SKIN MICROBIOME RESEARCH AND THE EFFECT OF N-ACETYL CYSTEINE ON BIOFILM OF ENTEROBACTER CLOACAE

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ABSTRACT

Chronic wounds affect about seven million people in the United States and up to 2% in developed countries globally. Understanding the skin microbiome and the three different types of chronic wounds will allow for a greater understanding of the pathogenesis of chronic wounds and biofilm formation. Numerous research studies suggest that N-acetylcysteine (NAC), an antioxidant, can heal chronic wounds in diabetic mice. Studying the biofilm of different bacteria and working on a more developed understanding of the various mechanisms will help us develop more efficient and cost-effective methods of healing chronic wounds. A practical approach is the application of NAC in preventing biofilm development and formation. A specific bacterium will be isolated in this study, Enterobacter cloacae, from a chronic wound in a sterile manner out of the db-/db- (diabetic) mouse model. Using the method of crystal violet staining, I will be quantifying the effect of the NAC on the bacteria and the biofilm. This research will contribute to the development of a set of experiments for the bacteria Enterobacter cloacae. Additionally, I will perform comprehensive research on the skin microbiome and the environment of venous, pressure, and diabetic ulcers. This research will allow the Martins-Green Lab to study other bacteria strains in biofilm to ultimately develop a wound dressing that can dismantle biofilm in chronic wounds of diabetic patients and enable the wound to heal.

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TABLE OF CONTENTS

Table of Contents

PART ONE

THE EFFECT OF N-ACETYL CYSTEINE ON BIOFILM OF ENTEROBACTER CLOACAE

INTRODUCTION

Diabetic patients are more prone to chronic wound formation due to the high blood glucose levels causing stiffening of the arteries and narrowing of the blood vessels. The chronicity of the wound is due to the body's inability to follow through with the 'normal' wound healing mechanism. The wound's failure to properly heal provides an ideal environment for opportunistic bacteria to take over through biofilm development. Within the biofilm environment, different factors and molecular mechanisms allow it to withstand topical antibiotic treatments. A fundamental operation is the quorum sensing system, which allows communication between bacteria and allows for the activation or inhibition of survival genes.[3] Understanding the molecular mechanisms inside the biofilm and those involved with wound healing will allow for a more holistic approach to discovering a treatment for chronic wounds.

The normal wound healing process consists of multiple stages with interactions between different dermal cells, proteins, and controlled angiogenesis. [12] Wound healing consists of three main stages that overlap: inflammation stage, proliferation stage, and remodeling stage. [12] After tissue injury, the trapped cells within the clot trigger an inflammatory response releasing various chemoattractants. [13] Once the inflammation phase begins, neutrophils and macrophages dominate the wound and remove bacteria and other foreign materials by releasing enzymes. During the proliferation phase, the fibroblasts help produce the extracellular matrix by stimulating collagen, proteins, and glycoproteins. [12] The old and new tissue then work together to heal the ulcer. Throughout the remodeling phase, the wound has closed, and scarred tissue remodeling results in decreased blood flow and cell content to the area. [12] Various growth factors are essential in mediating and controlling cellular interactions that occur during wound

healing. These growth factors influence proliferation and the migration of inflammatory cells and protein synthesis. [2] Studies have shown that diabetic patients tend to have decreased levels of some of the growth factors found in the normal wound healing process. For instance, the Insulin-like Growth Factor is reduced in one of the epidermis layers in diabetic skin. Insulin-like growth factors can induce the activity of specific proteins that strengthen the wound. [2] Identifying the growth factors, such as the Insulin-like Growth Factor, plays an essential role in understanding interactions and components that execute a role in wound healing or chronic wound development.

Quorum sensing systems have been found to trigger the formation of biofilms. These microbial communities utilize quorum sensing molecules or autoinducers that control the different regulatory networks. The quorum-sensing system mediates the biofilm formation in Pseudomonas aeruginosa. [10] Additionally, the formation of biofilm extracellular matrix required cell-to-cell communication, which is exerted by signaling molecules. Quorum sensing autoinducers can turn on signaling molecules and transcriptional regulators, resulting in virulence and biofilm formation. [10] In comparison to the quorum-sensing pathway of Pseudomonas aeruginosa, Enterobacter Cloacae has fewer genes turned on. The number of activated genes indicates that it may be a weaker biofilm-inducing microorganism. [21 & 22] To further study NAC's mechanism on the biofilms of microorganisms, it is essential to consider developing future studies of its impact on the quorum-sensing systems and the Extra Polymeric Substances (EPS) located within the biofilm.

Impairment of the wound healing process in diabetic patients results in the formation of infection by biofilm-forming bacteria. Enterobacter Cloacae is a gram-negative bacterium and is widely spread in the environment. Enterobacter Cloacae can be found in humans' gastrointestinal

tract and different food products. [25] This bacterium can form a biofilm that adheres to various surfaces and is difficult to remove or kill. Many factors play a role in the strength of the formation of biofilm. These factors include the pH, temperature, osmolarity, and nutrients present in the medium. [25] The biofilm formation process is complex, and for this to occur, the bacteria must undergo different stages. This process starts with a planktonic stage when the bacteria adhere to the surface and then proceeds through the irreversible stage which occurs when the bacteria cannot easily be removed. [38] In a study evaluating the effect of different conditions on the sustainability of biofilm formation for the Enterobacter cloacae strain, researchers found four different biofilm phenotypes after incubation of the bacteria for 24 hours. [25] The study observed that the strains adhered to the plate well when incubated for 48 hours rather than 24 hours. These results suggest that the Enterobacter cloacae biofilm takes longer to grow and develop. This investigation also suggests that the biofilm Enterobacter cloacae strains can be grown in broth or agar mediums. [25] These results of this study are significant because one of the substantial growth methods utilized for Enterobacter cloacae in this set of experiments was the use of LB broth.

This study aims to understand Enterobacter cloacae's biofilm formation and the effect of a compound known as N-acetyl cysteine (NAC) on biofilm and bacteria growth. The methodology utilized to study this was the application of NAC to mature (developed biofilm) and immature biofilm (to bacteria without well-developed biofilm). Crystal violet staining was performed to quantify the results of this experiment.

MATERIALS & METHODS

This research study will use Enterobacter cloacae samples obtained from chronic wounds from db-/db- mice. Bacterial samples were frozen in glycerol at -80ºC. We will be using bacteria culture techniques to study the biofilm of Enterobacter cloacae. Before applying NAC treatments, the biofilm of the bacteria was grown in LB broth to compare observations to biofilms of other bacteria. Using OD absorbance reading, a growth curve of the bacteria, and an experiment to count CFU of bacteria at different concentrations over a period of time were performed. After completing observations of the bacteria, different NAC concentrations were applied. Before applying the NAC to the bacteria and biofilm, the solid NAC was dissolved into phosphate-buffered solution (PBS) and filter-sterilized using a 0.1-micron syringe filter. Dissolving the NAC ensures that there is little to no contamination in the experiment. To ensure that all experiments were performed uniformly and prevent contamination, the LB media was autoclaved, and diluted bacteria were added at a specific CFU. Using proper microbiology lab techniques, we carefully applied the bacteria to the 96-well plate and applied NAC as necessary based on the concentrations examined.

After adding the controls and bacteria onto the plate, we added 20ul of NAC into each well with the respective concentrations. Thus far, we have solidified that the higher concentrations of NAC, above 35 mg/ml and up to 120mg/ml, affect Enterobacter Cloacae's biofilm.

Following the NAC experiments, we will hopefully study the extracellular polymeric substances (EPS) of Enterobacter cloacae biofilm. These experiments will explore the interaction of the chemical compound NAC with the biofilm's EPS. Investigating the EPS can be done using different probes, suspending the cells through high-speed centrifugation in the biofilm, and

extracting the supernatant. Using various chemical solutions, we can purify the supernatant solution to remove soluble EPS and other intracellular macromolecules. The purification process will then allow us to extract the proteins and the other polymeric substances and run them on an SDS PAGE gel.

RESULTS

This study's main aim is to observe the effect of NAC on Enterobacter Cloacae as a biofilm-forming bacteria. Understanding the mechanisms within the biofilm will allow us to understand possible treatments for chronic wounds. The first step is to find an optimal concentration to ensure accuracy and consistency in all experiments performed. This started with a serial dilution to find the most optimal concentration for future investigations and establish OD600 CFU standard for the experiments. The OD600 was confirmed by conducting a growth curve at different time intervals allowing us to see the most appropriate concentrations. Based on the graph provided in **Figure 1A**, the line that follows an ideal growth curve for bacterial microorganisms suggests that the bacterial growth at 10^{6} CFU concentration is the most optimal.

To further study the biofilm formation for Enterobacter cloacae, a CFU Timed Biofilm experiment was performed. As shown in **Figure 1B**, the greater the concentration of bacteria, the greater the biofilm formation. This experiment also indicates that CFU or biofilm formation increases over time. In comparison to the 12hr and 24hr biofilm, there is a significant difference between the OD value. As the concentration of Enterobacter samples increases, the thicker and more apparent the biofilm. These observations are presented in **Figures 1C, 1D, and 1E.** This suggests that the higher the concentration of the bacteria found in a sample, the thicker and more opaque the biofilm.

To further investigate Enterobacter biofilm, an immature biofilm NAC experiment was performed to understand how the NAC compound influences and interacts with the biofilm. By immediately adding the NAC treatment before biofilm formation, we studied this compound's effectiveness with this particular bacterial strain. As shown in **Figure 2A**, the results of this experiment suggest that as NAC concentration increases, there is a slight decrease in the

absorbance reading. The OD590 indicates that there is an increased reading for the 6hr treatment than the 0hr. The difference in the data is due to the greater amount of time that biofilm and bacteria had to grow, allowing the biofilm to become more resistant to chemical treatment.

After studying NAC's effect on immature biofilm, we designed an experiment to study the effect of NAC treatment on mature biofilm. The mature biofilm is a result of allowing the bacteria to grow for 24hrs before treatment. As shown in **Figure 2B**, the longer the NAC treatment interacts with the biofilm, the more effective it is. Although more experimental evidence is needed, there does seem to be a slight difference between the 6hr and 24 hr OD readings, suggesting that the NAC effectiveness as a drug is time-dependent. **Figure 2C and 2D** provide the biofilm formation for the different time points. As provided by the results of this set of experiments, the higher the concentration of the NAC drug, the less opaque or weaker the biofilm formation.

CONCLUSION

Biofilms are strong microbial communities, consisting of a diverse assortment of bacterial communities known to cause infections. Chronic wounds are caused by biofilm communities that are difficult to dismantle with antibacterial treatments and host responses. The N-acetyl-cysteine(NAC) is an antioxidant effective at inhibiting biofilm formation and destroying biofilms. [37] NAC is a molecule derived from the amino acid cysteine; it decreases biofilm formation, inhibits bacterial adherence, and reduces EPS and cell viability production. [37] NAC's various medicinal benefits and uses provide evidence for its potential to treat diabetic chronic wounds.

In this study, we studied the effect of NAC on the biofilm model system of Enterobacter cloacae. Different concentrations of NAC were applied to study the impact of this compound intracellularly. The results of this investigation indicate that there is a chance of NAC inhibiting Enterobacter cloacae adherence. The ability of NAC to inhibit the formation of biofilm suggests that the NAC potentially reduces the production of EPS in the biofilm of Enterobacter cloacae. The results provided from these sets of experiments suggest that the NAC compound can potentially affect the formation of biofilm and bacteria. To further understand the effect of NAC on the biofilm of Enterobacter cloacae, the experiments performed would be repeated, and other investigations would be established. These experiments include applying different NAC concentrations with varying pH to understand the relationship between the biofilm's pH and pKa. Another experiment that would be beneficial to study the effect of NAC on the biofilm at a cellular and molecular level is to break down the biofilm. This can be done by centrifuging the biofilm and running the pellets on an agarose gel, enabling us to study the biofilm's protein and DNA. The EPS plays a significant role in the function, structure, and development of the

microbial aggregates. This research would allow for the observation and analysis of the Extracellular Polyacrylamide Structures (EPS) and NAC interaction.

To further advance the study of NAC's effect on the biofilm of Enterobacter cloacae, different molecules with analogous structures can be applied to determine the functional group that works to dismantle the biofilm. These molecules include N-acetyl cysteine amide (NACA), N-acetyl serine (NAS), cysteine (Cys), and glutathione (GTH). Another significant set of experiments that can be done to investigate the intracellular mechanisms is to examine the quorum sensing between the bacterial communities. Following the quorum sensing mechanism is an alternative approach to study the bacterial communication system. [3] Studying the quorum sensing mechanisms and detecting the signal molecules may identify an inhibitor to act as an antibiofilm agent. These potential experiments may aid in developing a treatment plan for the dismantling of chronic wound biofilm.

FIGURE LEGEND

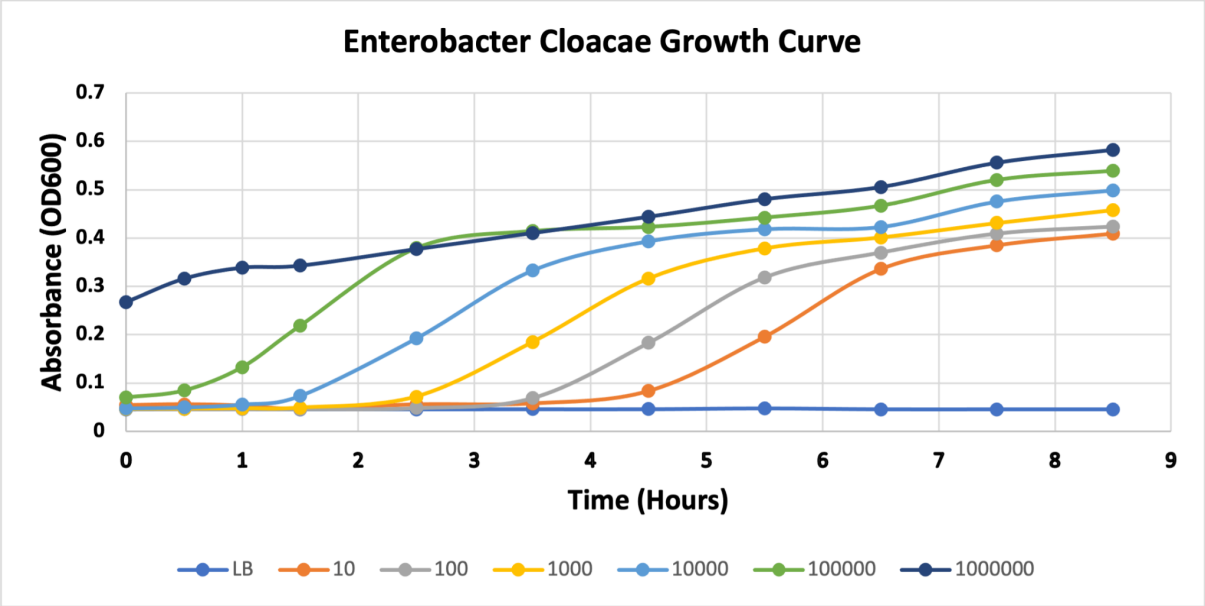

Figure 1A: Growth Curve of Enterobacter cloacae at different concentrations

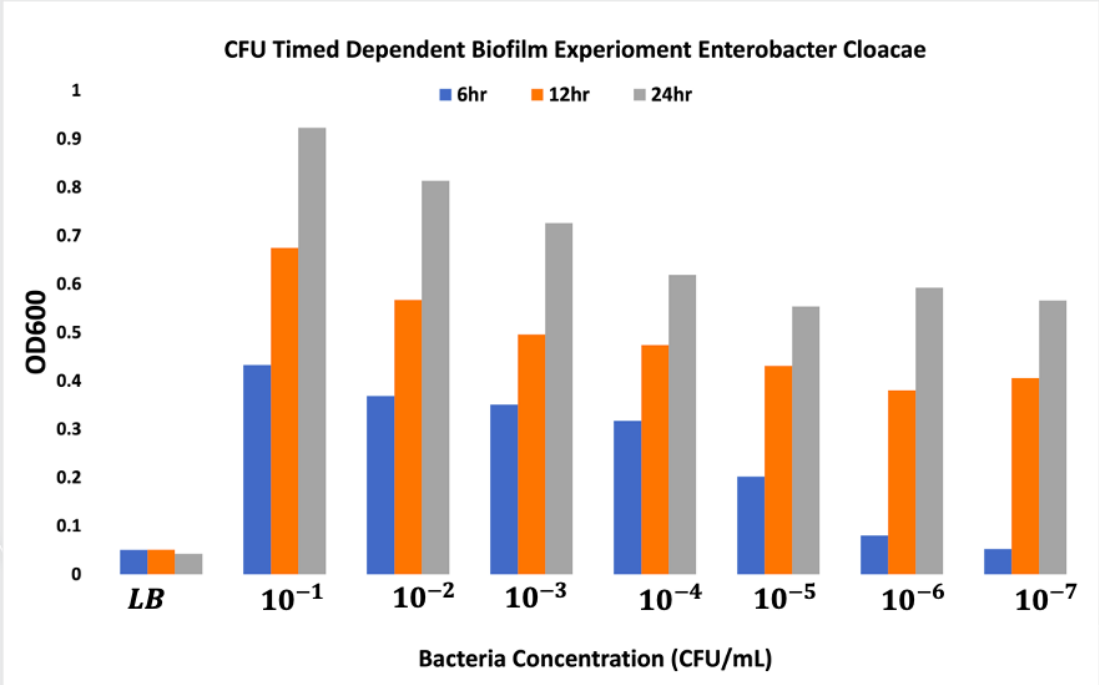

Figure 1B: Measure OD600 at different concentrations of Enterobacter cloacae growth at different time points

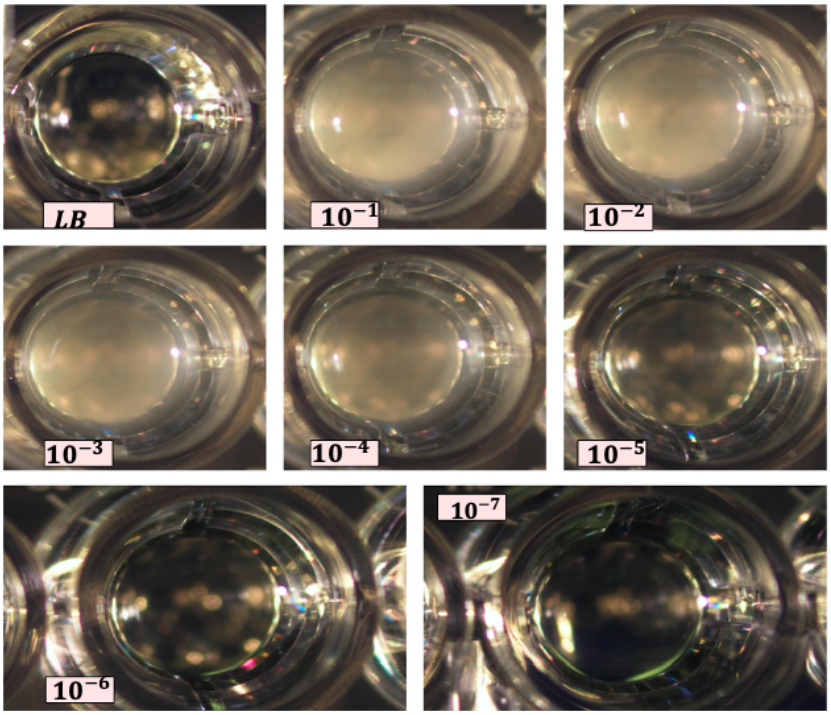

Figure 1C: 6 hour 96-well plate biofilm formation for all bacterial concentrations (CFU/mL)

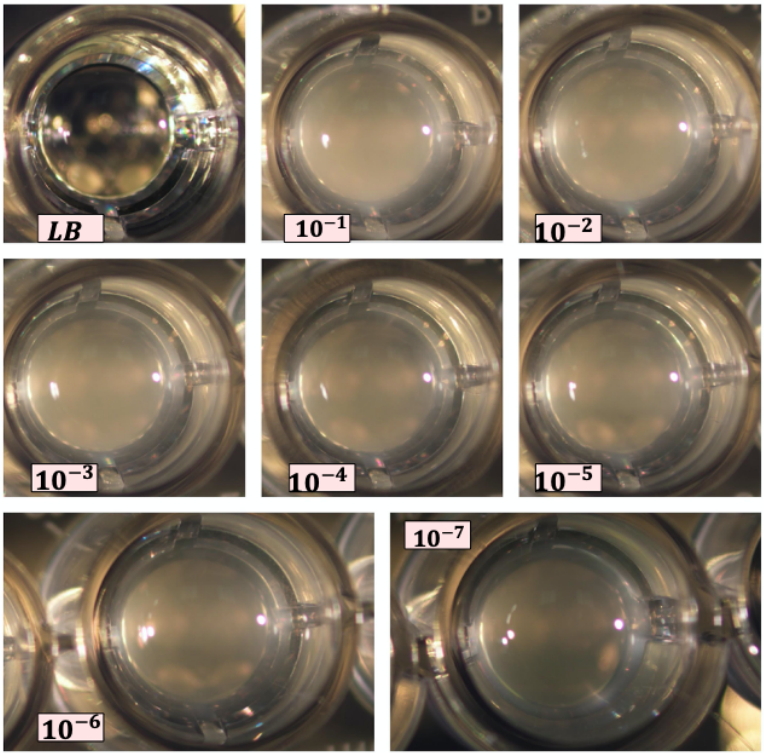

Figure 1D: 12 hour 96-well plate biofilm formation for all bacterial concentrations (CFU/mL

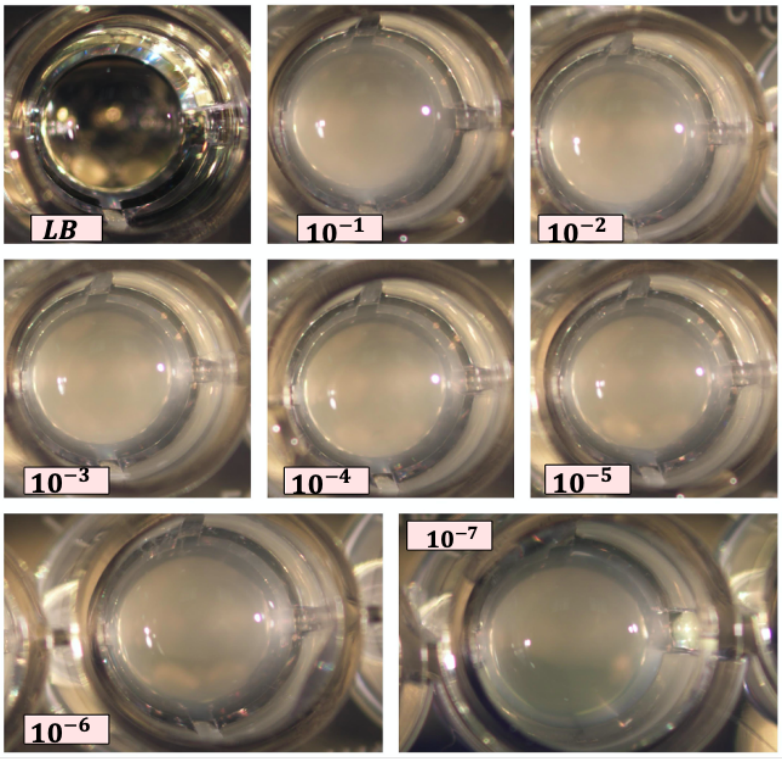

Figure 1E: 24 hour 96-well plate biofilm formation for all bacterial concentrations (CFU/mL)

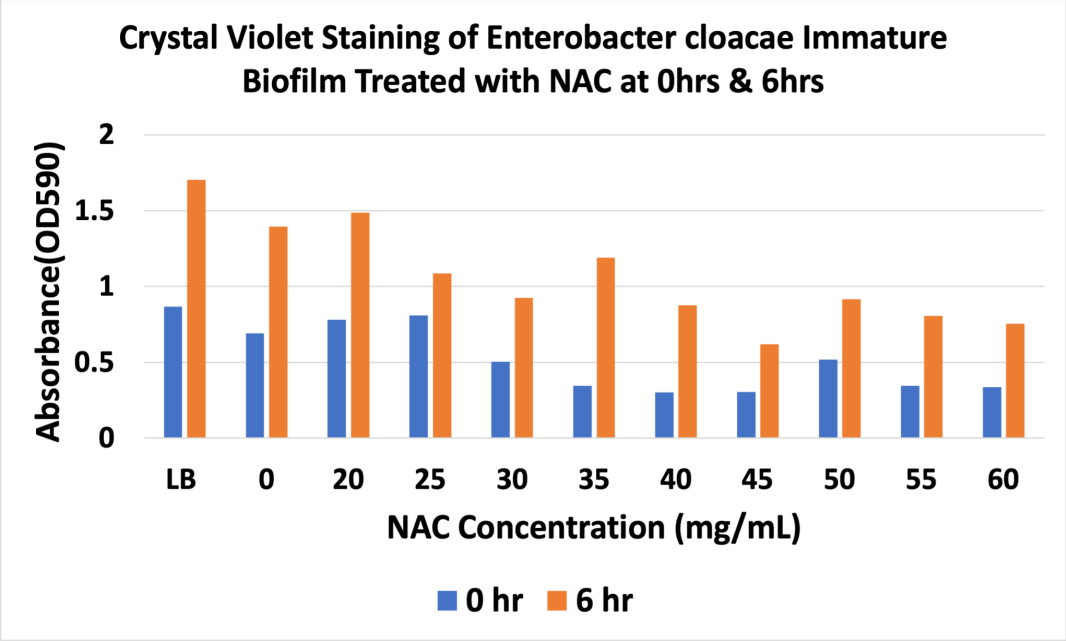

Figure 2A: Absorbance of Enterobacter cloacae at different concentrations of NAC. NAC applied prior to biofilm growth to observe effects of the compound on bacterial growth

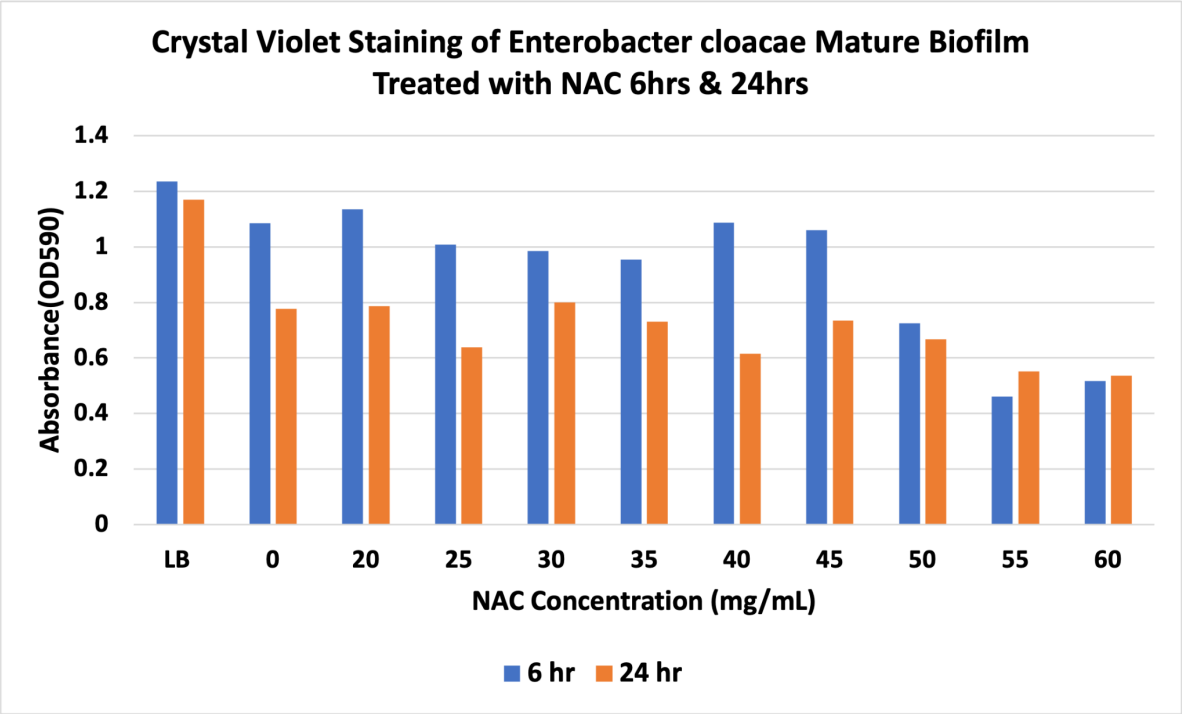

Figure 2B: Absorbance of Enterobacter cloacae at different concentrations of NAC. NAC applied during and after biofilm formation to observe effects on biofilm formation.

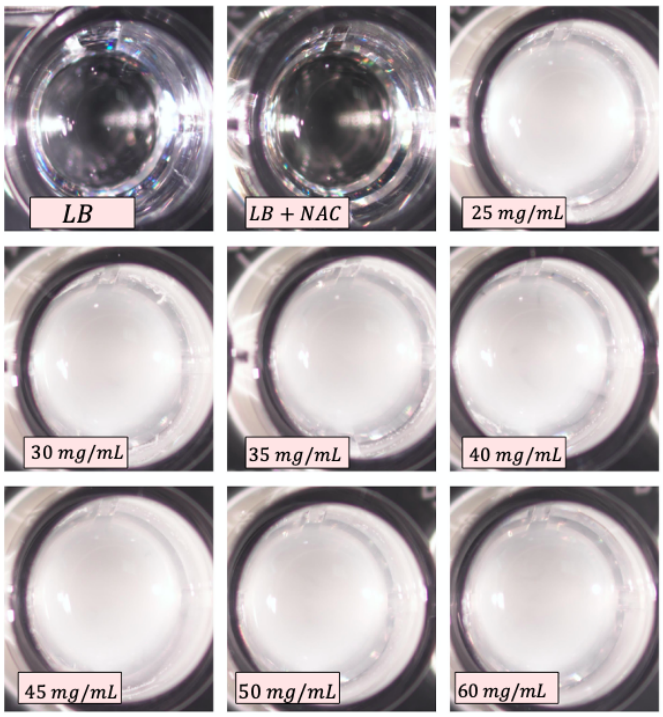

Figure 2C: 6 hr mature biofilm treated with NAC at different concentrations

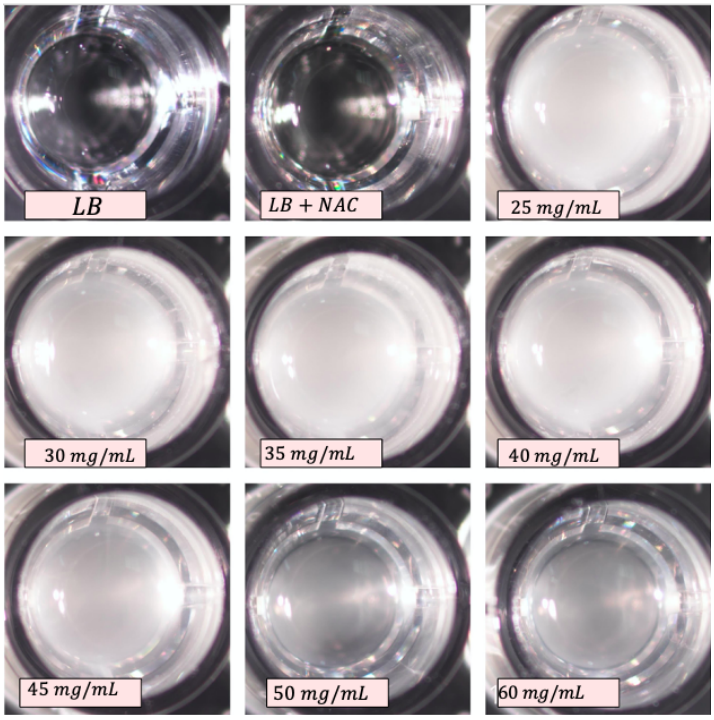

Figure 2D: 24 hr mature biofilm treated with NAC at different concentrations

PART TWO
SKIN MICROBIOME RESEARCH
INTRODUCTION

The human skin is flourishing with microorganisms that have adapted to utilize the nutrients available on the skin. These microorganisms can produce molecules that can inhibit the colonization of other organisms. Different body sites provide different microenvironments that vary in pH, temperature, and sebum content. Areas that are known to be moister have exceedingly more sweat glands that play a crucial role in thermoregulation. Sweat plays a role in thermoregulation and contains antimicrobial molecules that inhibit the colonization of microbes. Sebaceous glands secrete sebum, a hydrophobic lubricating substance that provides a shield to the hair and skin.

Different regions in the body have various microbial species. For instance, sebaceous sites are dominated by Propionibacterium species, while Staphylococcus and Corynebacterium dominate moist areas and the bends of elbows and feet. [5] There are four skin sites with major microenvironments: glabella sebaceous, antecubital fossa, volar forearm, and toe web-space. [5] The skin microbiota has adapted to utilize the nutrients in our sweat and sebum and the essential proteins and lipids found on the cuticle. By consuming these nutrients, the bacteria can produce free fatty acids contributing to the bacteria's adherence to the skin. The microorganisms found on the skin heavily depend on the condition of the skin. Changes in skin health result in changes in the skin microbiota. The microorganisms that colonize the skin also affect the healing of chronic wounds in populations with diabetes or obesity. [11] Individuals with diabetes and poor blood glucose control are associated with greater Staphylococcus spp. and Streptococcus spp. [11] Poor control of diet and health can also affect the microbial diversity and abundance of specific bacteria.

The skin's microbiome contains specific bacterium-bacterium interactions that play a role in the protection of the skin. For example, Staphylococcus aureus biofilm formation can be inhibited by Staphylococcus epidermidis, which produces AMPs known to protect the skin from pathogenic bacteria. AMPs are antimicrobial peptides that have a diverse class of molecules and are made to kill pathogens directly. [28] Different methodologies were performed to identify the microorganisms. Many research papers utilized 16S rRNA/rDNA sequencing to understand the microbiome of chronic wounds further. In particular, Wolcott et al. found that the wound microbiota comprises a significant barrier to healing the chronic wound. [35] Using 16S rDNA pyrosequencing, they analyzed the composition of the bacterial communities present in the samples of patients with chronic diabetic foot ulcers, venous leg ulcers, non-healing surgical wounds, and decubitus ulcers. This study included samples from 2963 chronic wound patients. The samples collected were at high risk for complications. The results of this study found that each wound had similar levels of bacterial diversity. [35] Additionally, there were no significant differences in the abundance of bacterial species in wound types. [35] The most prevalent genera found within the microbiota of chronic wounds in this study are Staphylococcus and Pseudomonas.

Chronic wounds are defined to be wounds that do not heal within three months. The three main types of chronic wounds are venous ulcers, pressure wounds, and diabetic foot ulcers. Venous ulcers mainly occur in the presence of venous disease, primarily in the legs. The pathogenic mechanisms of this type of ulcer are due to incompetent veins or valves in the legs due to impaired muscle function and venous pooling. [11] The pathogenic mechanisms responsible for pressure and diabetic ulcers are various causes that can be associated with lymphatic blockage and cell deformation. [11] These mechanisms lead to tissue inflammation,

which triggers toxins release that makes the tissue more susceptible to damage.[11] The chronicity of the wound is due to the formation of biofilm, persister cells, and the ability of the polymorphonuclear leukocytes to create chronic tissue inflammation. [13] The formation of deep and superficial biofilm and the variety of pathogens found contribute to the chronicity and the pathogenesis of wounds.

Proteolytic activity by multiple bacterial species isolated from chronic venous leg ulcers degrades matrix substrates [33]

In this study, the bacteria cultured from chronic wounds from leg ulcers were identified and tested for proteolytic activity. The methodology utilized involved isolating bacteria from six subjects and plating them on specific agar plates to identify the isolates. 16S rRNA sequencing and PCR were employed to identify the particular bacterial pathogen. The results found 13 bacterial isolates that expressed proteolytic activity. Six of the isolates were gram-positive bacteria. These included Staphylococcus aureus, Enterococcus faecalis, Staphylococcus epidermidis, Streptococcus agalactiae, Corynebacterium, and Streptococcus bovis. Seven of the isolates were gram-negative. These included Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Morganella morganii, Klebsiella pneumoniae, Bacteroides fragilis, and Serratia marcescens. Several bacteria are shared between intact skin and venous ulcers, including Staphylococcus epidermidis, Enterococcus faecalis, Streptococcus intermedius, Enterococcus species, Viridans, Streptococci, Proteus mirabilis, Pseudomonas aeruginosa, and Escherichia coli. The bacterial species identified in chronic leg ulcers remained constant over time.

Aerobic and anaerobic microbiology of chronic venous ulcers [4]

This study aimed to establish the aerobic and anaerobic bacteriology of chronic venous leg ulcers. This study had 43 specimens from a group of 41 patients. Facultative or Aerobic bacteria were present in 18 specimens, anaerobic bacteria were present in 3 specimens, and a mixture was present in 22 specimens. The methodology implemented utilized the swab technique to recover anaerobic and aerobic bacteria from samples. There was 97 isolates total of 64 aerobic or facultative bacteria, and 33 were anaerobic bacteria. The main anaerobic bacteria were

Peptostreptococcus spp, Bacteriodes fragilis, Propionibacterium, and Prevotella spp. The prominent aerobic organisms were Staphylococcus aureus and Escherichia coli. The most common organisms found in these samples were Pseudomonas aeruginosa and Staphylococcus aureus. A few isolates were also a part of the normal skin flora, such as Peptostreptococcus spp and Propionibacterium.

Microbiological profile of chronic ulcers of the lower limb: a prospective observational cohort study [23]

The main objective of this study was to determine the bacteriology of chronic limb ulcers. This study had a cohort of 39 patients with lower limb ulcers of over one month. The techniques to extract samples were utilizing superficial swabs and punch biopsy. This study found that the most common organism was Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus spp, and Corynebacterium. The swab and biopsy results shared at least one common organism. Venous ulcers made up 51% of the chronic limb ulcers and tended to be larger than the other ulcers.

Biochemical Association of Metabolic Profile and Microbiome in Chronic Pressure Ulcer Wounds [1]

This paper utilized a systems biology approach to study biopsies from chronic pressure wounds using analysis with 1H NMR spectroscopy. Researchers found that the bacterial microbiota profile was shaped by secretory, structural, and immunological changes. In this study, the main goal was to examine the wound's metabolic environment to understand how this contributes to the make-up of bacterial communities that colonize the wound. Wounds were accumulated from patients between 23 to 63 years of age; the wounds' causes and locations vary. The methodology applied was to extract DNA from the chronic wound samples and amplify the 16S rRNA region using PCR. Metabolite extraction was performed by homogenizing the samples and analyzing the components using NMR spectroscopy. Researchers found that three main bacterial species colonized the pressure ulcer - Firmicutes, Proteobacteria, and Actinobacteria. Although Staphylococcus and Pseudomonas are known to be the most opportunistic pathogens in chronic wounds, results showed that only one patient had a significant amount of Staphylococcus. The metabolites present at different regions of the wound (top or bottom) varied. There was an increased amount of creatine phosphate, malonate, and glycolate at the top of the wound. The metabolic extraction showed eight significant metabolic processes; protein biosynthesis, amino acid metabolism, ammonia recycling, urea cycle, and citric acid cycle. This paper suggests that focusing on the metabolic environment may be a potential intervention to inhibit pathogenic bacteria.

The Cutaneous microbiome in hospitalized patients with pressure ulcers [34]

This paper claims to be the first to demonstrate that the cutaneous microbiome is altered in patients with pressure ulcers. This research aimed to study the microbiome of the skin of patients with and without pressure ulcers. This study included 15 patients with pressure ulcers and 15 control ulcers. To analyze the microbiota, researchers obtained skin swabs from intact and specific areas. The obtained swabs were sequenced, and the three most abundant groups were Firmicutes, Proteobacteria, and Actinobacteria. The data suggest that regions with sacral pressure ulcers in normal patients' skin have a different microbial composition than patients without pressure ulcers. Patients with pressure ulcers have a less varied skin microbiota in comparison to patients with no pressure ulcers.

Additionally, results suggest that the most abundant species in patients with pressure ulcers are Staphylococcus aureus and unclassified Enterococcus. This study was able to identify eight species associated with pressure ulcer occurrence. Researchers suggest that the abundance of one of the eight species is associated with a higher chance of developing pressure ulcers.

Skin physiology and its microbiome as factors associated with the recurrence of pressure injuries [26]

This study aims to study the factors associated with recurrent pressure injuries by focusing on skin physiology and the skin microbiome. The methodology that was executed focused on utilizing 16S rRNA sequencing and PCR. The samples were extracted from 30 patients, 8 of whom had recurrent pressure injuries. Eight patients developed a recurrent pressure wound with a recurrence rate of 26.7%. The results from this study found lower hydration in the stratum corneum and higher Staphylococcus spp. Abundance was observed on the healed sites of the recurring pressure ulcers. The methodology utilized was not enough to distinguish between

the different species of Staphylococcus. Researchers suggest that dry skin may correlate with a higher risk for superficial wounding. The moisture content in the stratum corneum may affect the shape and elasticity of the skin, thus influencing the skin's ability to withstand the formation of recurrent pressure ulcers. This study compiled data on skin pH and sebum content of patients. Results showed that the skin pH of patients with recurrent pressure injuries is higher than patients with non-recurrent pressure injuries. Due to the small sample size, the causal relationships between the changes may be unclear.

A longitudinal study of diabetic skin and wound microbiome [15]

This study aimed to determine whether there are differences in skin microbiome between individuals with and without diabetes. Researchers also investigated the diversity and abundance of microorganisms within chronic wounds or skin microbiomes to understand the skin microbiome of chronic wounds further. To do this, researchers had a sample size of eight individuals with diabetes and eight control. They swabbed the skin on the soles of both feet and the chronic wounds (if there were any) at six time points over ten weeks. The microorganisms were identified using 16S rRNA. It was determined that diabetic skin was significantly less diverse than control skin for richness and diversity. The prominent diabetic foot skin communities found were to be Staphylococcus, Acinetobacter, Corynebacterium, Enterobacteriaceae. The significant microorganisms found in control skin were Staphylococcus, Acinetobacter, Kocuria, Corynebacterium, and Micrococcus. Although the top ten most abundant OTUs were similar, the microbiome from diabetic skin was significantly different from that of the control group skin. This study found a significant reduction in alpha diversity and decreased stability of the diabetic skin compared to non-diabetic skin. Due to the small sample size and limited data, the researchers speculate that increased antibiotics may decrease diversity. This decreased diversity has been correlated with disease and inflammation. This indicates that the microbiome diversity is an indicator of health, signaling how resilient the microbiome environment is.

One step closer to understanding the role of bacteria in diabetic foot ulcers; characterizing the microbiome of ulcers [30]

This study aimed to examine the microbiome of new and recurrent diabetic foot ulcers using 16S amplicon sequencing. Sequencing the 16S rRNA allows for the identification of bacterial species that may be critical chronicity-inducing microorganisms. In this study, swabs were taken from 20 patients who had not received antibiotics for three months. The researchers hypothesized that the microbiome between the new and recurrent ulcers would be different, which would impact the wound's ability to be managed appropriately. Results showed that 11 samples were positive for microbial growth while nine swabs did not have significant growth. It was also found that there is no significant difference between the bacterial population of the new and recurrent ulcers. The most prominent microorganism found in 40% of samples of diabetic ulcers was Staphylococcus aureus. Additionally, there was a high incidence of Pseudomonas, Enterobacteriaceae, and Enterococcus in moderate to severe ulcers. This study suggests that a larger sample and a more intense methodology are needed to further study the complexity of bacterial population in diabetic foot ulcers.

The microbiome of diabetic foot ulcers: a comparison of swab and tissue biopsy wound sampling techniques using 16S rRNA gene sequencing [32]

This study aimed to determine the concordance between tissue and swab samples from diabetic foot ulcers using 16S rRNA sequencing. Along with comparing the two methods, the researchers also studied the different sampling methods regarding clinical information. The biopsy method is more invasive and expensive, and the swab is a topical method. The samples were collected from diabetic foot ulcers and were sampled using 16S rRNA gene sequencing.

Results revealed that tissue biopsies had a greater diversity of bacteria in comparison to the swabs.

In contrast, swabs had a higher frequency of occurrence of bacteria from known genera and potential pathogens. There was a significant difference between the two sample methods in the potential pathogens of Staphylococcus spp., Streptococcus spp., Corynebacterium spp., Enterobacteriaceae, and Enterococcus. A significant finding was Staphylococcus spp. in all 20 swab samples along with Corynebacterium, Anaerococcus spp, and Finegoldias spp. Results showed that Pseudomonas spp was found in 45% of the tissue samples and 30% of swab samples. Both the swab and biopsy technique identified a similar range of pathogenic bacteria, but researchers found that the swab technique is more suitable for detecting pathogens in diabetic foot ulcers.

CONCLUSION

The skin flora is a diverse and complex system consisting of both beneficial and harmless microorganisms. Both the adaptive and innate immune responses respond and modulate with the skin's microbiota. An example of this is the presence of Propionibacterium acnes on the skin, which degrades sebum and releases fatty acids onto the skin. [28] These nutrients are taken in by other bacteria helping them become more nourished. Additionally, the most common microorganisms in the skin flora are Staphylococcus epidermidis, Actinobacteria, Corynebacterium, Propionibacterium, Brevibacterium, and Micrococcus. [28] Based on the research review between the three different types of chronic wounds venous, pressure, and diabetic, there seems to be an agreement between the microorganisms in the ulcers and the skin flora. The two common microorganisms present between all three types of wounds is Staphylococcus aureus and Pseudomonas aeruginosa. Identification of the different microorganisms within the chronic wounds will allow for understanding the organisms and factors that contribute to chronic wounds. Further studies on the three different types of ulcers can be done with a larger sample size and a more holistic approach to identifying the mechanisms of the skin microbiota in relation to the host's mechanisms of protection. Additionally, an extension of this work would be to study the quorum sensing mechanisms of the skin microbiota and the microorganisms within the wound to understand its development of chronicity.

REFERENCES

[1] Ammons, M. C., Morrissey, K., Tripet, B. P., Leuven, J. T., Han, A., Lazarus, G. S., . . . Copié, V. (2015). Biochemical Association of Metabolic Profile and Microbiome in Chronic Pressure Ulcer Wounds. *Plos One, 10*(5). doi:10.1371/journal.pone.0126735

[2] Blakytny, R., & Jude, E. (2006). The molecular biology of chronic wounds and delayed healing in diabetes. *Diabetic Medicine, 23*(6), 594-608. doi:10.1111/j.1464-5491.2006.01773.x

[3] Brackman, G., Cos, P., Maes, L., Nelis, H. J., & Coenye, T. (2011). Quorum Sensing Inhibitors Increase the Susceptibility of Bacterial Biofilms to Antibiotics In Vitro and In Vivo. *Antimicrobial Agents and Chemotherapy, 55*(6), 2655-2661. doi:10.1128/aac.00045-11

[4] Brook, I., & Frazier, E. H. (1998). Aerobic and anaerobic microbiology of chronic venous ulcers. *International Journal of Dermatology, 37*(6), 426-428. doi:10.1046/j.1365-4362.1998.00445.x

[5] Byrd, A. L., Belkaid, Y., & Segre, J. A. (2018). The human skin microbiome. *Nature Reviews Microbiology, 16*(3), 143-155. doi:10.1038/nrmicro.2017.157

[6] Dana, A. N., & Bauman, W. A. (2014). Bacteriology of pressure ulcers in individuals with spinal cord injury: What we know and what we should know. *The Journal of Spinal Cord Medicine, 38*(2), 147-160. doi:10.1179/2045772314y.0000000234

[7] Defoirdt, T., Brackman, G., & Coenye, T. (2013). Quorum sensing inhibitors: How strong is the evidence? *Trends in Microbiology, 21*(12), 619-624. doi:10.1016/j.tim.2013.09.006

[8] Dhall, S., Do, D. C., Garcia, M., Kim, J., Mirebrahim, S. H., Lyubovitsky, J., . . . Martins-Green, M. (2014). Generating and Reversing Chronic Wounds in Diabetic Mice by

Manipulating Wound Redox Parameters. *Journal of Diabetes Research, 2014*, 1-18. doi:10.1155/2014/562625

[9] Dhall, S., Do, D., Garcia, M., Wijesinghe, D. S., Brandon, A., Kim, J., . . . Martins-Green, M. (2014). A Novel Model of Chronic Wounds: Importance of Redox Imbalance and Biofilm-Forming Bacteria for Establishment of Chronicity. *PLoS ONE, 9*(10). doi:10.1371/journal.pone.0109848

[10] Dickschat, J. S. (2010). Quorum sensing and bacterial biofilms. *Natural Product Reports, 27*(3), 343. doi:10.1039/b804469b

[11] Drago, F., Gariazzo, L., Cioni, M., Trave, I., & Parodi, A. (2019, May 06). The microbiome and its relevance in complex wounds. Retrieved from https://link.springer.com/article/10.1684/ejd.2018.3486

[12] Edwards, R., & Harding, K. G. (2004). Bacteria and wound healing. *Current Opinion in Infectious Diseases, 17*(2), 91-96. doi:10.1097/00001432-200404000-00004

[13] Fivenson, D. P., Faria, D. T., Nickoloff, B. J., Poverini, P. J., Kunkel, S., Burdick, M., & Strieter, R. M. (1997). Chemokine and inflammatory cytokine changes during chronic wound healing. *Wound Repair and Regeneration, 5*(4), 310-322. doi:10.1046/j.1524-475x.1997.50405.x

[14] Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care, 4*(9), 560-582. doi:10.1089/wound.2015.0635

[15] Gardiner, M., Vicaretti, M., Sparks, J., Bansal, S., Bush, S., Liu, M., . . . Burke, C. M. (2017). A longitudinal study of the diabetic skin and wound microbiome. *PeerJ, 5*. doi:10.7717/peerj.3543

[16] González, J. E., & Keshavan, N. D. (2006). Messing with Bacterial Quorum Sensing.

Microbiology and Molecular Biology Reviews, 70(4), 859-875. doi:10.1128/mmbr.00002-06

[17] Grice, E. A., & Segre, J. A. (2011). Erratum: The skin microbiome. *Nature Reviews Microbiology, 9*(8), 626-626. doi:10.1038/nrmicro2619

[18] Guo, L., Zhao, B., An, Q., & Tian, M. (2015). Characteristics of a Novel Aerobic Denitrifying Bacterium, Enterobacter cloacae Strain HNR. *Applied Biochemistry and Biotechnology, 178*(5), 947-959. doi:10.1007/s12010-015-1920-8

[19] Han, G., & Ceilley, R. (2017). Chronic Wound Healing: A Review of Current Management and Treatments. *Advances in Therapy, 34*(3), 599-610. doi:10.1007/s12325-017-0478-y

[20] John, J. F., Sharbaugh, R. J., & Bannister, E. R. (1982). Enterobacter cloacae: Bacteremia, Epidemiology, and Antibiotic Resistance. *Clinical Infectious Diseases, 4*(1), 13-28. doi:10.1093/clinids/4.1.13

[21] Kanehisa, M. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research, 28*(1), 27-30. doi:10.1093/nar/28.1.27

[22] Kanehisa, M. (2019). Toward understanding the origin and evolution of cellular organisms. *Protein Science, 28*(11), 1947-1951. doi:10.1002/pro.3715

[23] Lim, T. S., Mwipatayi, B. P., Murray, R., Sieunarine, K., Abbas, M., & Angel, D. (2006). Microbiological Profile Of Chronic Ulcers Of The Lower Limb: A Prospective Observational Cohort Study. *ANZ Journal of Surgery, 76*(8), 688-692. doi:10.1111/j.1445-2197.2006.03832.x

[24] Lipsky, B. A., Richard, J., & Lavigne, J. (2013). Diabetic Foot Ulcer Microbiome: One Small Step for Molecular Microbiology . . . One Giant Leap for Understanding Diabetic Foot Ulcers? *Diabetes, 62*(3), 679-681. doi:10.2337/db12-1325

[25] Nyenje, M., Green, E., & Ndip, R. (2013). Evaluation of the Effect of Different Growth Media and Temperature on the Suitability of Biofilm Formation by Enterobacter cloacae Strains Isolated from Food Samples in South Africa. *Molecules, 18*(8), 9582-9593. doi:10.3390/molecules18089582

[26] Percival, S. L., Thomas, J. G., & Williams, D. W. (2010). Biofilms and bacterial imbalances in chronic wounds: Anti-Koch. *International Wound Journal, 7*(3), 169-175. doi:10.1111/j.1742-481x.2010.00668.x

[27] Rollins, B. J. (1997). Chemokines. *Blood, 90*(3), 909-928. doi:10.1182/blood.v90.3.909.909_909_928

[28] Schommer, N. N., & Gallo, R. L. (2013). Structure and function of the human skin microbiome. *Trends in Microbiology, 21*(12), 660-668. doi:10.1016/j.tim.2013.10.001

[29] Shibata, K., Ogai, K., Ogura, K., Urai, T., Aoki, M., Arisandi, D., . . . Sugama, J. (2020). Skin Physiology and its Microbiome as Factors Associated with the Recurrence of Pressure Injuries. *Biological Research For Nursing, 23*(1), 75-81. doi:10.1177/1099800420941100

[30] Smith, K., Collier, A., Townsend, E. M., O'Donnell, L. E., Bal, A. M., Butcher, J., . . . Williams, C. (2016). One step closer to understanding the role of bacteria in diabetic foot ulcers: Characterising the microbiome of ulcers. *BMC Microbiology, 16*(1). doi:10.1186/s12866-016-0665-z

[31] Tipton, C. D., Wolcott, R. D., Sanford, N. E., Miller, C., Pathak, G., Silzer, T. K., . . . Phillips, C. D. (2020). Patient genetics is linked to chronic wound microbiome composition and healing. *PLOS Pathogens, 16*(6). doi:10.1371/journal.ppat.1008511

[32] Travis, J., Malone, M., Hu, H., Baten, A., Johani, K., Huygens, F., . . . Benkendorff, K.

(2020). The microbiome of diabetic foot ulcers: A comparison of swab and tissue biopsy wound sampling techniques using 16S rRNA gene sequencing. *BMC Microbiology, 20*(1). doi:10.1186/s12866-020-01843-2

[33] Verbanic, S., Shen, Y., Lee, J., Deacon, J. M., & Chen, I. A. (2020). Microbial predictors of healing and short-term effect of debridement on the microbiome of chronic wounds. *Npj Biofilms and Microbiomes, 6*(1). doi:10.1038/s41522-020-0130-5

[34] Wert, L. A., Rensen, S. S., Soons, Z., Poeze, M., Bouvy, N. D., & Penders, J. (2020). The cutaneous microbiome in hospitalized patients with pressure ulcers. *Scientific Reports, 10*(1). doi:10.1038/s41598-020-62918-8

[35] Wolcott, R. D., Hanson, J. D., Rees, E. J., Koenig, L. D., Phillips, C. D., Wolcott, R. A., . . . White, J. S. (2015). Analysis of the chronic wound microbiota of 2,963 patients by 16S rDNA pyrosequencing. *Wound Repair and Regeneration, 24*(1), 163-174. doi:10.1111/wrr.12370

[36] Wysocki, A. B., Bhalla-Regev, S. K., Tierno, P. M., Stevens-Riley, M., & Wiygul, R. (2012). Proteolytic Activity by Multiple Bacterial Species Isolated From Chronic Venous Leg Ulcers Degrades Matrix Substrates. *Biological Research For Nursing, 15*(4), 407-415. doi:10.1177/1099800412464683

[37] Zafarullah, M., Li, W. Q., Sylvester, J., & Ahmad, M. (2003). Molecular mechanisms of N - acetylcysteine actions. *Cellular and Molecular Life Sciences (CMLS), 60*(1), 6-20. doi:10.1007/s000180300001

[38] Zurob, E., Dennett, G., Gentil, D., Montero-Silva, F., Gerber, U., Naulín, P., . . . Parra, C. (2019). Inhibition of Wild Enterobacter cloacae Biofilm Formation by Nanostructured Graphene- and Hexagonal Boron Nitride-Coated Surfaces. *Nanomaterials, 9*(1), 49.

doi:10.3390/nano9010049