 non-chronic and 10 chronic wounds. A *Firmicutes* species, *Staphylococcus xylosus,* was present in approximately in 36 non-chronic wounds 25 chronic wounds.

The collection of wound swabs was collected over approximately 2 years. About halfway through the sample collection, stricter biosecurity regulations were imposed on the conventional vivarium that housed the *db/db*$^{-/-}$ mice. In normal vivarium conditions, the mouse skin microbiota contained high percentage of *P. aeruginosa* in the bacterial population whereas in the more stringent conditions the mice skin contained no more than 3% of *P. aeruginosa* in the bacterial population in the wound microbiota. In the former cohort, we analyzed the progression of the microbiota in 20 mice with non-chronic wounds and 19 mice with chronic wounds. In the latter cohort, we analyzed the skin microbiota of 20 mice with non-chronic wounds and 18 mice with chronic wounds for a total of 39 mice in normal condition of the vivarium and 38 mice in more stringent conditions. We describe our findings below in **Figs. 3-6**.

Bacterial colonization of chronic wounds when *P. aeruginosa* was significantly present in the microbiota

At the species level, non-chronic wounds were populated with a diverse mixture of bacteria during wound healing (**Fig. 3A**). Biofilm-forming bacteria, such as *P. aeruginosa*, *E. cloacae*, and *S. xylosus,* were detected starting from D0 (skin microbiota) through D20. In these non-chronic wounds, *P. aeruginosa* was present in the wound as the wound healed but never at more than 18% of the bacterial population. *E. cloacae* on the other hand represented more than 25% of the bacterial population in wounds at D0, D3, D5, D10, D15 and D20. *S. xylosus* constituted less than 10% of the population all through wound healing. Despite the presence of these three biofilm-forming bacteria in the non-chronic wounds, biofilm never formed in the wounds of these mice, and the wounds went on to heal and close in ~20 days. Other common bacteria found throughout the healing of non-chronic wounds were *Corynebacterium frankenforstense*, *Acinetobacter* sp. and *Staphylococcus epidermis* at very low relative abundance. These bacterial species have the potential to also form biofilm, but do not in healing non-chronic wounds. Many other bacterial species were found throughout the course of healing, including *Aerococcus urinaeequi*, *Enterococcus gallinarum*, *Cutibacterium acnes*, *Massilia oculi*, *Escherichia coli*, *Lactobacillus crispatus*, *Lactobacillus murinus*, and *Lactobacillus johnsonii.*

When wounds were treated with inhibitors for catalase and glutathione peroxidase to induce chronicity, a dynamic shift was observed in the wound-associated bacterial population (**Fig. 3B**). Whereas the population of *P. aeruginosa* was less than 20% of the

wound associated community within the first 3 days after wounding, the population

increased to greater than 50% by D15 and continued to increase to D20. *E. cloacae*

represented 20-30% of the wound-associated bacteria when *P. aeruginosa* was abundant.

We measured the alpha diversity of wound-associated communities using the

Shannon diversity index (**Fig. 3C**), a measure of both species' richness (number of taxa)

and evenness, which is a measure of the relative abundances of each of the species. Low

indices indicate lower levels of diversity, found typically in infections (e.g., one

microorganism dominated and caused disease). High indices indicate higher levels of

diversity, found typically in stable, healthy communities. This analysis showed that

diversity in non-chronic wound microbiome was high in comparison to the chronic

wound microbiome (p-value < 0.0001). After wounding, the diversity in non-chronic

wounds decreased between D0 and D10. As the wound began to heal, the diversity of the

microbiome began to increase again. Conversely, the bacterial diversity of chronic

wounds with high levels of OS exhibited a precipitous decrease. By D3, once biofilm

production was observed, the bacterial diversity in the wound continued to decrease and

did not recover.

Whereas relative abundance measurements showed general trends in bacterial

population dynamics, striking inter-individual trends in bacterial communities were

observed, despite the mice having the same genetic background and living in the same

environment (**Fig. 4**). Non-chronic wounds were colonized by many different species

with the entire community exhibiting dynamic day-to-day variations (**Fig. 4A-C** shows

examples of wounds from three mice). The wounds of these mice healed well and in a

timely manner. In one mouse, temporary blooms of *S. xylosus* and *E. cloacae* at D1, D2, D15, and D20 were observed (**Fig. 4A**). In another mouse (**Fig. 4B**), *P. aeruginosa,* whose population exceeded 25% of the bacterial population in D2 and even more in D10, was unable to dominate in the wound in the absence of high OS levels. No biofilm formation was observed (**Fig. 4B**). In a third mouse, *P. aeruginosa* could colonize the non-chronic wound with ~98% relative abundance yet was unable to become biofilm-forming without high levels of OS (**Fig. 4C**). Note that this profile was very similar in composition and trend of a chronic wound containing *P. aeruginosa* biofilm (**Fig. 4F**).

Analysis of the bacterial composition in individual mice with chronic wounds showed evidence of bacterial interactions between *S. xylosus*, *E. cloacae*, and *P. aeruginosa* (**Fig. 4D, E, F**). In these three examples, chronic wounds were strongly populated with *S. xylosus* and *E. cloacae;* however, these bacterial species were unable to compete for dominance when *P. aeruginosa* was present. In the first example, the chronic wound was dominated by *S. xylosus* starting from D2. Sometime between D15 and D20, there was a change in the dominating bacteria. Within 5 days, *P. aeruginosa* entirely out-competed *S. xylosus* from the wound bed (**Fig. 4D**). In the second chronic wound, *E. cloacae* dominated the wound-associated community at D1. Starting from D5, *E. cloacae* was rapidly overtaken by *P. aeruginosa* (**Fig. 4E**). In another chronic wound, the microbiome became populated by *P. aeruginosa* by D1. No other bacteria took over to form biofilm (**Fig. 4F**). Numerous species identified in the chronic wound microbiome of our *db/db$^{-/-}$* mice are also represented in human chronic wounds (**Table 1**).

Bacterial colonization of chronic wounds when *P. aeruginosa* was present in the microbiota at < 3% of the bacterial population

A cohort of mice were identified to have *P. aeruginosa* present < 3% in the wound microbiome (n=38) (**Fig. 5,6**). In these mice, the strongest colonizer of the wound was *E. cloacae,* whose presence was found in high percentages in both non-chronic (n=20) and chronic wounds (n=18) (**Fig. 5**). Except for D0, *E. cloacae* was present on average 25-55% in non-chronic wounds. (**Fig. 5A**). However, despite the species' strong presence in the non-chronic wounds, biofilm formation could not be visually identified, and the wounds healed in a timely manner. This indicated that *E. cloacae,* much like *P. aeruginosa*, was unable to form biofilm in a wound in the absence of high levels of OS. Other bacteria commonly found in non-chronic wounds when *P. aeruginosa* was virtually absent were *C. frankenforstense, Enterococcus faecalis, Streptococcus sp.*, and *Bacillus paralicheniformis.* In chronic wounds, the colonization of the wound by *E. cloacae* was stronger with biofilm formation initiating between D3 and D5 (**Fig. 5B**). In D0 and D1, the population of *E. cloacae* was 15-18%. However, starting from D2, the percent population began to increase. By D5, *E. cloacae* population had increased to 56%. After D10, the percent composition of *E. cloacae* in the wound began to stabilize with D15-20 wounds consisting of >70% abundance. Other bacterial species, such as *E. faecalis*, *B. paralicheniformis, L. murinus,* and *C. frankenforstense,* were found in the chronic wound with *E. cloacae*. However, their populations rarely exceeded 10%. Shannon index analysis between non-chronic wounds and chronic wounds showed that the diversity of

the bacteria between the two types of wounds was significantly different (p-value < 0.0001) (**Fig. 5C**).

Individual mouse profiles showed distinct bacterial trends in non-chronic and chronic wounds (**Fig. 6**). In non-chronic wounds, *E. cloacae* could be found during healing and even when the wound is healed. Some wounds had *E. cloacae* in a few days after wounding (**Fig. 6A, B**), while other wounds had *E. cloacae* colonization throughout healing (**Fig. 6C**). However, biofilm formation was never observed in these wounds. In chronic wounds, the presence of *E. cloacae* was more common in higher % (**Fig. 6D-F**). Most chronic wounds presented with strong colonization by *E. cloacae* (**Fig. 6E, F**). Of those wounds, most showed complete domination of the wound with *E. cloacae* (**Fig. 6F**) showing that without the presence of significant amounts of *P. aeruginosa*, *E. cloacae* had the ability to become a strong biofilm-forming colonizer. Specific bacterial interactions were also observed with *E. cloacae*, *S. xylosus,* and *Streptococcus* sp. (**Fig. 6E**). In the absence of *P. aeruginosa*, *E. cloacae* was able to out-compete *S. xylosus* (**Fig. 6E**) in a similar manner found with *P. aeruginosa* (**Fig. 4D**).

Bacteria that colonize chronic wounds can be used as indicators of chronicity

Indicator species analysis was used to determine which bacterial species in the wound model were strongly associated with non-chronic and chronic wounds. Indicator values were calculated as (*fidelity* X *specificity*), where the fidelity of a species was its relative abundance and specificity of a species was the relative frequency or the number of samples within the group where the species was detected (**Fig. 7A** and **Fig. 8**). Thus, this analysis considered both the strength and generality of colonization by microbes

across different environments. Several bacterial species were found to be consistently associated with chronic wounds compared to non-chronic wounds (**Fig. 7**). Wound tissues that were infected with aggressive biofilm-forming pathogens, such *P. aeruginosa* (**Fig. 7B**), underwent significant damage especially when strong biofilm was present. *P. aeruginosa* became a strongly indicator of chronic wounds after D2 and by D20, chronic wounds are fully developed. *P. aeruginosa* was able to also colonize non-chronic wounds, but the indicator values in these healing wounds were significantly lower in comparison to the values representing the species colonizing biofilms in chronic wounds. Similarly, *E. cloacae* could also colonize both non-chronic and chronic wounds until it was the predominant bacterial species in the wound community in some mice (**Fig. 7C**) but was more strongly associated with chronic wounds. *C. frankenforstense* colonization in the wound began shortly after injury and indicator values in the wound increased until it peaked at D15. Unlike *P. aeruginosa* and *E. cloacae*, this bacterial species did not frequently colonize non-chronic wounds (**Fig. 7D**). *Acinetobacter* sp. (**Fig. 7E**) had a similar indicator values profile as *P. aeruginosa* (**Fig. 7B**). The bacterial trend in chronic wounds increased exponentially after D2 and remained high once biofilm began to form, protecting and supporting the bacteria so it could continue to infect the wound. In non-chronic wounds, the indicator values were very low, suggesting that this species did not colonize the wound well, perhaps due to competition with other species in the wound as the wound heals. *S. xylosus* was a biofilm-former that could quickly infect a chronic wound once an injury was made as specified by high indicator values at D2 and D3 (**Fig. 7F**). However, as seen in individual bacterial mouse profiles (**Fig. 4D, 6E**), stronger

biofilm-forming bacteria such as *E. cloacae* and *P. aeruginosa* could out-compete *S. xylosus* until it virtually disappeared from the chronic wounds. Indeed, the indicator value began to decrease at D3, at the same time when *P. aeruginosa, E. cloacae* and *Acinetobacter* sp. began to dominate the wound. After D5, the indicator values between chronic wounds and non-chronic wounds were not significantly different. In non-chronic wounds, *S. xylosus* slowly increased, but much like *C. frankenforstense* and *Acinetobacter* sp., it did not strongly colonize the wound. The indicator value trends of *B. paralicheniformis* was higher shortly after injury but was unable to exist in a community with other strong biofilm-formers (**Fig. 7G**) much like *S. xylosus* (**Fig. 7F**).

Bacteria that colonize non-chronic wounds can be used as indicators of healing

A number of bacterial species were found to be significantly associated with resolving wounds (**Fig. 8**). One of these bacterial species *C. acnes*, formally known as *Propionibacterium acnes*, is an abundant commensal Gram-positive anaerobic bacteria found on human skin [51], especially in the sebaceous glands. *C. acnes* has been associated with skin conditions such as acne and an opportunistic pathogen in post-operative infections in humans [51]. However, this species was not commonly associated with human chronic wounds. The indicator value for *C. acnes* in the days following injury increased only in non-chronic wounds while in chronic wounds the value remained very low, showing that *C. acnes* was not significantly associated with chronic wounds in this model system (**Fig. 8B**). While the indicator value trend increased in non-chronic wounds, the indicator value of *C. acnes* was also dynamic over the course of wound healing. *Achromobacter* sp. consisted of non-fermenting Gram-negative bacteria. The indicator

value for *Achromobacter* sp. increased significantly starting from D2 in non-chronic wounds compared to chronic wounds (**Fig. 8C**). The high indicator value was sustained through wound healing. Similar to *C. acnes*, the indicator value for *Achromobacter* sp. was very low in chronic wounds, suggesting it cannot survive in a population with other biofilm-forming bacteria. *Delftia* sp., Gram-negative aerobes, had indicator values consistently high healing wounds compared to chronic wounds (**Fig. 8D**). Although *Delftia* sp. could be found in human chronic wounds, they are not common [15]. *Streptococcus* sp. were Gram-positive species and could be commonly found in human chronic wounds (**Table 1**). The *Streptococcus* sp. in the wound model was significantly associated with non-chronic wounds (**Fig. 8E**). While the indicator values between non-chronic and chronic wounds were similar between D0 and D2, the values were higher in non-chronic wounds compared to chronic wounds from D10. *Streptococcus* sp. was not significantly associated with chronic wounds in the same manner as the other species previously shown above. *E. coli*, a Gram-negative facultative anaerobe, was significantly associated with non-chronic wounds (**Fig. 8F**). Indicator value analysis showed that *E. coli* had significantly higher indicator values throughout the 20 days. Conversely, indicator values for chronic wounds significantly decreased after 72 hrs. *S. epidermis* was a Gram-positive bacterium that was found prevalently in human skin microflora. In the wound model, *S. epidermis* was significantly associated with non-chronic wounds, especially in the early days between D1 and D5 (**Fig. 8G**). After D5, this species began to disappear until D15 at which time it began to return in both non-chronic and chronic

wounds. In chronic wounds, indicator values were low, suggesting that this bacterial species was not highly associated with the microenvironment of chronic wounds.

Comparison of indicator values for bacteria present in chronic and non-chronic wounds

To identify bacterial species that were associated with a microenvironment that would lead to either chronic wound development or wound healing, the dataset was divided into "early" timepoints between D0 and D5 and "late" timepoints between D5 and D20 (**Table 2**). Indicator value analysis showed that several bacteria, *S. xylosus*, *Bacillus* sp., and *E. faecalis*, were significantly associated with the early wound microenvironment that resulted in chronic wound development. Conversely, *P. aeruginosa*, *C. frankenforstense*, *E. cloacae*, and *Acinetobacter* sp. were identified as late indicators. These bacteria significantly associated with the microenvironment of fully developed chronic wounds that have strong biofilm formation. Several species were identified to be highly associated with the wound microenvironment of non-chronic wounds, including *Delftia* sp., *L. murinus*, *Turicibacter* sp., and *S. epidermis*. These bacteria species were identified to be significant later during the healing process.

Discussion

Sequencing the bacterial ITS rRNA gene over the course of healing or chronic wound development, revealed that the wound microbiome was complex, dynamic, and could be altered or disturbed by high levels of OS. When wounds had normal levels of OS as part of the wound healing program, the wound microbiome was diverse and dynamic with many bacterial species present. Although pathogenic bacteria species could

be found in the healing wounds, these species did not form biofilm in the wound. When inhibitors for antioxidant enzymes were administered, resulting in excessive levels of OS induced beyond the scope of the normal wound healing process[36], detrimental changes to the wound microenvironment supported the colonization of opportunistic biofilm-forming bacteria such as *P. aeruginosa*, *E. cloacae*, and *S. xylosus*. As a consequence, while several bacteria species were present in the wound the first few days after injury, competition between commensal bacterial species unable to withstand these conditions and the strong biofilm-forming opportunistic colonizers resulted in a decrease in overall diversity of the wound microbiome over time with biofilm development. Individual wound profiles of healing and chronic wounds demonstrated that mice with identical genetic backgrounds could have distinct and personalized bacterial colonization depending on the levels of OS in the wound. Although the same species dominated chronic wounds, no two wound profiles were the same. Indicator value analysis showed that several bacteria found in the model were highly associated with either healing wounds or wounds that developed into chronic wounds. These bacteria followed different time trends in healing wounds and chronic wounds, illustrating the complexity of bacterial interactions that resulted in these distinct patterns. Bacteria highly associated with non-chronic wounds may play an important role in wound healing, by stimulating the immune response or initiating wound healing processes. Bacteria that have been identified as bioindicators of healing wounds may also play a role in interacting with opportunistic pathogens, to control their growth and prevent them from strongly colonizing the wound. A number of bacterial species sequenced in the model were also

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found in human chronic wounds (**Table 1**), demonstrating the model's ability to recapitulate conditions for chronic wound development with the formation of biofilm from clinically relevant bacteria that was naturally found in the skin microflora.

Previously culture-based methods have been used to identify important wound pathogens. However, culture-dependent techniques were limited in their ability to evaluate complex microbiomes due to the variety of specific culture conditions required to grow different bacterial taxa[52]. Culture-independent metagenomic techniques has been used to describe the microbial diversity of complex communities, such as the wound-associated microbiome[53,54]. One of the most common methods is high-throughput sequencing of bacterial 16S ribosomal RNA (16S rRNA) genes[14,53,55]. In this study, the genomic ITS rRNA gene was targeted over traditional 16S rRNA sequencing in order to obtain taxonomical information about the bacterial community in the chronic wound model. The ITS region offered species and subspecies-level resolution because this region could harbor higher sequence variations[42]. While sequencing the 16S rRNA revealed the complicated nature of microbiomes, the data was commonly represented at the genus taxonomical level which made interpretation of the data difficult because bacteria acted as either pathogens or commensal bacteria at the species level. Other bacteria needed to be identified at the subspecies or strain levels to identify its status of pathogenicity[56].

Wound profiles of individual mice that showed strong biofilm-forming bacteria colonization, from species such as *P. aeruginosa* and *E. cloacae,* were capable of dominating the wound microbiome in non-chronic wounds and chronic wounds alike.

These two species are very important clinical bacteria found in chronic wounds of humans[57,58]. *P. aeruginosa* especially is a notorious biofilm-forming bacterium in human chronic wounds and is important in other diseases such as cystic fibrosis[59]. This is of concern because strong colonization of the wound should be indicative of infection and biofilm formation. However, analysis of bacterial trends using indicator value analysis showed one significant difference between bacteria prominently found in non-chronic wounds as opposed to chronic wounds: bacteria preferentially colonizing chronic wounds occupied the bacterial community at high abundances when compared to non-chronic wounds. In chronic wounds, biofilm created by the opportunistic pathogenic bacteria significantly enhanced the ability of the bacteria to colonize the wound. In the biofilm, nutrients and oxygen were effectively available for the bacteria harbored inside, allowing populations of many bacterial species to significantly increase as observed with *P. aeruginosa*, *E. cloacae*, *C. frankenforstense* and *S. xylosus*.

Analysis of the bacterial microbiome data revealed a subset of mice with low populations of the strong biofilm-formers such as *P. aeruginosa* and *Acinetobacter* sp. When *P. aeruginosa* was present in low percentage of the bacterial population, *E. cloacae* was able to dominate the wound microbiome. *E. cloacae* is a nosocomial opportunistic pathogen found in the environment as well as on human skin and is frequently identified in the biofilm of the wounds with some strains already harboring anti-microbial resistance genes[60]. In our model system, *E. cloacae* was present in high proportions in both non-chronic and chronic wounds. In wounds that did not have high levels of OS, *E. cloacae* did not form biofilm and we did not observe delays in healing.

However, when the wounds are administered inhibitors for catalase and glutathione peroxidase to induce high levels of OS and become chronic, the wounds formed strong biofilm[18,36]. These studies suggest that high levels of OS are necessary to activate or allow for the formation of biofilms by opportunistic pathogens and that the ability to adapt to these high OS conditions may determine which opportunistic pathogen is able to effectively colonize and develop biofilms in the wound.

In our *db/db*^{-/-} model, we were able to identify key pathogenic bacteria such as *P. aeruginosa*, *E. cloacae, C. frankenforstense, Acinetobacter* sp. and *S. xylosus* that are present in the biofilm of the chronic wounds. These bacteria are clinically relevant because they are found in human chronic wounds and create biofilm which complicates and significantly delays healing (**Table 1**). The infection and production of biofilm in our model is derived only from the manipulation of the redox state of the wound tissue in diabetic, obese mice, with the participating bacteria originating naturally from the skin microbiota[18,36]. The infection and biofilm production can be observed from the initial stages in the chronic wound model so that mechanisms of pathogenicity in these bacteria can be understood *in vivo*. This is a significant advantage since chronic wounds in humans are observed by physicians and wound care specialists weeks, sometimes months or even years, after the initial injury.

Several bacterial species that are used as probiotics to treat disease in humans are also present in the bacterial microbiome in our mouse model (**Table 3**). Microbiome studies in the gut have led to the development of probiotics, a cocktail of bacteria that can reverse disease progression by replacing harmful bacteria with beneficial ones. It has

been shown that bacterial interactions between *Lactobacillus plantarum* with *P. aeruginosa* in a burn mouse model lead to inhibition of *P. aeruginosa* colonization, resulting in better wound healing outcomes such as tissue repair and enhanced phagocytosis of *P. aeruginosa*[61]. Consuming probiotics supplements consisting of several *Lactobacillus* sp. were able to improve clinical outcomes in patients with diabetic foot ulcers[62]. Ethyl acetate extract of an *Achromobacter* sp. from entomopathogenic nematodes were found to have significantly antibacterial properties against biofilm-forming bacteria such as *P. aeruginosa* and *S. aureus*. Specific cyclic dipeptides, in combination with ampicillin, inhibited biofilm formation while also increasing production of anti-inflammatory cytokines from peripheral blood mononuclear cells[63]. Even though species such as *Achromobacter xylosoxidans* are implicated in nosocomial infections, infection with this species in skin is thought to be very uncommon[64]. However, this species was found to be infecting burn wounds and is an emerging pathogen of cystic fibrosis[65,66].

Whereas research in pathogenic bacteria have resulted in the identification of mechanisms in which bacteria may interfere with wound healing[67], mechanisms to understand the beneficial role of probiotics in wound healing are still in their infancy. Bacteria identified to be highly associated with non-chronic wounds (**Table 2**) may be beneficial by assisting in stimulating the immune system and wound healing processes. A combination of multiple bacterial species with different metabolisms may collectively strengthen the affect the bacteria has on wound healing, since interactions between commensal bacteria may lead to a more diverse set of active metabolites that can

stimulate the immune response or wound healing[68]. Bacteria identified as early

bioindicators may perform as putative probiotics since they are present in the wound

immediately after injury and can interact and compete with opportunistic pathogenic

bacteria to prevent them from colonizing the wound and forming biofilm. Bacteria

identified as early bioindicators of chronic wounds (**Table 2**) may have a significant role

in the presence of high levels of OS in establishing a wound microenvironment that

allows other biofilm forming pathogens to infect and colonize the wounds. These bacteria

are of particular interest because late bioindicators of chronic wounds are significant

bacterial species isolated and identified in human chronic wounds. To the best of our

knowledge, the microbiome of a wound immediately after injury that will develop into

chronic wounds has not been surveyed. Analysis on wound microbiomes of human

chronic wounds (**Table 1**) are currently done on mature chronic wounds, and thus, are

more similar with bacterial species identified as late chronic wound bioindicators.

Comparing the bacterial bioindicators of non-chronic wounds and acute/healing human

wounds may assist in focusing research to identify bacterial species that function as

putative probiotics, or identify metabolites, that when applied on wounds can stimulate

proper wound healing. Conversely, analysis of different types of human chronic wound

microbiomes in comparison to the bioindicators of chronic wounds found in our model

can focus efforts to understand how early chronic wound bioindicators interact with late

chronic wound bioindicators to lead to chronic wound development. In that endeavor, our

chronic wound model can be used to understand host–microbe interactions to identify

novel mechanisms, pathways, chemicals, or metabolites that can effectively prevent or reverse chronic wound formation.

The microenvironment of human chronic wounds is frequently manipulated in order to eliminate harmful bacteria and facilitate wound healing[69,70]. Wounds are often debrided to remove biofilm and dead tissue, and subsequently treated with wounds dressings to protect the wound and stimulate wound healing. Antibiotics are also administered in order to prevent further colonization of biofilm-forming bacteria[71,72]. Despite great efforts put into healing chronic wounds, treatment efficacy has been low with many wounds persisting despite antibiotic therapy, wound debridement, and applications of wound dressings. This is because the underlying cause of chronic wound development is poorly understood, and the treatments applied, and therapies used are not targeting the underlying cause. Human wound microenvironments are very complex and many cells that participate in wound healing are dysfunctional in human chronic wounds. Just as important as the wound parameters in patients are the bacteria that colonize the wound. Chronic wounds are rarely colonized by a single pathogenic species. Instead, several "cornerstone" species are observed, including common nosocomial species such as *P. aeruginosa*, *Enterobacter* sp., *Staphylococcus aureus* and *Acinetobacter baumannii.* Many species found in chronic wounds are also found in normal skin microflora. While these species may be detected because they are skin components, they may have a role in either promoting wound healing or contributing towards the development of non-healing wounds. Applying broad-spectrum antibiotics, while used to control the population of pathogenic species, also removes from the wound bed species that may positively

contribute to healthy healing through competition of pathogenic species. Also important, and underscored in this study, is the longitudinal nature of chronic wound development. Most microbiome studies in human chronic wounds are samples of the wound at only one time. In addition, the timing of sampling is crucial in the days following debridement, a common technique used to clear away necrotic and infected tissue to re-expose healthy tissue. Future microbiota studies in human chronic wounds would benefit from sampling several timepoints.

In conclusion, our studies demonstrate distinctive bacterial trends in the microbiome of healing non-chronic and chronic wounds in our diabetic mouse model. In non-chronic wounds, higher levels of bacterial diversity and dynamic colonization were observed over time as the wounds healed. Opportunistic pathogens, capable of forming biofilm, were present in the wound microbiota and did not make biofilm without increased levels of OS. In contrast, chronic wounds had low levels of bacterial diversity and were colonized by strong opportunistic pathogens that formed biofilm and out-competed other bacteria present in the wound microenvironment. Chronic wounds are colonized by *P. aeruginosa* when it is present in the microenvironment. Conversely, *E. cloacae* is the dominating wound bacterial species when *P. aeruginosa* is not present. Indicator value analysis showed that specific bacterial species are highly associated with non-chronic wounds compared to chronic wounds; these bacteria may potentially benefit or facilitate wound healing and the exclusion of pathogenic bacteria. The microbiome studies in this paper show that our chronic wound model provides the correct microenvironment that will enable the study of bacterial interactions that lead to either

wound healing or chronic wound development, potentially leading to new methods of

controlling biofilm in chronic wounds in order to support wound healing.

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Table 1. Bacteria found in human chronic wounds

Bacteria	References
Acinetobacter sp.	11,12,15,17,57,73
Anaerococcus sp.	15,74,75
Bacillus sp.	11,15,17,21,57
Corynebacterium sp.	15,17,73–76
Enterobacter sp.	11,12,15,57,75
Enterobacter cloacae	11,73
Enterococcus sp.	12,15,57,75,76
Enterococcus faecalis	11,15,73
Escherichia sp.	12,17,57,73,76
Escherichia coli	11,57
Finegoldia sp.	15,17,74
Finegoldia magna	15,75
Paenibacillus sp.	57
Peptoniphilus sp.	15,57,74,75
Porphyromonas sp.	74
Prevotella sp.	15,17,74–76
Propionibacterium sp.	15,17
Propionibacterium acnes	70
Pseudomonas sp.	11,12,15,17,57,75,76
Pseudomonas aeruginosa	11,15,73,76
Staphylococcus sp.	12,15,17,57,74,75
Staphylococcus epidermidis	15,76
Streptococcus sp.	12,15,17,57,75,76
Turicibacter sp.	15

Table 2. Bacterial bioindicators for non-chronic and chronic wounds

Early indicators of chronic wounds	p-value	Late indicators of chronic wounds	p-value
Staphylococcus xylosus	0.001	*Pseudomonas aeruginosa*	0.001
Bacillus sp.	0.001	*Corynebacterium frankenforstense*	0.033
Enterococcus faecalis	0.048	*Enterobacter cloacae*	0.001
Staphylococcus nepalensis	0.001	*Acinetobacter* sp.	0.036
Erysipelothrix rhusiopathiae	0.05		
Lachnospiraceae	0.045		
Mucinivorans hirudinis	0.045		
Early indicators of non-chronic wounds	**p-value**	**Late indicators of non-chronic wounds**	**p-value**
Delftia sp.	0.001	*Cutibacterium acnes*	0.001
Lactobacillus murinus	0.015	*Streptococcus* sp.	0.001
Turicibacter sp.	0.004	*Achromobacter* sp.	0.001
Staphylococcus epidermidis	0.026	*Escherichia coli*	0.014
Weissella paramesenteroides	0.001	*Lawsonella clevelandensis*	0.007
Paenibacillus sp.	0.04	*Lactobacillus crispatus*	0.049
Bacteroidales	0.004	*Pseudomonas* sp.	0.027
Lactobacillus sp.	0.005	*Corynebacterium choanis*	0.012
Massilia oculi	0.046		
Massilia sp.	0.003		
Planococcus sp.	0.029		

Table 3. Bacteria used as probiotics in humans

Bacteria	References
Enterococcus faecalis	77,78
Lactobacillus sp.	62,76,79,80
Lactobacillus johnsonii	81
Lactobacillus reuteri	78,79,81
Streptococcus sp.	80
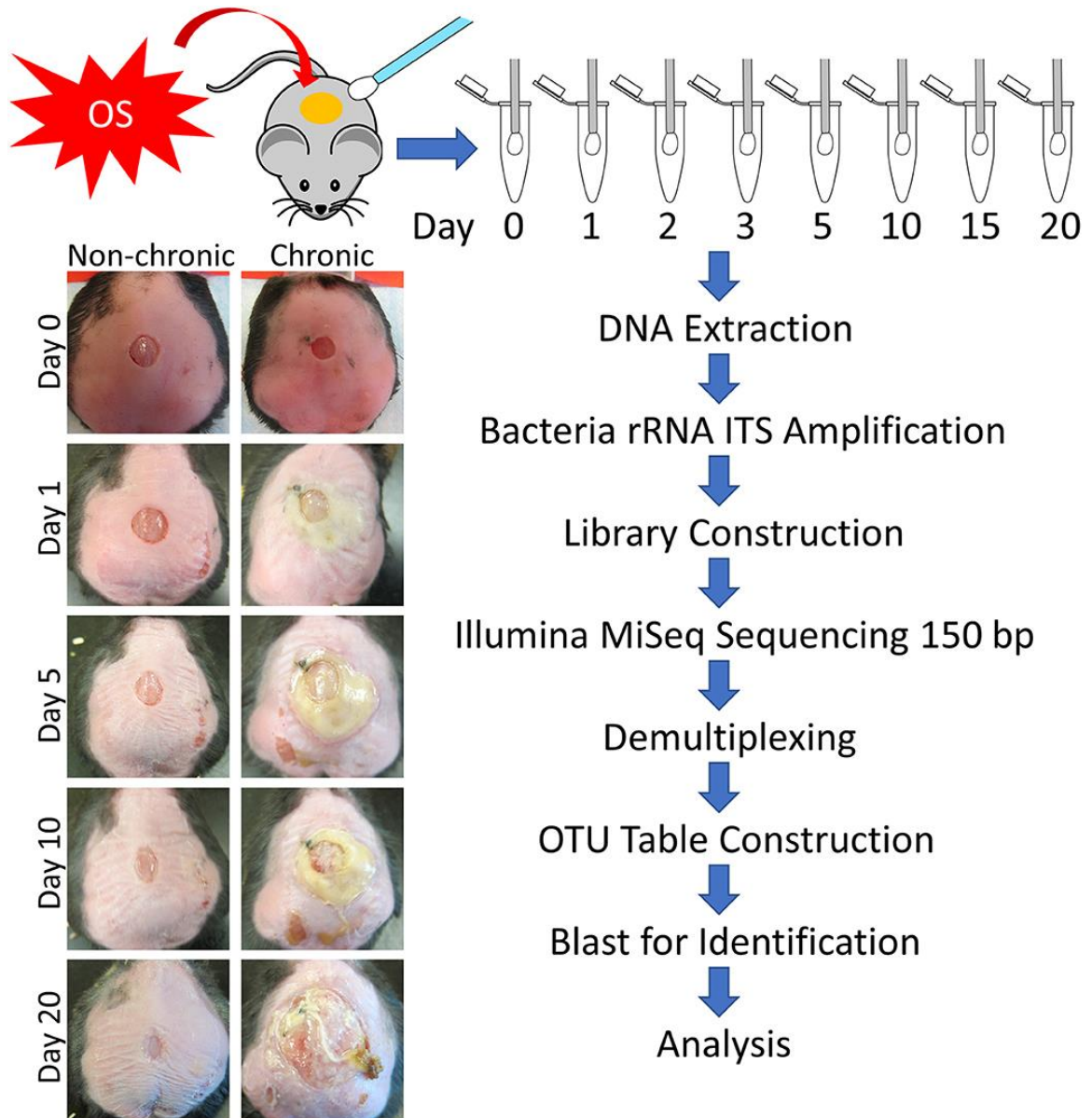
Figure 1. Flowchart of the experimental procedure to obtain bacterial counts and wound types. Sterile swabs were used to sample the microbiota from non-chronic and chronic wounds from injury until D20. Non-chronic wounds healed around day 20 whereas chronic wounds became fully chronic with biofilm formation by D20. After DNA extraction, the bacterial ITS rRNA region was PCR amplified for each sample. Libraries were sequenced by Illumina MiSeq with 150bp single end reads, followed by demultiplexing and OTU table construction for bacterial identification.
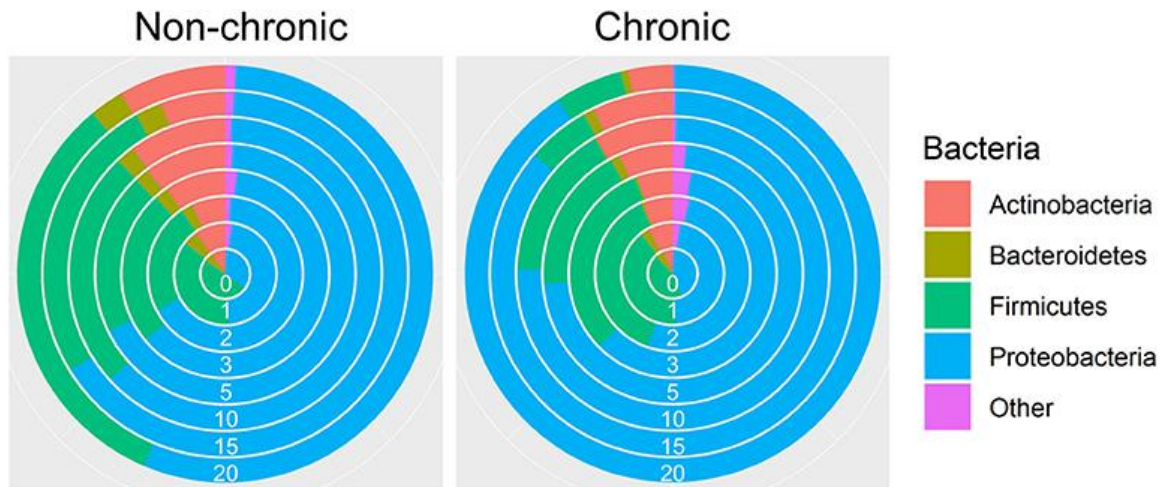
Figure 2. Bacterial diversity in the chronic wound model. Bacteria identified in the non-chronic and chronic wounds crossed phylum lines. Most of the bacteria sequenced were found in the Actinobacteria, Firmicutes and Proteobacteria phylum. While proportions of the phylum did not change much in non-chronic wounds as the wound healed, the proportion of Proteobacteria, which consisted of *P. aeruginosa* and *E. cloacae*, increased over time in chronic wounds. Non-chronic wound, n=40; chronic wound, n=37.

A Non-chronic Wound

B Chronic Wound

C Shannon Index

Legend

- *Enterobacter cloacae*
- *Pseudomonas aeruginosa*
- *Streptococcus sp.*
- *Staphylococcus xylosus*
- *Enterobacteriaceae*
- *Corynebacterium frankenforstense*
- *Cutibacterium acnes*
- *Escherichia sp.*
- *Turicibacter sp.*
- *Escherichia coli*
- *Enterococcus gallinarum*
- *Lactobacillus johnsonii*
- *Lactobacillus murinus*
- *Acinetobacter sp.*
- *Achromobacter sp.*
- *Weissella paramesenteroides*
- *Bacillus sp.*
- *Aerococcus urinaeequi*
- *Bacillus paralicheniformis*
- *Staphylococcus epidermidis*
- *Exiguobacterium sp.*
- *Lactobacillus reuteri*
- *Enterococcus faecalis*
- *Massilia sp.*
- *Faecalibaculum rodentium*
- *Massilia oculi*
- *Helicobacter hepaticus*
- *Staphylococcus hominis*
- *Streptococcus sp.*
- *Staphylococcus nepalensis*
- *Bacilli*
- *Pantoea vagans*
- *Candidatus Arthromitus sp.*
- *Paenibacillus sp.*
- *Alistipes finegoldii*
- *Pantoea sp.*
- *Bacteroidales*
- *Lactobacillus crispatus*
- *Ralstonia sp.*
- *Pantoea agglomerans*

Figure 3. Bacterial composition in non-chronic and chronic wounds when *P. aeruginosa* was present in the microbiota. The average % relative abundance of bacterial OTUs was calculated across the top 100 OTUs for wound cohorts. The top 40 most abundant bacteria are shown in the legend. (**A**) Non-chronic wounds had a diverse bacteriome that included pathogenic bacterial species. However, biofilm formation was not visibly detected, and the wounds healed in approximately 20 days. (**B**) Chronic wounds were composed of a less diverse bacteriome and over time became dominated by a few pathogenic bacteria such as *P. aeruginosa* and *E. cloacae*. (**C**) Alpha diversity measured with Shannon Index confirmed that chronic wounds had much less bacterial diversity in the wounds compared to non-chronic wounds (p-value < 0.0001). Non-chronic wound, n=20; chronic wound, n=19. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.
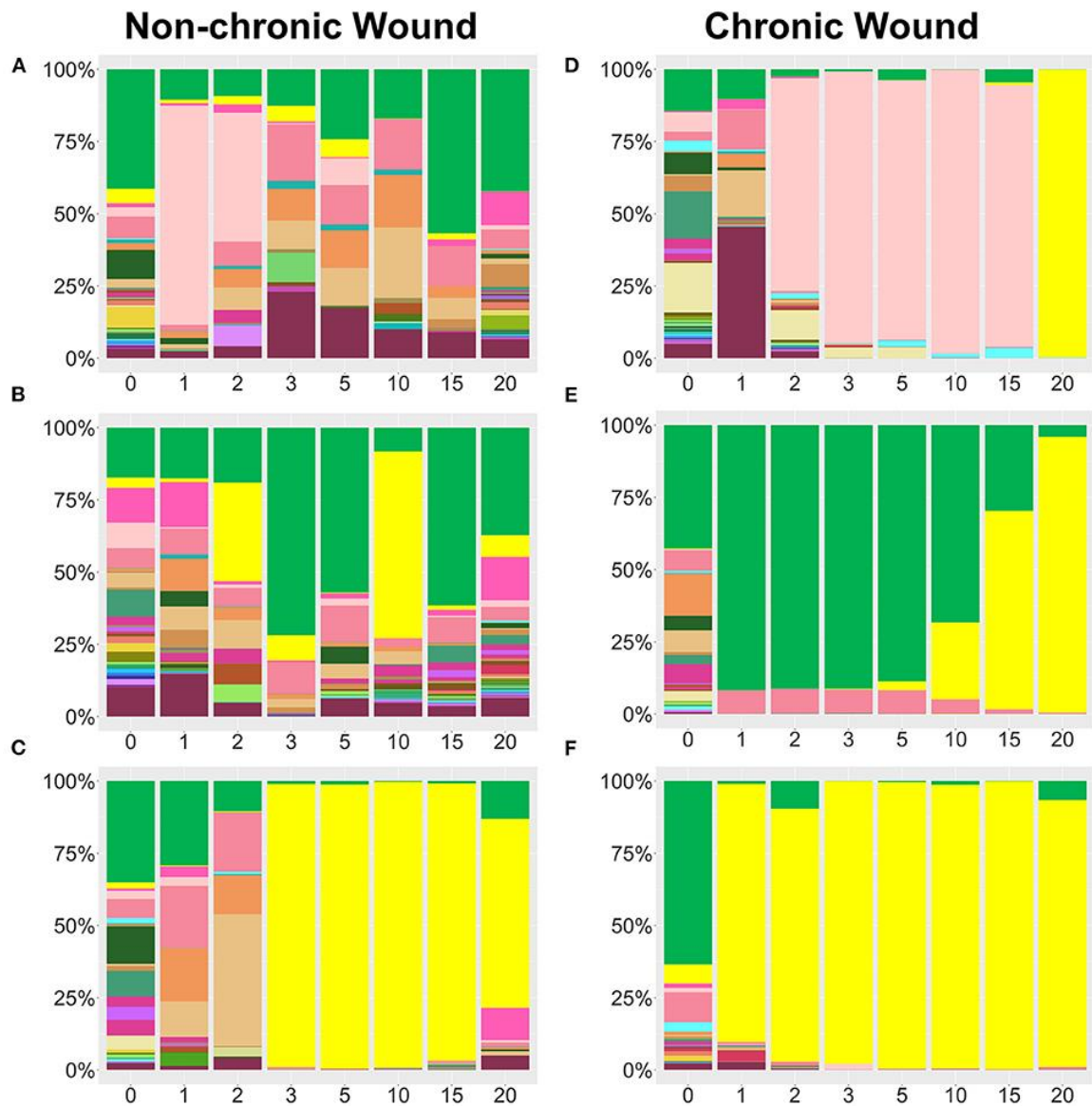
Figure 4. Examples bacterial profiles from non-chronic and chronic wounds. (**A-C**) Examples of non-chronic wound bacterial profiles three individual mice showed differences in bacterial percentages in the absence of high levels of OS in the wound. Species legend is shared with Fig. 3. Non-chronic wounds may have pathogenic bacteria in the wound, but they do not form biofilm and the wounds will ultimately heal. *P. aeruginosa* was abundant in (**C**), but, in the absence of high levels of OS, no biofilm formed. (**D-F**) Individual profiles of bacteria in chronic wounds show distinct bacteriome patterns as pathogenic bacteria colonize a wound in the presence of high levels of OS. *P. aeruginosa, S. xylosus,* and *E. cloacae* predominate over other bacteria and form biofilm as the chronic wounds develop.

A Non-chronic Wound

B Chronic Wound

C Shannon Index

Legend
- *Enterobacter cloacae*
- *Pseudomonas aeruginosa*
- *Streptococcus sp.*
- *Staphylococcus xylosus*
- *Enterobacteriaceae*
- *Corynebacterium frankenforstense*
- *Cutibacterium acnes*
- *Escherichia sp.*
- *Turicibacter sp.*
- *Escherichia coli*
- *Enterococcus gallinarum*
- *Lactobacillus johnsonii*
- *Lactobacillus murinus*
- *Acinetobacter sp.*
- *Achromobacter sp.*
- *Weissella paramesenteroides*
- *Bacillus sp.*
- *Aerococcus urinaeequi*
- *Bacillus paralicheniformis*
- *Staphylococcus epidermidis*
- *Exiguobacterium sp.*
- *Lactobacillus reuteri*
- *Enterococcus faecalis*
- *Massilia sp.*
- *Faecalibaculum rodentium*
- *Massilia oculi*
- *Helicobacter hepaticus*
- *Staphylococcus hominis*
- *Streptococcus sp.*
- *Staphylococcus nepalensis*
- *Bacilli*
- *Pantoea vagans*
- *Candidatus Arthromitus sp.*
- *Paenibacillus sp.*
- *Alistipes finegoldii*
- *Pantoea sp.*
- *Bacteroidales*
- *Lactobacillus crispatus*
- *Ralstonia sp.*
- *Pantoea agglomerans*

Figure 5. Bacterial composition in non-chronic and chronic wounds when *P. aeruginosa* was not present in the microbiota. (**A, B**) Average % bacterial composition was calculated for the top 100 OTUs for wound cohorts without *P. aeruginosa* present in the skin microbiome. The top 40 most abundant bacteria are shown in the legend. (**A**) Non-chronic wounds have a diverse microbiome with strong colonization by *E. cloacae,* a biofilm forming bacteria, yet the wounds do not develop biofilm. (**B**) Bacterial diversity in the chronic wounds decreases over time, with the wound becoming dominated by *E. cloacae*. (**C**) Alpha diversity measured by the Shannon Index shows that chronic wounds have less diversity in the wounds compared to non-chronic wounds (p-value < 0.0001). Diversity in non-chronic wounds decreases at start of wound healing and then stabilizes once the wound heals. Diversity in chronic wounds drops precipitously as bacterial infection leads to biofilm formation that harbors only a few species. Non-chronic wound, n=20; chronic wound, n=18. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.
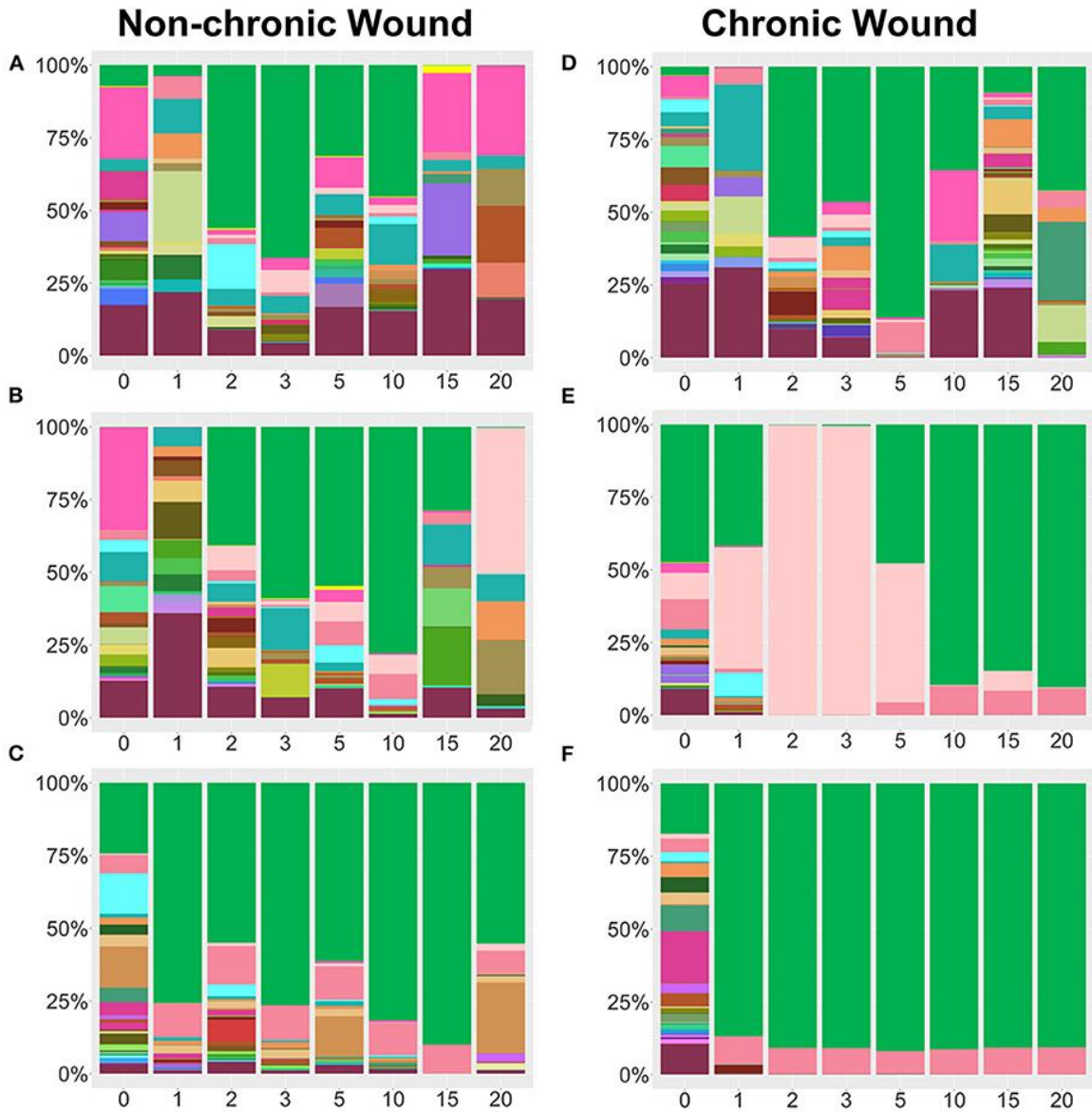
Figure 6. Examples of individual mice bacterial wound profiles from non-chronic and chronic wounds in the absence of *P. aeruginosa* in the bacteriome of the skin. (**A-C**) Examples of three individual non-chronic bacterial wound profiles show differences in percentages of the individual bacteria in the absence of high levels of OS in the wound. Species legend is shared with Fig. 5. Despite of the abundance of *E. cloacae*, a biofilm forming bacterium, formation of biofilm does not occur, and wounds heal. (**D-F**) Individual profiles of bacteria in chronic wounds in the presence of high OS show distinct microbiome patterns with biofilm forming bacteria such as *E. cloacae* and *S. xylosus* predominating in the wound. These wounds contain biofilm. Non-chronic wound, n=20; chronic wound, n=18.
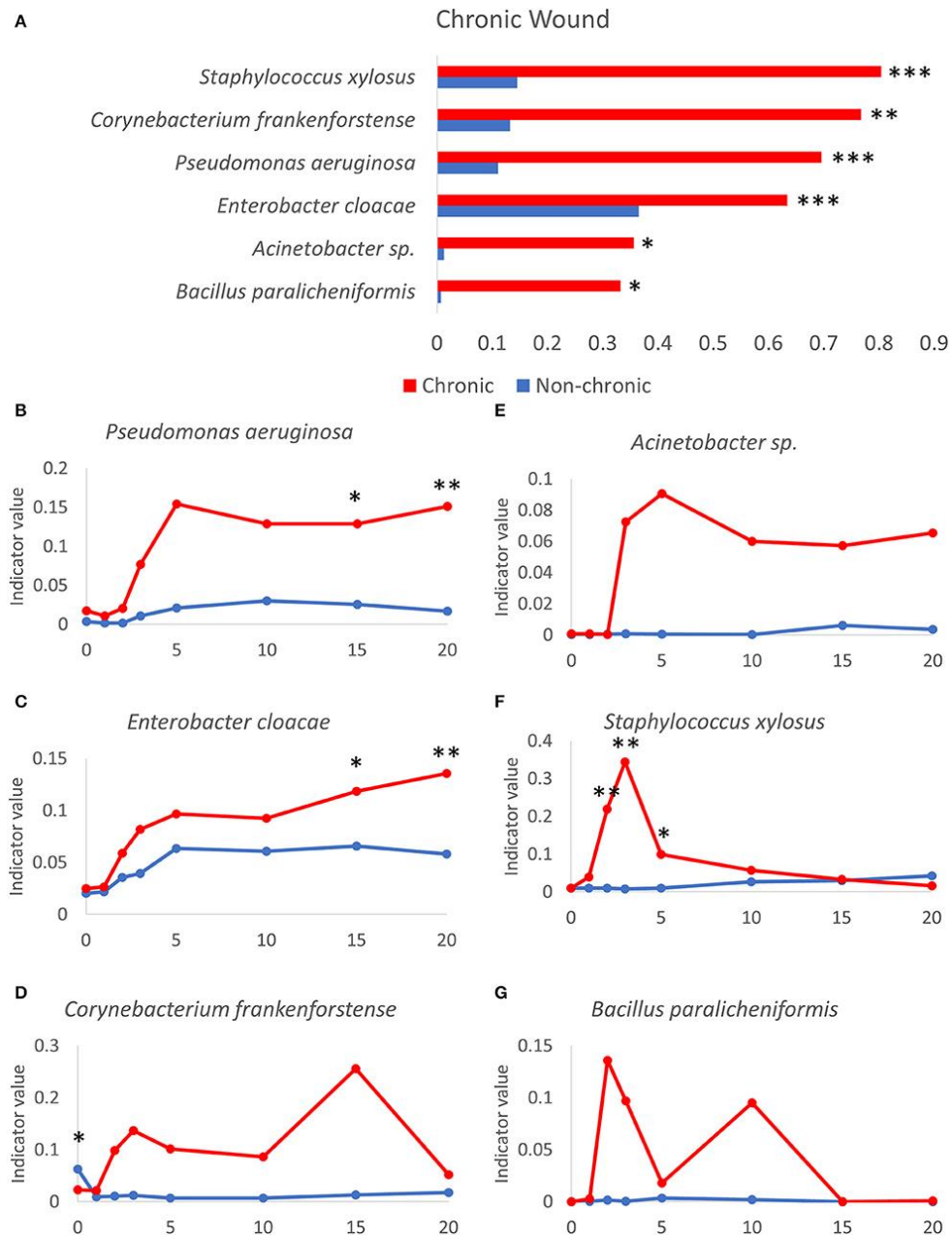
Figure 7. Bacteria that participate in the microbiome profile of chronic wound microenvironment. (**A**) Several bacteria that can form biofilm are highly indicative in chronic wounds compared to non-chronic wounds. Indicator values of non-chronic (blue lines) and chronic (red lines) wounds are shown for the following bacteria over time as chronic wounds develop. Gram-negative biofilm forming bacteria: (**B**) *P. aeruginosa*, (**C**) *E. cloacae*, (**D**) *C. frankenforstense*, (**E**) *Acinetobacter* sp., (**F**) *S. xylosus*. Gram-positive biofilm-forming bacteria: (**G**) *B. paralicheniformis*. Non-chronic wound, n=40; chronic wound, n=37. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

A Non-chronic Wound

Cutibacterium acnes ***
Streptococcus sp. ***
Achromobacter sp. ***
Escherichia sp. ***
Delftia sp. ***
Escherichia coli ***
Staphylococcus epidermidis ***
Turicibacter sp. **
Streptococcus sp. **
Massilia oculi **
Bacteroides uniformis **
Lawsonella clevelandensis ***
Pseudomonas sp. ***
Faecalibaculum rodentium *
Weissella paramesenteroides **
Clostridium. saccharolyticum *
Blautia hansenii ***
Ralstonia sp. *
Porphyromonas endodontalis *
Marinilactibacillus sp. **
Geobacillus sp. *
Porphyromonas gulae **

■ Chronic ■ Non-chronic

B *Cutibacterium acnes*

C *Achromobacter sp.*

D *Delftia sp.*

E *Streptococcus sp.*

F *Escherichia coli*

G *Staphylococcus epidermidis*

Figure 8. Bacteria that participate in a diverse microbiome profile typically found in the non-chronic wound microenvironment. (**A**) A number of bacteria are significantly associated with non-chronic wounds using indicator species analysis. Indicator values of non-chronic (blue lines) and chronic (red lines) wounds are shown for the following bacteria over time as non-chronic wounds heal: (**B**) *Cutibacterium acnes*, (**C**) *Achromobacter* sp., (**D**) *Delftia* sp., (**E**) *L. murinus*, (**F**) *Streptococcus* sp., (**G**) *Escherichia* sp.*,* (**H**) *Escherichia coli* and (**I**) *Staphylococcus epidermidis*. Non-chronic wound, n=40; chronic wound, n=37. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Chapter 3

Skin Microbiota and its Role in Health and Disease with Emphasis in Wound Healing and Chronic Wound Development

Summary

Skin has evolved into a protective barrier in the body to withstand physical, chemical, and biological assaults from pathogens. When the skin is compromised, the body activates the highly complex process of cutaneous wound healing to quickly close the wound, eliminate invading microbes and strengthen the damaged area to restore the protective functions of the skin. Investigations using high-throughput sequencing platforms have begun to study the complex and diverse communities of microbes on the skin, the skin microbiota. The skin microbiota consists of trillion of microbes across bacterial, viral and fungal families with different communities colonizing different locations of the body. The unique genetic, metabolic, and proteomic profiles of each microbial community may impact health, disease and infection on the skin. How host-microbe interactions support healthy skin and wound healing is an urgent topic of research because dysbiosis, perturbations to the skin microbiota, may result in the development of human skin diseases and aliments, including the development of chronic wounds. Studies have now shown that skin aliments such as atopic dermatitis, acne vulgaris, rosacea, psoriasis and even some skin cancers have skin microbiota different from normal skin. While common wound pathogens of chronic wounds have been known through culture-dependent methods, it is now appreciated that the community of biofilm-forming microbes differ with some uniqueness across different etiology of wounds such as venous ulcers, pressure ulcers, and diabetic foot ulcers. Understanding how specific skin microbes play an important role in wound healing and overall health of the skin may lead to the next generation of therapeutics where the skin microbiota is manipulated

through probiotics, prebiotics and skin microbiota transplants in order to prevent and treat skin aliments and chronic wounds.

Introduction

Skin is the largest organ in the human body. It is a barrier that protects the organs from the environment, functioning as an interface between the outside and the inside of the body. The cellular structure and composition of the skin evolved to act as a physical, chemical, and biological barrier with support from both the innate and adaptive immune system[1-5]. A myriad of chemical and physical pollutants, toxins, and biological agents, such as pathogens, are barred from entering the body and causing damage. If the integrity of the skin is compromised, the body relies on the highly complex process of cutaneous wound healing, to quickly close and strengthen the damaged area to restore the protective functions of the skin.

Residing on and in the body is a complex and diverse community of microbes called the microbiota[6-10]. Trillion of microbes, across the bacterial and fungal families, use a wide range of aerobic and anaerobic metabolic pathways to survive. Also, viruses inhabit various locations of the body, some are pathogenic to humans and others are pathogenic to bacteria (also called bacteriophages)[5,8,10–12]. Single- and multi-cellular parasites are also present in the body and contribute to the microbiota. The collective genome of the microbiota (termed the microbiome) results in unique metabolic and proteomic profiles specific to each location of the body. The contributions and impact of the microbiome on health and disease are still under investigation[4,13,14].

A considerable effort is being made to study the role of the microbiota in skin-related infections, disorders, and disease[15–17]. In particular, how host-microbe interactions are specifically relevant to wound healing is an urgent topic of research because specific microbes may play an important role in wound healing and overall health of the skin. If the homeostasis between human skin and the microbiota is disturbed, dysbiosis may result in the development of a number of human skin diseases and aliments, including the development of chronic wounds.

Historically, many important wound microbes were identified by relying on isolation and culturing protocols under artificial laboratory conditions, emphasizing only a small fraction of the entire skin microbiota. The limited growth conditions that can be replicated in culture cannot capture and sustain the growth of the majority of microbes, greatly limiting the ability to identify and describe the whole microbiota[13,18–20]. In recent years, advances in high through put DNA sequencing platforms have led to the identification of many microbes in normal healthy human skin and have characterized the microbiome of wounded skin[21–24]. This review discusses how the relationship of the skin and the residing microbiota play a role in skin disease, normal wound healing, and on chronic wound development. References cited were chosen as examples of several possible references for the same subject.

Skin Structure and Function

The skin is important in carrying out very important functions, such as in protection, preservation of water and electrolytes, and regulation of temperature, water, and fat storage, in addition to playing a major role in the endocrine and immunological

systems[25,26]. It is the largest organ in humans and functions as an interface between the connective tissue/organs and the environment, meaning it must be waterproof and protect the body against environmental stresses. It is a continuously developing organ composed of three main structures, the epidermis, the dermis, and the hypodermis, and also includes other anatomical structures such as hair follicles, nails, sebaceous, and sweat glands[27–29]. The skin is mostly acidic and dry but different areas of the body have different topography.

The epidermis is the outer layer of the skin, and it interacts directly with the environment. It is a physical barrier, resisting penetration by microorganisms and potential toxins while retaining moisture and nutrients inside the body. The thickness of this layer depends on the location of the body; for example, the skin is thin on the eyelids and thick on the palms of the hands and soles of the feet[26]. The epidermis is predominantly composed of keratinocytes and melanocytes but includes other types of cells such as Langerhans and Merkel cells[29]. Because this layer of the skin interacts with the environment, the epidermis is continuously undergoing renewal as these cells undergo terminal differentiation followed by cell death. The epidermis is composed of sublayers called the stratum corneum, stratum granulosum, stratum spinosum, and basal layer based on the morphology and location of the keratinocytes[28,29]. The most superficial layer of the skin, the stratum corneum, functions as the major constituent of the skin barrier. This layer is made up of corneocytes, which are flat, enucleated dead keratinocytes that have undergone terminal differentiation. These cells are connected together by desmosomes which help create the cornified envelop. In combination with proteins and lipids, these

cells create an insoluble barrier that has a critical role in the skin barrier[27]. Underneath the

stratum corneum is the stratum granulosum, which is a cell layer that contains live

keratinocytes that arise as differentiated cells from the layer below, the stratum spinosum,

and ascend outwards from the basal layer, a layer of rapidly proliferating columnar-

shaped keratinocytes[27–29]. The basal keratinocytes are attached to the basement lamina

which separates and connects the epidermis to the dermis. The basement lamina is a thin

extracellular matrix sheet that contains three structural components: the lamina lucida, the

lamina densa, and the sublamina densa[27–30]. These layers are composed of collagen types

IV, laminin, entactin, and fibronectin that are anchored by filaments composed of

collagens type VI, VII, XV, and XVIII and chondroitin sulfate proteoglycans, to the

underlying dermis[30]. The basal lamina plus the molecules connecting it to the underlying

dermis constitute the basement membrane[28–32].

Beneath the basement membrane is the dermis, which is composed of the

papillary dermis and reticular dermis. The papillary dermis is a thin layer of loosely

arranged collagen fibers beneath the basement membrane, while the reticular dermis is a

thick layer of collagen bundled that align parallel to the surface of the skin. 70% of the

dermis is made up of collagen fibers which gives the skin its strength and toughness.

Elastin fibers on the other hand, maintain the elasticity and flexibility of the skin[27–29,33].

Throughout the collagen and elastin fibers, proteoglycans provide viscosity and hydration

to the extracellular matrix. The dermis primarily contains fibroblasts and myeloid and

lymphoid immune cells that reside in and maintain the dermal layer[26]. One immune cell

type present in the dermis is the mast cell. Mast cells are important in both innate and

adaptive immune responses. They have an important role in chemoattracting other immune cells by secreting chemokines and cytokines. These mast cells also secrete histamine during allergic responses[29]. In addition, lymph and blood vessels are also present in the dermis. These vessels have important homeostatic functions because they provide nutrients for the epidermis and dermis and support immune processes. In addition, the dermis contains a network of sensory nerves that has an important role in maintaining cutaneous homeostasis and interfacing with the environment. It is also a critical sensory organ that allows for the perception of touch, pain, and temperature[28,29]. Moreover, sebaceous glands, located in the dermis, are connected to hair follicles, forming the pilosebaceous unit, and secrete lipid-rich sebum. Sebum is a hydrophobic coating that protects and lubricates the skin and hair and provides antibacterial shielding.

The skin also contains sweat glands[26]. There are two types of sweat gland; they are distinguished by their development, morphology and function. Eccrine sweat glands constitute the majority of these glands and are distributed nearly throughout the entire body, whereas apocrine sweat glands are present in a just few areas on the body[26,28,29]. Eccrine sweat glands have an important thermoregulatory function and can respond to emotional stimuli. Under stress such as heat, eccrine sweat glands are capable of producing up to 10 liters of sweat per day. Sweat is a clear, odorless solution which contains salts such as sodium, calcium, chloride, and potassium. Inorganic and organic chemicals such as urea, lactate, amino acids, and bicarbonate are also present in smaller concentrations. Larger proteins structures such as immunoglobulins, make up less than 1% of sweat by weight[34]. Apocrine sweat glands are present in limited areas: the axilla,

perianal region, areolae, periumbilical skin, prepuce, scrotum, mons pubis, and labia minora[35]. They produce a sterile, thick, milky, and odorless fluid that contains many proteins, fatty acids, carbohydrates, ammonia, lipids, and ferric ions. This secretion can undergo bacterial decomposition and potent odorous compounds are produced as a result.

Beneath the dermis is the hypodermis or subcutaneous tissue which contains fibroblasts, adipose cells, and macrophages[27–29]. A fibrous network of connective tissue provides structural support for additional nerve cells, blood vessels, and lymphatic vessels located within the hypodermis. The subcutaneous adipocytes give the hypodermis some characteristics of an endocrine tissue because these adipocytes can produce hormones such as adiponectin, which regulates glucose levels and leptin which regulates hunger.

Skin Microbiome Diversity in Different Parts of the Body

Using a variety the techniques, scientists have found that the skin microbiome consists of both benign, commensal, microbes that contribute to the health of the skin, and pathogenic microbes that can cause infection and disease if not controlled[4,5,11,12,36]. The microbes present in skin include bacteria, fungi, and viruses. Commensal bacteria have recently been shown to stimulate the adaptive and innate immune system and directly or indirectly prevent pathogens from causing infections[4,37–40]. Therefore, understanding how the skin microbiota colonizes different sites in the body may provide insight in the pathophysiology of cutaneous infections and could explain how the balance between skin health and disease can be modulated by the microbiota.

Specific dermatological disorders manifest themselves at different skin sites. As discussed above, the skin provides many niches in which large populations of microbes are subjected to a variety of ecological pressures including humidity, temperature and pH[4,9,11,36]. High temperature and high humidity are associated with increased quantities of bacteria and increased diversity[2]. These ecological factors contribute to the uniqueness of the various skin structures such as the hair follicles and sebaceous, eccrine, and apocrine glands that produce a variety of antimicrobial peptides and lipids that can chemically control and regulate the population of microbes on the skin. Competition between microbial species is important for development and maintenance of a healthy microbiome[11,26,41,42].

The bacterial species that populate the different regions of the skin are directly related to the microenvironment[2]. Due to the complexity of the microenvironment, the normal microbiota of the skin is very complex and likely developed through the cooperation of commensal bacteria and competitive exclusion of pathogens[43]. Major bacterial families that are present on the skin include Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%) with Actinobacteria more abundant on skin[13]. Major bacterial contributors at the genera level to the skin microbiota are *Corynebacterium*, *Propionibacterium* and *Staphylococcus*[13].

Areas of the skin with abundant sebaceous glands, such as on the face, are predominantly populated by *Propionibacterium acnes* and *Staphylococcus* species. *Propionibacterium acnes* is a common skin commensal bacterium and has encoded in the genome multiple lipases that degrade lipids in sebum[4]. In areas of the skin that are

considered moist such as the axilla, the predominate members of the microbiota are *Propionibacterium, Corynebacterium* and *Staphylococcus* species. On the palm of the hand, *Propionibacterium* (31.6%), *Streptococcus* (17.2%), *Staphylococcus* (8.3%), *Corynebacterium* (4.3%); and *Lactobacillus* (3.1%) are present[44,45]. The dominating bacteria in the pilosebaceous units, or pores, on the nose, is *Propionibacterium acnes* which accounts for 87% of the clones. Other bacteria isolated from the pores include *Staphylococcus epidermidis*, *Propionibacterium humerusii,* and *Propionibacterium granulosum*[46].

Areas of the skin that are not characterized by high moisture content or sebum production have a mix of bacterial populations from the Proteobacteria and Flavobacteriales[4]. On the forearm, *Propionibacterium* (22.0%), *Corynebacterium* (19.0%), *Staphylococcus* (11.1%), *Streptococcus* (5.8%), *Acinetobacter* (3.7%), and *Finegoldia* (1.3%) can be found[47]. On the skin surrounding the ankles, common bacteria have been identified include *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, *Chryseobacterium*, *Acinetobacter*, *Methylophilus*, *Acidithiobacillus*, *Segetibacter*, *Wautersiella* and *Psychrobacter*[4]. On the skin of the feet, the microbiota is dominated by bacteria from the genera *Staphylococcus*, *Acinetobacter*, *Kocuria*, *Corynebacterium* and *Micrococcus*[4]. Using phylogenetic marker 18S rRNA, predominant fungi species detected belonged to the *Malassezia* genus, including the most frequent *isolates Malassezia globosa*, *Malassezia restricta*, and *Malassezia sympodialis*[11]. These are lipophilic microbes that are associated with areas of the skin that produce significant

levels of sebum[48]. Little is known about the role of viruses as a part of the human skin and is still under investigation[14].

Microbial Dysbiosis in Skin-Related Diseases

An altered cutaneous microbiome has been shown to contribute to the high susceptibility of the skin to infections. Bacteria on the skin have important functions for skin health and healing[5,49,50]. However, the impact on the health and disease of skin by these microbes is still under investigation[4,13,14]. When disturbed, the skin microbiota can play a role in disease progression of many skin ailments including atopic dermatitis, acnes vulgaris, rosacea, psoriasis, and some skin cancers (**Table 1; Fig. 1**).

Atopic dermatitis: Atopic dermatitis is a chronic skin disorder that affects ~15% of US children and also affects adults[51]. This disorder is commonly associated with irritated skin that is colonized and/or infected by *Staphylococcus aureus*. The irritated skin can be found on almost any part of the body but is commonly localized on the antecubital fossa and the popliteal fossa. This skin disease can be effectively treated with topical or systemic antibiotics and steroids. Medicated baths are also used to decrease bacterial burden on the affected skin.

Acne vulgaris: Acne vulgaris is the most common skin disease in the US and affects 80% of the population[52]. Acne develops frequently on the face and upper torso and most often affects adolescents. It occurs when hair follicles and sebaceous glands are clogged and inflamed, resulting in the formation of comedones, papules, pustules, nodules, and/or cysts. Lesions of different stages frequently coexist, often in the same location. Nodules and cysts can be quite painful and may require medical attention and scarring as a result

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of these lesion can be a source of significant emotional distress. Treatment options can involve a combination of topical and systemic medications directed at treating or preventing the formation of acne. These treatment options include reducing sebum production, comedone formation, inflammation, bacterial counts and at normalizing keratinization. *Propionibacterium acnes* is a dominant skin bacterial species, in this disease. Other bacteria present in acne are Corynebacteria, Propionibacteria and Staphylococci[53].

Rosacea: Approximately 3% of the world population suffers from rosacea. Rosacea is commonly associated with flushing, but also presents itself as inflammatory nodules, non-transient erythema, papules, pustules, and telangiectasia[54,55]. There are four clinically based subtypes of rosacea: erythematotelangiectatic, papulopustular, phymatous, and ocular. Patients will experience the onset of rosacea usually after 30 with increased prevalence in women and individuals with fair-skin. In addition to genetic elements, other risk factors that contribute to rosacea include increased alcohol consumption and excessive UV exposure. While there is a genetic predisposition for rosacea, dysbiosis of the affected skin microbiota may be contributors. Rosacea occurs when both the innate and adaptive immune system is dysregulated (associated with chronic inflammation) which leads to the dysbiosis of microbes on the skin. Specifically, disturbances to the skin include altered TLR2 expression, high levels of the serine protease kallikrein 5, and abnormal expression of the cathelicidin antimicrobial peptide LL-37[56]. Bacterial species found to be present on the skin of rosacea patients include *Bacillus oleronius* and *Staphylococcus epidermidis*[55].

Psoriasis: Affects 2% of the world population and results from a combination of genetic and environmental factors. The pathology of this disorder includes hyperkeratosis, inflammation, hyperproliferation of keratinocytes, and increased angiogenesis. Although little is known about the role of the skin microbiome in psoriasis, the overall diversity of the microbiota overlaying psoriasis lesions was found to be greater than in healthy skin[53]. Firmicutes are predominant, whereas Actinobacteria are underrepresented in psoriatic lesions in comparison to healthy skin. It is not known whether these alterations in the bacterial population in psoriasis are a consequence of the disease or contribute to its pathogenesis. *Staphylococcus*, *Micrococcus,* and Planococcaceae, were the most abundant bacteria identified in patients with psoriasis[57].

Skin cancers: The association between microbiota and skin cancer is a recent proposition[58,59]. Although no pathogen has been found to cause or initiate the development of skin cancers, patients with certain chronic skin diseases may have an altered skin microbiota with colonization of specific pathogenic bacteria that increase the risks for developing cutaneous malignancies. For example, the microbiome of cutaneous melanomas and benign melanocytic nevi has been characterized by the presence of *Propionibacterium* as the most common bacterial genus in these lesions[60]. Common skin colonizers such as *Staphylococcus* and *Corynebacterium* are found in skin cancer, but various bacterial taxa not typically found on human skin were also identified. These bacterial genera include *Halomonas*, *Salinicola* and *Shewanella.*

The Microbiome and Normal Cutaneous Wound Healing

Wound healing is a dynamic process that involves cellular and molecular processes that must be precisely regulated both temporally and spatially. Healing of the skin is organized into several overlapping stages that require the concerted activation, proliferation, differentiation, and migration of many different cell types that reside in the skin tissue. The major stages of cutaneous wound healing include hemostasis, inflammatory phase, proliferative phase, and remodeling phase.

Hemostasis requires the release of factors that attract and activate circulating platelets to injured endothelial cells, forming the initial plug to stop the loss of blood. Clotting is achieved with additional molecular cascades to form fibrin in order to fortify the platelet plug with platelets trapped in the clot. The platelets release growth factors and chemokines into the immediate wound environment. Hemostasis is achieved once blood is no longer lost from the damaged blood vessels in the wound. At the same time blood vessels begin to dilate, allowing oxygen, nutrients, and chemotactic cytokines and leukocytes such as neutrophils, monocytes and macrophages into the wound tissue. Activated platelets also secrete growth factors that stimulate the keratinocytes at the wound edge to proliferate and migrate to re-epithelialize the wound.

The first inflammatory cells recruited to the wound bed are neutrophils[61]. Neutrophils clear the wound bed of cellular debris and microorganisms by secreting proteolytic enzymes such as matrix metalloproteases, elastase, reactive oxygen species, and cytokines[62,63]. Neutrophils also produce antimicrobial peptides (AMPs) which are multi-functional molecules that are capable of directly killing pathogens and stimulating

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cytokine production[64–68]. Some of these cytokines attract monocytes, which are leukocytes that circulate in the blood but when in the tissue differentiate into macrophages that play a critical role in the initiation, maintenance, and resolution of inflammation. Inflammatory macrophages (M1) play an important role in antigen presentation and have phagocytic abilities to clear dead neutrophils, bacteria and the debris that neutrophils leave behind in the process of killing pathogens[69]. Important genes involved in macrophage function include Toll-like receptors (TLRs), complement receptors, and Fc receptors, allowing macrophages to sense and respond to their environment[66,70,71]. They are also considered immunomodulators because they produce various cytokines and growth factors such as TNFα, IL-1, IL-6, IL-8, and IL-12. In conjunction with leukotrienes, prostaglandins increase vascular permeability and additional recruitment of inflammatory and immune cells.

Successful wound repair requires resolution of the inflammatory phase[72]. In the process, M1 macrophages secrete factors that result in either attracting a new wave of monocytes that in the tissue differentiate in M2 macrophages, or that stimulate the M1 macrophages to become M2 macrophages. Pro-healing M2 macrophages are cells that resolve inflammation and prepare the tissue for the next phase of healing. This can be achieved by downregulation of inflammatory molecules by anti-inflammatory cytokines such as IL-10 and TGFβ1 or upregulation of anti-inflammatory molecules such as IL-1 receptor antagonist. The mechanism of controlling the termination of the inflammatory phase of wound healing is still under investigation[69].

The inflammatory phase of wound repair overlaps with the proliferative phase

which involves re-epithelialization, formation of the healing tissue and angiogenesis.

During this phase, new tissue forms composed of keratinocyte, fibroblasts, macrophages,

lymphocytes, and endothelial cells that work in concert to rebuild the new tissue[73,74]. To

begin the repair of either damaged or missing dermal tissue, fibroblasts and endothelial

cells migrate and proliferate beneath the newly formed epidermis to generate new dermal

tissue called granulation tissue[69,75,76].

Fibroblasts can proliferate and differentiate into myofibroblasts, which assist

wound healing by contracting the healing tissue. They also secrete extracellular matrix

molecules, such as collagen, which contribute to strengthen the tissue and begin the

formation of the scar. Angiogenesis, a process by which new blood vessels develop by

proliferation and migration of endothelial cells, bring nutrients and oxygen to the wound

site to support the reconstruction of the healing tissue[69,75,76].

Remodeling is the final stage of wound healing and follows the completion of re-

epithelization and granulation tissue formation. The excess cells and ECM present in the

healing tissue now require to be removed for complete formation of the scar. This

primarily occurs by cell apoptosis[69]. Apoptosis, a form of cell death that is tightly

regulated and controlled, allows for the removal of the remaining inflammatory cells,

excess fibroblasts, and endothelial cells as well as ECM, without causing additional

damage to the wound tissue. The tensile strength of the tissue is enhanced by

myofibroblasts remodeling of various components of the extracellular matrix so that the

healing tissue becomes stronger and the skin barrier is re-established to protect the newly formed tissue from the environment[76,77]

In contrast to what is currently understood by those outside the wound healing field, microbial presence in the wound is critical for proper wound healing. A sterile wound does not lead to a better healing outcome. This is because microbes induce neutrophil and macrophage infiltration which, when properly regulated, clear wound of pathogens and activate proper healing.

Host-microbe interactions studies in skin further stress the complex relationship between wound healing processes and the skin-residing microbes[16,17]. For example, patients with diabetes are at risk of developing wounds that become infected and chronic. This increased risk may stem from a dysbiosis of the skin microbiota resulting from a different activated innate immune response. Studies surveying the skin over the toe-web space in 41 patients showed that the microbial composition and bacterial growth is different in patients with diabetes (ref?). Out of the 220 microbial colonies isolated from all samples, 95% of all colonies isolated were gram-positive bacteria (ref?). Gram-negative bacteria and fungal colonies accounted for only 2.7% and 0.9% respectively. *Staphylococcus epidermidis* was highly prevalent among diabetic patients. 77.5% of diabetic patients had *Staphylococcus epidermidis* compared to 53.7% of patient without diabetes. On the other hand, *Bacillus* species were isolated from 34% of patients without diabetes; only 15% of diabetic patients was found to have *Bacillus* species (ref?).

The importance of the microbiome in the skin for proper healing has become more and more apparent. Microorganisms on the skin, whether commensal or pathogenic, have

a close relationship with wound healing because they interact with the immune system, and they can play a major role in determining the strength of the inflammatory response. However, if the inflammatory response of wound healing is not properly regulated, chronic wounds can develop.

One study surveying the bacterial microbiome in acute wounds shows that as wounds heal, the relative abundances of *Bevibacterium*, *Microbacterium*, *Mycobacterium* and *Paracoccus* species significantly change over time. A reciprocal relationship between the relative abundance of *Staphylococcus* and *Propionibacterium* was found to be significant 21 days post-injury. The relative abundance of *Staphylococcus* decreased as the abundance of *Propionibacterium* increased. The increase in *Propionibacterium* was positively correlated with collagen levels in the wound as a result of stimulating the immune system. Ultimately, predominant genera in wounds that heal are similar to that of healthy skin. How the process of wound healing leads to this outcome is still under investigation.

Several animal models have been used to elucidate the fundamentals of host-microbe interactions during wound healing. Using a diabetic mice model for cutaneous wound healing, wounds swabbed during the course of healing show that the wound microbiome is very complex and dynamic as the wound begins to heal and ultimately close[78]. The diverse microbiome of the healing wounds includes *Cutibacterium acnes*, *Achromobacter*, *Delftia*., and *Escherichia coli*. Results from these studies have only scratched the surface of the complexity between the microbiota and the host during cutaneous wound healing.

The Microbiome and Cutaneous Chronic Wound Development

Chronic wounds develop as a result of defective wound healing regulation, that is, deviation from the highly coordinated processes describe above that are involved in wound healing. Chronic wounds are characterized by having increased levels of oxidative stress, chronic inflammation, damaged microvasculature, and abnormal collagen matrix deposition[78,79]. Most chronic wounds occur in patients with underlying pathologies or systemic diseases, leading to an aberrant and/or impaired immune response. Tissues that are ischemic, necrotic, and hypoxic have high levels of oxidative stress that contributes for a microenvironment that is conducive to colonization and proliferation of potentially pathological microbial populations.

Oxidative stress, both endogenously and exogenously produced in the wound, can delay wound closure, providing time for infection to occur and further prevent healing in a timely manner. During wound healing, if the inflammatory phase becomes chronic, the host tissue undergoes extensive damage due to a continuous influx of inflammatory cells that release cytotoxic enzymes, increase in free oxygen radicals, and inflammatory mediators, resulting in cell death. The physiological balance between tissue destruction and tissue reassembly is significantly disturbed, stemming from prolonged inflammation and overall catabolic proteolytic enzyme activity[80]. They are observed in humans with chronic metabolic diseases such as diabetes, which have many complications related closely to elevated levels of oxidative stress.

The most common chronic wounds found in patients are venous and arterial ulcers, pressure ulcers, and diabetic foot ulcers. In these wounds, the healing process is

complicated by pathogenic opportunistic bacteria and fungi that take advantage of host

nutrients that are leeched in the destructive inflammatory microenvironment. These

microbes contribute to the damaging of the host tissue when they form biofilm[19,49,69,81–84].

These biofilms are recalcitrant to conventional antibiotic therapies because the structure

of the biofilm decreases the efficacy of antibiotic therapy by significantly decreasing their

diffusion rate. The biofilm extracellular polymeric substance (EPS) also helps the

bacteria in the biofilm evade the host innate and adaptive immune system[85–87].

In the destructive microenvironment of chronic wounds, biofilm-forming bacteria

take advantage of host nutrients and contribute to the damage of the host tissue. These

biofilms are difficult to control and remove because the EPS which is composed of

proteins, DNA, RNA, lipids and polysaccharides allows bacteria harbored within it to be

tolerant to conventional antibiotic therapies and evade the host's innate and adaptive

immune response. A multitude of aerobic and anaerobic bacterial species across the

phylogenetic tree, aggregate into sessile microcolonies that harbor within these biofilms.

Each species in the biofilm differs significantly from their planktonic counterparts in their

morphology, mode of communication, and metabolism with very different nutritional

demands and roles. At the same time, stochastic processes and nonuniform gene

expression can lead to the appearance of subpopulations of bacteria with different

phenotypes and environmental responses. The biofilm provides a unique environment to-

cell signaling by the production of, and detection of quorum-sensing molecules, which

promote collective behavior such as optimizing nutrient acquisition and regulation of

virulence, leading to sustained pathogenicity in the wound[84,88]. They can also evade the host's innate immune response[89,90].

In addition, many bacterial species rely on quorum sensing to regulate much of their physiological processes; it is also a central mechanism in regulating the social activities of a population[91,92]. This mechanism relies on small diffusible signaling molecules produced by bacterial cells to detect and respond to other bacterial cells in the population[92]. Quorum sensing allows bacteria to organize the individual activities of a single bacterium in favorable microbial interactions in order to achieve population-level virulence in bacterial infections[92]. Their use of auto-inducer signal molecules significantly increases virulence by mediating subsequent transcription of hundreds of virulence genes. Studies have shown that quorum sensing is necessary for pathogenic bacteria to reach a critical cell density that overwhelms the immune system or defense mechanisms through the expression of virulence factors, initiating the symptoms of infection and resulting in the development of the infectious disease. Quorum sensing has a close relationship with bacterial biofilms because the development of biofilm is induced and regulated by many quorum sensing genes in addition to environmental factors in the host[92]. *Pseudomonas aeruginosa* is a common opportunistic pathogen highly associated with various chronic infections, including various etiologies of chronic wounds. A prominent feature of *P. aeruginosa* is to use multiple quorum sensing systems to increase virulence and establish infection quickly by evading the host innate and adaptive immune systems.

Specific Chronic Wounds and Their Wound Microbiome

The microenvironment of chronic wounds provides optimal conditions that can support different bacterial species with multiple abiotic and nutritional needs. Sequencing for bacterial 16S rRNA in several chronic wound studies show that no single microorganism has been identified to be the causal agent of infection/biofilm in chronic wounds, adding to the complexity of chronic wounds. Multiple studies surveying cutaneous chronic wounds in humans show that chronic wounds are colonized by many bacterial species (**Table 2; Fig.1**). Various types of chronic wounds can be colonized by the same bacterial genus and species. Common bacteria found in human chronic wounds include, but are not limited to Corynebacteria, Bacteroides, *Peptoniphilus sp.*, *Fingoldia sp.*, *Anaerococcus sp.*, *Streptococcus sp.*, *Serratia sp.*, Clostridiales, and Streptococcaceae. Many of the bacteria identified in human wounds have also been identified in a diabetic chronic wound murine model. Non-healing chronic wounds in 5–6-month-old diabetic mice had wounds colonized by biofilm-forming bacterial species such as *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Corynebacterium frankenforstense*, and *Acinetobacter sp*[93].

Venous ulcers: Venous ulcers form as a result of poor blood circulation and chronic venous insufficiency[94] and develop due to poor venous pressure or sustained venous hypertension because valves in the calf are unable to maintain proper blood pressure[94,,95]. Up to 50% of venous ulcers form because of superficial venous insufficiency and/or incompetent perforating veins[95]. They usually form on the calf and ankle, with 95% of venous ulceration in the gaiter area of the leg. The wound bed of the ulcer usually has a

fibrinous layer mixed with granulation tissue and in general it has an irregular wound edge[95]. Patients with history of smoking or diabetes are at risk of developing arterial ulcers. Other important risk factors include hypertension, and obesity. Venous leg ulcers are often infected; predominant microbes found in these ulcers include *Staphylococcus aureus*, *Corynebacterium, Pseudomonas aeruginosa*, *Serratia* and *Streptococci* bacteria.

Pressure ulcers: Pressure ulcers are injuries of the skin or underlying tissue that occur as a result of pressure alone or in combination with shearing forces over bony prominences[94]. Age is an important risk factor for pressure ulcers with elderly or disabled patients. Patients who constantly reside in a bed or wheelchair can present with pressure ulcers. These ulcers start developing when soft tissues, most commonly between a surface and a bony prominence is compressed for 2–6 h, resulting in tissue damage[94]. Specifically, the formation of an ulcer follows sustaining high pressure and shear forces in aged skin. Aged skin contains reduced levels of elastin, and friction leads to superficial erosions and blisters, resulting in hypoxia and tissue necrosis. When the wound opens it becomes exposed to damaged tissue, to excess moisture from urine, sweat, and even fecal matter[94]. Patients with diabetes mellitus are associated with increased risk of developing pressure ulcers. Other risk factors include paralysis, cardiovascular diseases, unconsciousness, malnutrition, and fecal incontinence. Pressure ulcers are often colonized by many bacteria species[96–98]. Common bacteria include *Streptococcus*, *Peptoniphilus, Anaeroccocus, Gemella*, *Staphylococcus*, *Corynebacterium*, Enterobacteraceae , *Anaerococcus*[97], *Acinetobacter*, *Finegoldia*, *Micrococcs*, *Enhydrobacter*, *Prevotella*, *Lachnospiraceae*, *Blautia*, *Lactobacillus*, and *Enterococcus.*

Diabetic foot ulcers: Diabetic foot ulcers are commonly found in the foot and are high-risk cutaneous wounds for patients with diabetes mellitus, resulting in high incidence of hospitalization and increased risk for amputation if the antibiotic treatments are not effective in controlling infections. They are the result of nonhealing lesions due to neurological disorders, diabetic neuropathy, and peripheral vascular disease of lower limbs[94]. The presence of peripheral neuropathy significantly increases the risk of developing diabetic foot ulcers because lacking proper sensory greatly reduces the patient's awareness of a mechanical trauma in the foot[97]. Patients cannot feel the pressure, pain and discomfort normally elicited by an injury and when the injury is not addressed, ulceration is the result[94]. Common causes for diabetic foot ulcers are neuropathy and ischemia but complications from pathology of diabetes itself, including altered regulation in blood flow and impaired oxygenation of extremities, greatly increase the risk of developing diabetic foot ulcers. Most patients with diabetic foot ulcers show symptoms of infection and may require hospitalization. Diabetic foot ulcers are colonized by a variety of bacteria species. This includes *Acinetobacter.* [81–83,99,100], *Bacillus*[83,85,100,101], *Enterobacter*[81–83,99,100,102], *Escherichia.*[81,82,85,99,100,103], *Propionibacterium*[83,85,101], *Enterococcus*[82,83,100–103], *Pseudomonas*[82,83,85,99,101–103], and *Staphylococcus*[82,83,85,100–102].

Common Techniques for Surveying the Skin Microbiota

To begin understanding the nature of the skin microbiota and its effect on skin function, new methods and technologies were developed to survey the microbiota. Methods to identify primary infectious microbes previously relied on culture-dependent

methods. This led the identification of important wound pathogens that commonly colonized wounds with impaired healing. These culture-dependent techniques, while still commonly used today in the health clinic, were dependent on the use of specific media that catered to the growth needs of the microbe. Thus, this method cannot be reliably used to evaluate complex multi-metabolism-dependent microbial communities, which need specific culture conditions to grow. For example, strictly anaerobic microbes cannot grow in conditions where oxygen is present in high concentrations, and thus, require a strictly anoxic environment or very low oxygen concentration in growth chambers to facilitate growth. Other microbes may require specific unknown metabolites to grow and would consequently never grow artificially in media. Accordingly, microbes requiring the complex microenvironment of human skin will not consistently grow in simplified artificial media and will be missed in these culture-dependent surveys.

To overcome the inability to evaluate complex microbiomes, culture-independent methods that do not need specific culture conditions required to grow different bacterial taxa, were developed[104]. Culture-independent metagenomic techniques has been used to describe the microbial diversity of complex communities, such as the wound-associated microbiome[105,106]. One of the most common methods is high-throughput sequencing of bacterial 16S ribosomal RNA (16S rRNA) genes[19,105,107]. Fungal species can be identified by sequencing the 18S rRNA. Results from these methods showed that the microbiome of wounds are complex and cross both bacterial and eukaryotic domains. Dynamic changes in relative abundances between these microbes over time are shown to correspond to healing and impaired wounds.

Other techniques of culture-independent methods such as sequencing the internal transcribed space (ITS) or shotgun metagenomic sequencing are now being used to survey complex wound microbiomes across domains with dynamic interactions between microbes over time corresponding to healing and non-healing wounds[14,19,21,49]. In the ITS method, the genomic ITS rRNA gene is targeted over traditional 16S rRNA sequencing in order to obtain taxonomical information about the bacterial community. The ITS region offers species and subspecies-level resolution because this region could harbor higher sequence variations[108]. While sequencing the 16S rRNA revealed the complicated nature of microbiomes, the data was commonly represented at the genus taxonomical level which made interpretation of the data difficult because bacteria act as either pathogens or commensal bacteria at the species level. Other bacteria need to be identified at the subspecies or strain levels to identify its status of pathogenicity[109].

Skin Microbiota Can Be Manipulated to Prevent or Treat Skin-Associated Aliments

The composition of commensal bacterial species is relatively stable over time but can be perturbed during infection or disease. Recently, interest in manipulating the microbiota has come to play to study potential therapeutic strategies for the treatment and study of diseases and improve the health of patients affected by skin ailments. The goal is to limit the growth of a few pathogenic species and support the growth of commensal or beneficial bacterial species to improve overall health. Thus, manipulations of the microbiota may directly promote healing by reducing the presence of pathogens and enhancing the abundance of beneficial species in the affected area. Probiotics, microbiota transplants, and even prebiotics have been heavily researched in the gut microbiota and

have been used to treat many gut-related conditions and diseases. While recent research has shown a strong relationship between skin health and probiotics, the mechanisms by which probiotics, facilitate improved skin health remains unclear.

Probiotics: Live microbial supplements may be beneficial to health by improving the microbiota community of the skin[43]. Providing beneficial skin probiotics may promote homeostasis by influencing both the skin tissue and cells as well as the bacteria that reside on the skin[7]. Because the skin is a complex ecosystem with many interactions between microbes and skin cells, probiotics may be beneficial in treating skin diseases or ailments that are caused by an imbalance of microorganisms[2]. Both diseased and healthy skin may be affected by probiotic treatment. Research on skin health benefits of probiotics indicates that oral consumption might reduce skin sensitivity and support skin immune function. Oral ingestion of several probiotic Lactobacillus species, including *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus casei*, and *Lactobacillus bulgaris* improve the health of the skin. Benefits include improving the skin immune system, decreasing the neurosensitivity of reactive skin, and reducing transepidermal water loss[52]. Lactobacilli bacteria is a major group of probiotics that can modulate the host immune response in the gut by binding to pattern recognition receptors expressed on a number of immune cells and other tissues including the intestinal epithelium. Research has also shown that probiotics can help maintain skin homeostasis by protecting against UV-induced skin damage[110]. How probiotics can affect other skin ailments such as psoriasis and rosacea are yet to be explored[2].

Skin microbiota transplant: While success has been found in the gut, the most prominent example of this procedure has been shown with the treatment of the antibiotic-resistant bacteria *Clostridium difficile* within the gut microbiome with the help of fecal transplantation[111]. Following this successful treatment, many projects have been focused on developing treatments for gut diseases through the use of microbiome transplants. Efforts to manipulate the skin microbiome have been made in using microbiome transplants as a novel therapeutic approach for skin diseases and conditions. *Cutibacterium acnes* and its strain diversity represents a major part of the human skin microbiome, and specific bacteria strains are associated with acne vulgaris. An effort was made to determine if this subpopulation of species can modulate at the strain level. It has been shown that the human skin microbiome composition can be modulated through approaches similar to those used in fecal transplantation of the gut microbiome. Probiotic solutions from donor microbiomes have been applied onto healthy volunteers, whose skin microbiome was monitored during and after the treatment[112]. The dose of bacteria to be applied played an important role in the capacity of modulation. Indeed, temporary modulation of the *Cutibacterium acnes* population at the strain level was feasible without a negative reaction of the host. It is important to note that all subjects underwent dermatological inspection in order to establish that no adverse effects were detected.

Prebiotics: Prebiotics are chemicals or metabolites that are beneficial to commensal bacteria of the gut and skin in order to improve the health of the gut and skin[113,114]. This means that prebiotics need to be specific or targeted enough to only impact commensal bacteria, as opposed to the pathogenic bacteria, in order to have a positive effect on the

organ or tissue. This is because the beneficial or commensal bacteria should be selectively stimulated to grow over the bacteria that can potentially cause disease or infection. Prebiotics can be as simple as simple sugars or molecules but can be as complex as oligomers such as galactooligosaccharides. Examples of prebiotics include glycerin which supports *Cutibacterium acnes*. The cosmetic industry has long utilized extracts from natural sources, such as plants and yeast, to stimulate cellular processes in the skin to enhance the appearance of skin[113,114]. Research on plant extracts such as ginseng, black currant, and pine, can be formulated into cosmetic products to be applied to the skin. This research has been conducted to begin elucidating the role of plant extracts on skin microbiota. Daily application of such products for three weeks showed inhibition of the growth of *Propionibacterium acnes* while the growth of coagulase-negative staphylococci bacteria that are beneficial to the skin was not affected[114].

Perspective

Thus far, studies have focused on understanding how different cell types, extracellular matrix components, and signaling molecules play a role in maintaining healthy skin and how they contribute to cutaneous wound healing. However, studies on the contributions of the skin microbiota to skin health, disease, and impaired wounds have lagged behind even though microbes are acknowledged as an important factor in these processes. Whereas key infectious microbes were identified through culture-dependent methods, identifying the myriad of microbes residing in the skin and wound has been a challenge because different microbes have specific, most often time unknown, growing conditions that have not yet be replicated in culture. In the past decade, after

appreciating the complexity of the gut microbiota and its role in gut health and disease, there was a strong push to describe the skin microbiota and to define the microbiota present in healing and in chronic wounds. With the development and advancement of high-throughput sequencing platforms and the support of large databases, physicians and scientists were able to show how complicated and complex the microbial community of the skin is.

Once microbes that significantly contribute towards wound healing or chronic wound development are known, experiments that elucidate the functional role of the microbes must be conducted. With shotgun metagenomic sequencing platforms, and with bioinformatics and statistics support becoming more prevalent, the information from these sequencing runs provides insight beyond taxonomical identification. Gene-level metagenomic profiles can characterize not just the identification of the microbe, but also the genetic profile of the microbiota. Genes important for virulence, biofilm formation, quorum sensing, antibiotic resistance, and host-defense evasion, can be identified as potential targets for new drugs and antibiotics. Several publicly available databases that include bacterial genes can help identify key processes and signaling pathways in order to generate new hypotheses that can be explored both *in vitro* and *in vivo* using animal models in order to study mechanisms.

Other large data-driven experiments such as transcriptomics experiments, can identify genes that are activated and transcribed during infection or toxic stress in response to drug therapeutics. Proteomic and metabolomic studies can provide

information about how microbes are able to evade host innate and adaptive immunity and provide insight into which proteins or metabolites should be targeted in future studies.

Altogether, the future to understanding how microbes contribute towards healthy skin and skin-based ailments will result from hypothesis-driven experiments researching the host-microbe interactions between the skin of the host and the microbial community that resides on the skin. With the discovery and treatment with antibiotics, many bacterial infections, which previously had high infection, morbidity, and mortality rates, can be treated. Whereas the battle against primary pathogens continues, the number of antibiotic-resistant pathogens are on the rise. In addition, the number of cases involving opportunistic pathogens in patients with compromised innate and adaptive immune systems will continue to increase dramatically. It will be necessary to study how the health of patients, who may or may not exhibit symptoms of a disease or condition, will interact with pathogenic members of the microbial community to result in infection.

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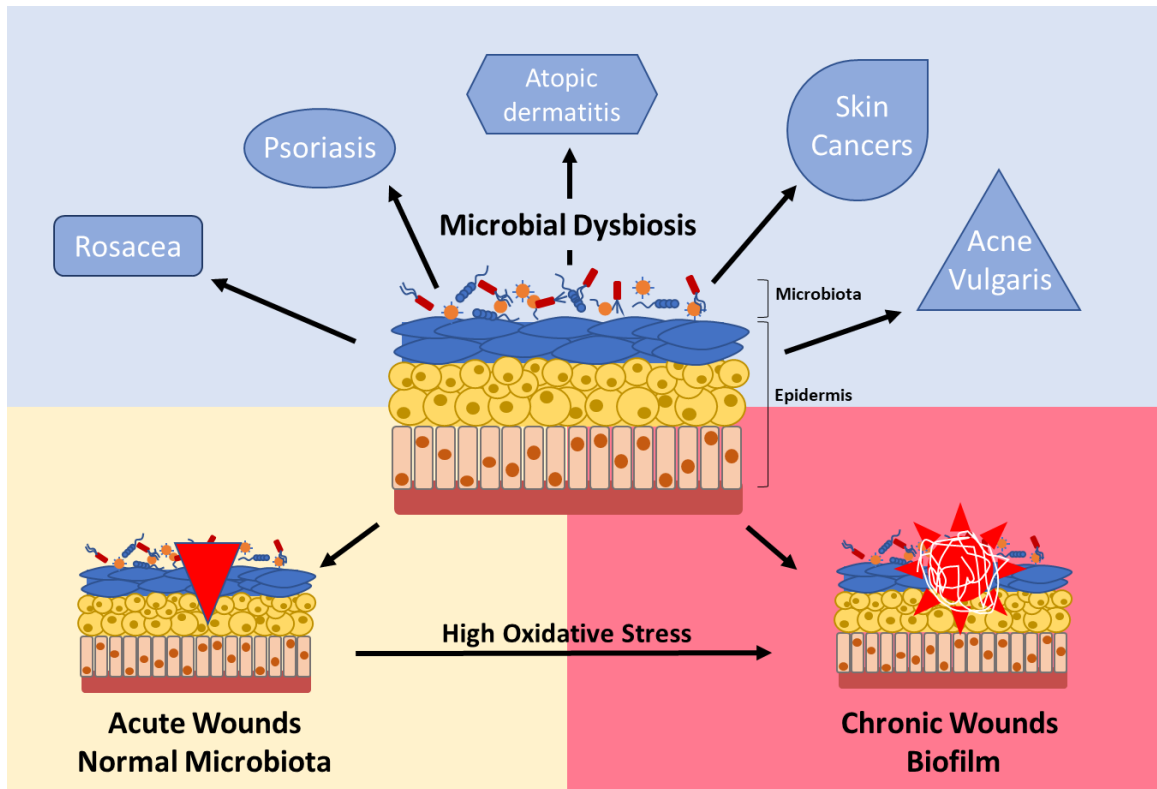
Figure 1: **Top**: **The skin microbiota plays a key role in maintaining skin integrity and health.** However, microbial dysbiosis is commonly seen across many skin-related diseases, including Rosacea, Psoriasis, Atopic Dermatitis, Acne Vulgaris, and Skin Cancers. **Bottom left**: Normal microbial presence in the wound tissue is critical for proper healing as bacteria initiate key inflammatory responses such as those performed by neutrophils and macrophages. **Bottom right**: Under high oxidative stress conditions, chronic inflammation sets in causing extensive damage to the wound tissue. This, in addition to the presence of pathogen opportunistic bacteria, hinders the tissue's ability to fight of infection and results in the formation of biofilm (white wiggles) on a wound that fails to heal.

Dissertation Summary

In this dissertation, levels of oxidative stress and the skin/wound bacteriome were determined to be factors that contribute towards the initiation and development of chronic wounds in an obese and diabetic mouse model. Using the *db/db*$^{-/-}$ mouse model, I showed that that the degree of chronic wounds development and strength of bacterial biofilm formation, was initiated when specified levels of specific inhibitors for catalase and glutathione peroxidase were administered in the mouse (Chapter 1). The development of chronic wounds was directly proportional to the endogenous levels of oxidative stress in the wound tissue. Treatment of low dose of 3-amino-1, 2, 4-triazole (ATZ) to inhibit catalase and low dose of mercaptosuccinic acid (MSA) to inhibit glutathione peroxidase led to the development of wounds that healed in a slightly delayed timeframe compared to wounds that healed with basal levels of oxidative stress. Wounds had higher diversity of bacteria present without the formation of biofilm. When the wounds heal, the quality of healing was similar to that of wounds with basal levels of oxidative stress. Wounds treated with higher doses of ATZ and MSA lead to delays in wound closure, significantly increasing the time it took for the wounds to undergo re-epithelialization. There was higher degree of tissue damage, and the wound area was significantly larger with strong bacterial biofilm formation. When wounds were treated with high doses of inhibitors to significantly increase oxidative stress levels, wounds did not close, and biofilm formation was observed to initiate within 2–3 days after wounding. The effect of oxidative stress on the bacterial community that colonize the wounds was found to be inverse and dose dependent. Wounds with higher levels of oxidative stress showed decreases in the

diversity of the wound microbiome with dynamic shifts that favored fewer but stronger biofilm-forming bacterial species colonizing the wounds. In contrast, wounds with low levels of oxidative stress had higher bacteria diversity that potentially aided in proper wound closure. Moreover, when mature bacterial biofilms, transplanted from another chronic wound, are place on new excision wounds without increasing the levels of oxidative stress, wounds were able to close and not develop into chronic wounds. On the other hand, high oxidative stress levels in the wound tissue in the absence of the skin microbiome do not lead to chronic wounds.

In the second chapter, sequencing the bacterial ITS rRNA gene over the course of healing or chronic wound development, revealed that the wound microbiome was complex, dynamic, and could be altered or disturbed by high levels of oxidative stress. The bacterial community of the wounds were sequenced by the bacterial rRNA internal transcribed spacer (ITS) gene of the wound microbiota from wound initiation to fully developed chronic wounds. We found again that wound microenvironment with high levels of oxidative stress had significantly less microbiota diversity that stimulated biofilm-forming bacteria to colonize and form biofilm. In contrast, the microbiota of non-chronic wounds is very diverse, and the bacteria present on the wound does not form biofilm. Even though some of the species are biofilm-forming bacteria, they are present in the wound during healing and closure. Using indicator species analysis, which considers a species' fidelity and specificity, certain bacterial species were determined to be strongly associated with healing wounds or chronic wounds. Several clinically relevant species that are present in human chronic wounds, such as *Cutibacterium acnes*,

Achromobacter sp., Delftia sp., and *Escherichia coli*, were highly associated with healing wounds. These species may possibly be used as probiotics to treat chronic wounds in humans. *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Corynebacterium frankenforstense*, and *Acinetobacter sp*. were associated with chronic wounds. These bacteria are clinically relevant meaning that they are also found in chronic wounds of patients to significantly delay healing.

In the third chapter, the interaction between the skin and the skin/wound microbiota and how the relationship affects health and disease is reviewed. The skin being a protective barrier of the body has the important role of withstanding physical, chemical, and biological assaults from pathogens. Should the skin be injured and prevented from fulfilling this role, the skin is capable of healing itself by activating the highly complex process of cutaneous wound healing. Normal healing will lead to quick wound closure, elimination of microbes and re-fortify the damaged area to restore the protective functions of the skin. The relationship between the skin and the microbiota is discussed over several skin ailments (atopic dermatitis, acne vulgaris, rosacea, and psoriasis) and wound types (venous ulcers, pressure ulcers, and diabetic foot ulcers), especially about the microbiota. The review closes with a review on an exciting new generation of therapeutics: manipulation of the skin microbiota through probiotics, prebiotics and skin microbiota transplants as a method of preventing and treating skin ailments and the different wounds etiologies.

Dissertation Conclusion

From my work on using the chronic wound model, two important factors are determined to be the driver for chronic wound initiation and development: Oxidative stress and microbes on the skin. Oxidative stress in normal skin is an absolute critical component of and regulator of cutaneous wound healing. In healthy skin, normal or basal levels of oxidative stress after injury establish an environment that is conducive for the initiation of the wound healing program involves the four overlapping phases of healing: homeostasis, inflammation, granulation tissue formation, and tissue remodeling. When highly reactive oxygen and nitrogenous species levels are abnormally high, DNA, proteins, metabolites and lipids in the wound can be indiscriminately damaged. If this damage to basic macromolecules cannot be reversed in a timely manner, this signals to cells and tissues to abandon and forego the highly concerted program of wound healing and instead undergo unregulated cell death in the form of necrosis, which is in itself a damaging process for wound tissue. The skin is colonized by many species of microbes, spanning across the domains, from bacteria and fungi to viruses and protists. While investigations into the skin microbiota is ongoing, it can be concluded that not all of the species colonizing the skin are pathogens and detrimental to skin. Many species are neutral or commensal while other may aid in the protective role of the skin. What can also be concluded is that, excluding primary pathogens that cause illness or disease even in healthy patients, many of the pathogens found in the skin microbiota are opportunistic, that unless the environmental conditions are conducive for infection, the potential pathogens will not infect the skin.

Many clinical factors have been identified as increasing the risk of chronic wound formation. The findings in this dissertation show that high oxidative stress levels and skin microbiota are both needed for the initiation and development of chronic wounds. Of the two factors, I propose that oxidative stress is very critical and a fundamental determining factor in providing a microenvironment conducive for chronic wound initiation and development. Skin ailing from high levels of oxidative stress or conditions that contribute towards increased levels of oxidative stress or inflammation alters the skin microbiota, decreasing diversity and increasing the risk of opportunistic pathogens in the native skin microbiota community to colonize and infect the skin. After microbial infection has been established, the complex interaction between wound microenvironment and the pathogens prevents healing and hinders conventional wound therapies. A better understanding of oxidative stress and the pathogenic bacterial interactions evolving in chronic wound microenvironment may lead to better solutions for chronic wound.