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REVIEW

The role of the microbiome in nonhealing diabetic wounds

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Wound healing is a highly coordinated and complex process, and there can be devastating consequences if it is interrupted. It is believed that, in combination with host factors, microorganisms in a wound bed can not only impair wound healing but can lead to stalled, chronic wounds. It is hypothesized that the wound microbiota persists in chronic wounds as a biofilm, recalcitrant to antibiotic and mechanical intervention. Cultivation-based methods are the gold standard for identification of pathogens residing in wounds. However, these methods are biased against fastidious organisms, and do not capture the full extent of microbial diversity in chronic wounds. Thus, the link between specific microbes and impaired healing remains tenuous. This is partially because local infection and, more specifically, the formation of a biofilm, is difficult to diagnose. This has led to research efforts aimed at understanding if biofilm formation delays healing and leads to persistent and chronic infection. Circumventing challenges associated with culture-based estimations, advances in high-throughput sequencing analysis has revealed that chronic wounds are host to complex, diverse microbiomes comprising multiple species of bacteria and fungi. Here, we discuss how the use of genomic methodologies to study wound microbiomes has advanced the current understanding of infection and biofilm formation in chronic wounds.

Keywords: microbiome; chronic wound; next-generation sequencing; biofilm; antibiotic resistance

Introduction

Skin disease affects approximately 85 million people, or 25% of the entire population of the United States, resulting in direct medical costs of $75 billion and lost opportunity costs of $11 billion per year.[1] Although there is a wide range of pathologies, cutaneous infections account for the greatest proportion of these medical costs. Furthermore, infections, wounds, burns, and ulcers are responsible for the greatest number of skin disease deaths (~30%) after skin cancer.[1] This has resulted in what some consider a silent epidemic; the Association for the Advancement of Wound Care has gone so far as calling chronic wounds "The Most Important Health Problem You've Never Heard About."[2]

One major type of chronic wound is diabetic foot ulcer (DFU). In this review, we will discuss DFU chronic wounds as a prototypical example for studying subsequent wound infections. Infection has been the coup de grace for patients with DFUs because in most cases it is the final insult that precipitates amputation.[3–5] Over 30 million people in the United States and over 400 million people globally have diabetes.[6] Up to 25% of them will develop a DFU in their lifetime.[7–9] Each year, 2 million Americans seek care for DFUs, with inpatient costs alone exceeding $790 million.[10–12] Even more sobering than the economic impact is the associated morbidity and mortality. Within 5 years of ulceration, more than 50% die and 5% lose a limb.[13–16] DFUs are common, complicated, and costly. Following the advent of endovascular techniques to address underlying vascular disease, infection now commonly serves as the tipping point between limb salvage and amputation.[17–19]

Nearly half of DFUs become infected.[4] Clinical infection is defined by the presence of its cardinal signs: rubor, calor, tumor, and dolar.[20] These are grossly tangible signs of the host response to invading microbes. However, in the earliest stages, the

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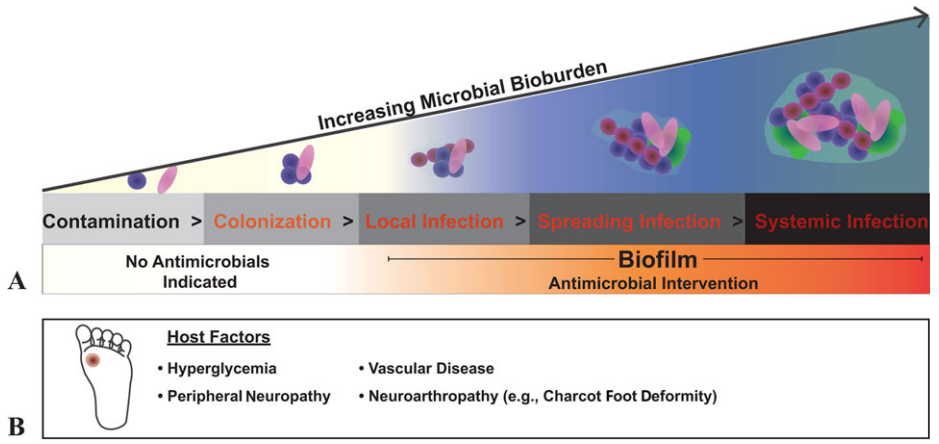

Figure 1. Factors contributing to impaired wound healing. (A) The wound infection continuum (adapted from Ref. 21). Microorganisms are detected in all wounds. Host defenses are sufficient to prevent tissue invasion during contamination. As a biofilm forms, microbes expand and begin to cause covert local infection. As the biofilm establishes, overt or classic signs of infection, such as erythema, swelling, and purulent discharge are apparent. If left untreated, the infection may spread and become systemic. Wound healing becomes delayed at the development of covert local infection. (B) Host factors contributing to impaired healing in diabetic patients. Hyperglycemia, peripheral neuropathy, vascular disease, and neuroarthropathy contribute to formation of a local microbiome and delayed healing.

host response often is not robust enough to mount a clinically evident sign of infection. Prior to this, there are pathophysiologic changes along an infection continuum: local wound contamination, colonization, early local infection (where there may be molecular evidence of a host response, but not clinical signs), late local infection (where clinical signs of infection are present), spreading infection, and systemic infection (Fig. 1).[21] Progress along this continuum is driven by the following factors: microbe interactions in a complex interkingdom microbial community (i.e., microbiome), virulence, and host response. The progress from colonization to early local infection, defined by molecular but not clinical evidence of a host response, represents a pivot point at which members of the microbiome may interact to form polymicrobial biofilms. Although early local infection and biofilm formation precedes clinically apparent infection, it may represent the optimal point to intervene. It is unclear whether the current antimicrobial armamentarium is up to the task of dismantling biofilm at this early stage. However, enzymatic or sharp debridement of the biofilm may prevent progression to clinically overt infection. Halting the progression from early to late local infection is critical because 50% of patients that develop clinically overt infection (i.e., late local infection) require amputation.[22] We must understand, diagnose, and treat microbial biofilms in DFUs to prevent clinical infection and subsequent amputation.

In this review, we describe the current understanding of the role of the microbiome in DFUs and describe recent methodological advances in the study of wound microbiomes. Understanding this complex, typically polymicrobial infection should result in improved management and limb salvage.

Host factors that shape the microbiome of DFUs

DFUs arise and persist due to a constellation of host factors, which also shape their microbiomes. These host factors include hyperglycemia, peripheral neuropathy, vascular disease, and neuroarthropathy (Table 1). Poorly controlled diabetes results in hyperglycemia, which drives the remaining host factors. It independently promotes the establishment of a microbiome by (1) creating a surplus nutrient source for bacteria and fungi and (2) decreasing innate immunity.[23] Specifically, hyperglycemia leads to poor chemotaxis, phagocytosis, and lysis of bacteria and fungi by neutrophils due to low production of superoxide and myeloperoxidase.[24–26] Peripheral neuropathy, including sensory, motor, and autonomic deficits, is an underlying comorbidity in the majority of ulcers.[27,28] Decreased sensation

Table 1. Host factors that shape the microbiome of diabetic foot ulcers

Host factor	Impact
Hyperglycemia	• Creates a surplus nutrient source for microbes • Decreases innate immunity; specifically leads to poor chemotaxis, phagocytosis, and lysis of microbes by neutrophils due to low production of superoxide and myeloperoxidase[23–26] • Drives remaining host factors
Peripheral neuropathy	• Sensory neuropathy exacerbates minor traumas[29] • Autonomic neuropathy leads to skin desiccation and microfissures, which provide a portal of entry for microbes[30,31] • Autonomic neuropathy leads to microvascular shunts, where the most superficial layers of the skin are relatively ischemic and the deeper layers are warmer than usual, promoting the establishment of anaerobes[30,31] • Motor neuropathy weakens the anterior shin muscles, leading to increased forefoot pressure and subsequent tissue damage[29]
Vascular disease	• Accelerates tissue damage • Delays wound healing • Impedes delivery of systemic antimicrobials and the host immune response
Neuroarthropathy	• Abnormal pressure distribution predisposes to skin breakdown

exacerbates minor traumas and motor weakness, especially in the anterior shins, and results in increased forefoot pressure, leading to tissue breakdown.[29] Autonomic dysregulation leads to desiccation of the skin, and the resulting microfissures provide an entry portal for pathogens. Furthermore, autonomic dysregulation also causes microvascular shunts, where the most superficial skin layers are relatively ischemic and the deeper layers are warmer than usual, establishing an excellent milieu for incubation, especially of anaerobes.[30,31] Lack of blood flow resulting from vascular disease accelerates tissue damage, delays wound healing, and impedes delivery of antimicrobials and the host defense response. Finally, neuroarthropathy leads to biomechanical changes in the feet, including collapsed arches and Charcot foot deformities. These malformations cause abnormal pressure distributions over new, boney prominences that predispose to skin breakdown. To summarize, skin integrity is compromised, local tissue is ischemic, a surplus of glucose serves as a nutrient source, and the innate immune system is blunted. These host factors prime the ulcer bed for microbiome colonization, impact the formation of biofilms, and influence the probability of clinical infection.

Biofilms and chronic wounds

Similar to humans and other social beings, microbes do not live in isolation. In natural ecosystems, bacteria preferentially adhere to surfaces and assemble into complex aggregate communities.[32] This occurs as a multistage process, beginning with cell adhesion through sensing their proximity to a surface and other bacterial cells. Structurally, biofilms are distinct microcolonies, often encapsulated by extracellular matrix, and may host one or many different species. A commitment to the biofilm lifestyle results in a complete metabolic shift and profoundly different phenotype from cells growing planktonically.[32,33] Within the biofilm architecture exists a highly organized and structured system, with water channels for efficient cycling of nutrients and the small signaling molecules used for bacterial communication.[34,35] Oxygen availability and pH also vary across a gradient in the biofilm, and sessile heterogeneous cell populations exist together in different metabolic states.[34,35] Dormant cells in a very low metabolic state have been shown to reside in the oxygen-poor center of microcolonies.[36,37] Referred to as persister cells, these cells are tolerant to antibiotic exposure by removing or reducing the small molecule antibiotic target.[38] The combination of a protective matrix and antibiotic tolerance allows microbes within the biofilm to limit the access of host immune factors and thwart an antimicrobial attack. These properties lead to chronic, recurrent infection and persistent local inflammation.[38–40] This has led the National Institutes of Health (NIH) to announce biofilms as medically important, attributing over 80% of human infections to biofilms. DFU beds are also often colonized by complex communities of microbes (microbiomes) that

can form biofilms, interact with the host immune system, and blunt healing responses.[21,41]

Despite the growth in laboratory biofilm research and the development of biofilm-based wound care concepts, interpreting the link between biofilm and clinical outcomes in wound care persists as an ongoing debate.[21,42] While there is consensus that biofilm impedes wound healing, detection and diagnosis of biofilm in wound tissue is difficult. A plethora of *in vitro* biofilm research has made significant advances toward understanding the life cycle of biofilm-forming organisms, but microbial behavior in complex tissues such as a chronic wound is less certain. Biofilm is not visually distinguishable in the wound bed but it has been associated with slough, yellow devitalized tissue with dead cells and fibrin that can form on the surface of a wound. However, slough does not always equate to biofilm and although some clinicians will use the physical characteristics of slough (such as low-grade inflammation, presence of a shiny slime layer, or rapid reformation upon removal) to predict if it is biofilm, diagnosis remains ambiguous and standards of care widely vary across institutions.[43–45]

With a lack of diagnostic tests or validated biomarkers for biofilm, confirmation of biofilm in wound tissue is best accomplished by microscopy. In 2008, one of the first studies to assess whether biofilm is an indicator of chronic, but not acute, wounds was published. In this study, wound debridement specimens were imaged by scanning electron microscopy (SEM).[46] Biofilm was detected in 60% of the chronic wounds (mixed etiology) compared with 6% of acute wounds, a statistically significant difference. Clusters of coccoid bacterial cells were the most frequently imaged biofilms, however mixed species microcolonies were also detected.[46] More recently, Johani *et al.* obtained tissue biopsies after saline cleansing from 65 patients with DFU.[41] Specimens were processed for next-generation sequencing, fluorescent *in situ* hybridization (FISH), and SEM to confirm the presence or absence of biofilm. Biofilm was detected by either FISH or SEM in 100% of the specimens, and all wounds were found to host multiple species by either microscopy or sequencing.[41] Microscopy is a powerful tool to compliment *in vivo* biofilm studies, however it requires sophisticated equipment that is not readily available at the bedside and is limited in the identification of the microbial make-up of biofilms detected in tissue biopsies. Therefore, culture-independent molecular techniques (i.e., high-throughput DNA sequencing) have become the preferred method of analyzing microbial communities associated with DFU and other chronic wounds.

Culture-based characterization of chronic wound microbiomes

Until recently, the majority of studies focused on characterizing the microbes associated with polymicrobial wound infection and biofilm have been culture based. Multiple, independent, culture-based studies agree in their findings that Gram-positive cocci (GPC) are the most frequently isolated microbes from DFU. *Staphylococcus aureus* is consistently the most prevalent species (>50% of all wounds), followed by coagulase-negative *Staphylococci* spp. and *Streptococcus* spp.[47–50] *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), is often found in association with other Gram-positive pathogens and mixed anaerobic communities, but not *Pseudomonas aeruginosa*, a common misconception.[51–53] Because of *P. aeruginosa's* ability to form the archetypal mushroom-like biofilm colonies encapsulated in a protective extracellular matrix *in vitro*, it has been studied as a model organism of biofilm formation. It is also implicated in the pathogenesis of human infections involving biofilm, such as cystic fibrosis and burn wounds.[54–56] As a result, it has been commonly thought to also be associated with biofilm in chronic wounds such as DFU.[57–59] While *P. aeruginosa* is the most commonly isolated aerobic Gram-negative species identified in DFU, and while Gram-negative wound infection due to the presence of multidrug resistant and totally drug resistant organisms such as *Klebsiella pneumonia*, *Stenotrophomonas maltophilia*, and *Enterobacteriaceae* spp. can be deadly, these organisms are a much more serious concern for other types of wounds, such as burns.[60–62] Indeed, GPC are the major participants associated with chronic wound infection.

Assembly of GPC communities that also include mixed anaerobic microorganisms is likely underestimated, but may contribute significantly to healing outcomes. For instance, mixed aerobic–anaerobic infection was first described by Louie *et al.* in 1976, where deep wound samples were inoculated into several types of selective media at the bedside and

immediately placed into GasPak jars to allow for anaerobic growth. From this study an average of 5.8 species per wound specimen were isolated with mixed aerobic and anaerobic bacteria in 90% of the specimens.[63] Importantly, this early study emphasized the polymicrobial nature of the DFU environment, and suggested straightforward antibiotic regimens were likely to leave at least one isolate uncovered. The implication of anaerobic bacteria in diabetic foot infection and their effect on cell-mediated responses of keratinocytes and fibroblasts during wound healing is still not clear. This is largely due to the time-consuming nature of anaerobe isolation and systematic identification, resulting in an underrepresentation of anaerobic communities isolated from wound tissue, despite the evidence from large-scale targeted studies aimed at optimizing specimen collection and isolation of diverse species. For example, in a study of 74 wounds, Bowler and Davies[51] found 82% of clinically infected and 73% of noninfected wounds contained anaerobic bacteria. More recently, Citron *et al.*[53] collected DFU specimens into anaerobic transport tubes from 454 wounds, followed by incubation in an anaerobic chamber for up to 5 days. This resulted in the isolation of >1600 organisms, and the majority of DFUs were found to be polymicrobial (>80%), with over 40% of wounds harboring mixed aerobic–anaerobic growth. The average number of anaerobes identified per positive specimen was 2.3, with anaerobic cocci representing the largest percentage of isolates (*Finegoldia magna*, *Peptoniphilus asaccharolyticus, Peptostreptococcus anaerobius, Anaerococcus* spp.), followed by *Prevotella* spp., *Porphyromonas* spp., and the *Bacteroides fragilis* group. Historically, culture-based studies have unsuccessfully focused on associating a single pathogen or microbe to healing outcomes. Consequently, the polymicrobial landscape of DFU has been overlooked.

Although organisms such as *S. aureus* are strongly associated with DFU, the direct impact on clinical outcome is confounded by the difference between colonization and infection, which can be difficult to distinguish. This is challenging from a clinical point of view, especially if a *S. aureus* colonized wound is on trajectory toward healing. One promising way to potentially distinguish colonization from infection is phenotypic variation in *S. aureus* strains. Strains of a single species are divergent across their pangenome, resulting in distinct phenotypes corresponding to severity of pathogenesis. This has been demonstrated *in vitro*, different strains of *S. aureus* and *Streptococcus pyogenes* elicited widely different adapted immune responses in healthy human donor cells.[64] For patients with a DFU, strain variability could affect the structure of microbial communities via genes specific to modulating interactions with other members of the microbiome, and/or formation of stable biofilms in the wound tissue niche. Understanding the processes contributing to this variation could lead to novel biomarkers and antimicrobial targets, as well as the ability to decipher between colonization and infection, as heralded by the formation of a biofilm. High-throughput genomic approaches provide an opportunity to do this.

Culture-independent characterization of chronic wound microbiomes

The first culture-independent wound microbiome study[65] was published in 2008. It focused exclusively on DFU and used sharply debrided material (devitalized tissue removed with a scalpel using sterile technique) to amplify and sequence the V4 hypervariable region of the bacterial 16S ribosomal RNA (rRNA) gene, resulting in taxonomic assignment at the genus level. Unlike the culture-based studies described above, *Corynebacterium* spp. was identified as the most prevalent taxa (75% of the samples), but *Staphylococcus* spp. was still present in ~30% of the samples. These were followed by obligate anaerobes *Bacteroides* spp., *Peptonophilus* spp., *Finegoldia* spp., and *Anaerococcus* spp. These findings led the authors to conclude that culture-based studies are greatly biased and overestimate the importance of some organisms. They further point out that if cultured from a wound, *Corynebacterium* spp. are often considered a contaminant from normal skin flora, but given their prevalence in DFU and association with infections, they should not be overlooked. Subsequent studies have reached similar conclusions regarding the major taxa associated with DFU. *Staphylococcus* spp. and *Corynebacterium* spp. are highly prevalent, followed by mixed anaerobic communities.[52,66–70] DFU microbiomes are highly heterogeneous and polymicrobial, suggesting biofilm architecture in the tissue consists of complex multispecies consortiums, confounding treatment strategies because the increased species diversity of biofilms is also associated with

an increased resistance to antimicrobials.[71–74] It has now been well-established that traditional culture-based clinical microbiology underestimates the total microbial diversity of the wound bed. For example, traditional microbiological isolation typically yields an average of 2–10 species per sample, while molecular methods yield >15 species/sample on average.[68,75] Culturing overestimates the predominance of easy-to-cultivate microorganisms such as *Staphylococcus* species, but when the microorganisms are a minor constituent of the community they may go undetected, while anaerobes are consistently underrepresented.[68] Microbes from healthy skin and the environment are frequently detected together in the wound tissue, suggesting normal skin flora may be involved in virulence and disease progression.

To date, the leading methodology applied to the study of chronic wound microbiomes is PCR-based amplicon sequencing. This easily accessible technique involves PCR amplification of the bacterial 16S rRNA gene, a highly conserved gene containing hypervariable regions that are informative for taxonomic identification, followed by high-throughput DNA sequencing.[76–78] Fungal identification is achieved by sequencing the fungal rRNA gene operon, targeting the hypervariable internal transcribed spacer regions (ITS1 and ITS2) flanking the 5.8S rRNA gene (Fig. 2).[79,80] Development of software pipelines to ease the bottleneck of bioinformatics analysis, such as the Quantitative Insights Into Microbial Ecology (QIIME) and mothur, has led to the expansion of published microbiome studies via increased accessibility throughout the broad field of biology; the chronic wound care field is one such area with a high potential for translational microbiome research.[81,82]

Recent advances to overcome challenges in wound microbiome research

Although advances have occurred in characterizing the composition of chronic wound microbiomes using sequencing, a major challenge in the treatment of DFU that still remains is discerning which microbes or microbial community types interact with the immune response to disrupt healing pathways, and which act as neutral players, simply colonizing the tissue with little interaction and effects on healing. That is, researchers are still trying to decipher how to use the microbiome as a marker to identify colonization versus infection. There are many reasons for this. The inability to classify short sequence reads with confidence beyond the family or genus level limits the ability to identify pathogens, virulence factors, and antibiotic resistance markers. A wound colonized by MRSA is often regarded and treated differently than a wound colonized by *Staphylococcus epidermidis*. Amplicon-based sequencing studies are incredibly informative to decipher who is present in a complex

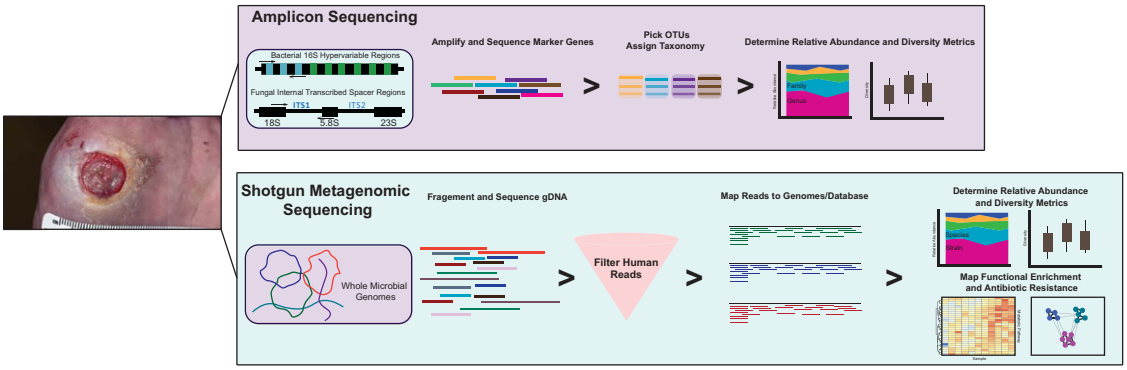

Figure 2. High-throughput sequencing pipelines to analyze chronic wound microbiomes. Amplicon sequencing requires PCR amplification of hypervariable regions in the bacterial 16S rRNA gene or hypervariable regions of the internal transcribed spacer (ITS) region in the fungal rRNA cistron. Amplicons are sequenced, followed by clustering of similar sequences into operational taxonomic units (OTUs). Representative sequences from each OTU cluster are then assigned a taxonomy using a reference database. Shotgun metagenomic sequencing involves fragmenting DNA extracts, followed by sequencing and filtering of human reads from raw sequences. Taxonomic identification is accomplished by mapping microbial reads to reference genomes or a database of marker genes. Partial genome assembly is possible, allowing insights into genomic function from individual members of the microbiome.

community and to monitor community dynamics in response to environmental cues. Amplicon sequencing does not, however, provide functional information to answer the question of what members of the microbiome are doing. Whole metagenome shotgun (WMS) sequencing involves extracting total DNA from the microbiome and sequencing the resulting fragments (the metagenome). WMS offers several advantages over amplicon sequencing. First, higher species resolution is possible and allows for profiling of strain-level variation. Second, composite genomes can be constructed to evaluate functional enrichment associated with different environments. Finally, WMS provides the opportunity to identify novel biomarkers within a genomic context and track antibiotic resistance genes, a powerful epidemiological tool. This is summarized in Figure 2. But applying WMS to study chronic wound microbiomes has drawbacks. The technique is much costlier and requires greater sequencing depth for a robust analysis. Skin and wound specimens are also plagued with high levels of human DNA contamination that is sequenced alongside microbial genomes. This represents a computationally intensive step that should not be underestimated; to efficiently filter human sequences from wound samples can mean discarding upwards of >95% of the raw sequence reads. To date there are no published chronic wound microbiome studies employing the use of WMS, but in the future, this could represent a very powerful technique to address some of the existing challenges in wound research discussed here.

Amplicon-based sequencing of the 16S rRNA gene also excludes microorganisms that are not bacteria, consequently limiting the information obtained in microbiome research. The vast majority of wound studies have excluded fungi from detection, although fungal foot infection in diabetic patients (including toenail infection) can lead to secondary bacterial infection and increased risk of a foot ulcer.[83,84] Culture-based studies have reported isolation of fungi from nearly 30% of DFU specimens, the vast majority cultivated as mixed bacterial–fungal flora.[85] To understand the prevalence and distribution of fungi in DFU, researchers have used ITS amplicon sequencing to investigate the role of fungi and their associations with not only clinical factors but interactions with their bacterial counterparts.[86] In a cohort of patients with neuropathic DFU ($n = 100$) that was followed longitudinally for 26 weeks, 79% of wounds were positive for fungi, and often contained multiple species (range: 1–20).[86] The mycobiome had high interpersonal variation, but increased fungal diversity was associated with complications (wound deterioration, osteomyelitis, or amputation) across the cohort. The stability of the mycobiome mirrored that of the bacteriome within the same patient, suggesting a communal response to changes in the microenvironment of the wound. Microbial communities comprising opportunistic and pathogenic fungi such as *Candida* spp., *Trichosporon asahii*, and *Rhodotorula* spp. have been detected in higher proportions from wounds resulting in an amputation and have been shown to be significantly associated with wound necrosis.[86] Furthermore, cultured isolates from the DFU exhibited the ability to form dense, three-dimensional biofilms with intimate fungal–bacterial interaction.[86] There are numerous examples in both the environment and medicine of fungi and bacteria interacting to form interkingdom biofilms (see Refs. 87 and 88). Enhanced resistance to antimicrobial treatment occurs when fungal hyphae provide a foundation for bacterial adherence,[89,90] secrete glycans and other polysaccharides, and sometimes actively penetrate tissue, enabling bacterial epithelial entry.[72,91,92] Intriguingly, a high proportion of saprophytic fungi have also been detected in DFU. *Cladosporium herbarum*, a ubiquitous organism thriving around the globe and an important agent of allergic disease, was the most prevalent species identified across the cohort reported by Kalan *et al.*[86] As a part of the oral, nasal, vaginal, and gut mycobiomes, this fungus has also found in clinical specimens of superficial and deep skin tissue.[93–98] The implications of *Cladosporium* spp. in DFU is still not clear; however, it may lead to new avenues of research for fungal infection in chronic wounds.

Another challenge facing wound microbiome research is the individuality of wound microbiomes. Much like the skin, gut, and oral microbiomes, wound microbiomes differ more between individuals than within individuals, both spatially (wound edge versus center) and temporally.[52,67,86,99–101] Furthermore, because chronic wounds can persist for years, a baseline state is difficult to define and this is particularly problematic for building accurate statistical models in microbiome studies. To

address some of these challenges, some studies have attempted to better control the patient cohort by including a single type of wound etiology (e.g., neuropathic DFU) and incorporated a longitudinal study design.[52,86,100,102] This has allowed researchers to apply community ecology principles to measure the stability of wound microbiomes over time and in response to perturbations and physiological markers. The effects of antibiotic use, mechanical debridement, blood glucose levels, or wound deterioration events on the microbiome can be monitored.[52,86,103] By shifting the focus from taxonomic profiling, in an attempt to identify causative agents, toward mathematical modeling of diversity and community dynamics within a single wound niche, it has been discovered that community stability is the best marker for poor healing. This was discovered by applying a Dirichlet multinomial mixture model approach to assign community types to DFU microbiota. In this study, DFU microbiomes were clustered into four community types. One major community type was dominated by *S. aureus* (23.5% of the community) and another was dominated by high relative abundances of *Streptococcus* spp. (64% of the community). Each of these two community types was associated with serum C-reactive protein levels and white blood cell count, which are markers of inflammation. DFUs unhealed after 26 weeks were also linked to the *S. aureus*-dominated community type. The other two community types were mostly heterogeneous, but without a single dominant organism.[52] Community type transitions over time could be measured because time series samples were collected. Healed wounds transitioned between community types every 1.6 visits (3.2 weeks), whereas wounds resulting in an amputation transitioned once every 3.08 visits (6.2 weeks). This was further confirmed by Markov chain visualization of community transitions to represent transition frequency over time. There was a highly significant difference in transition patterns between wound healing in less than 12 weeks than those greater than 12 weeks; notably, the slower healing wounds tended to self-transition to one of the community types dominated by *Streptococcus* spp. or *S. aureus*. That is, the these wounds became stalled in these community types, which suggests that the more stable a wound microbiome becomes, the more likely that the wound will remain unhealed.[52,103] This pioneering work provides further evidence to support the hypothesis that establishment of a biofilm is the tipping point between normal and stalled healing. Mature microbial biofilms are stable and can persist over long periods due to their recalcitrance toward perturbations, including antibiotic exposure.[34]

Antibiotics and the wound microbiome

The effects of antibiotic use on the human skin microbiome are virtually unknown, and very few studies have comprehensively examined how antibiotics affect wound microbiomes. Earlier, we highlighted the complexity of chronic wound microbiomes. They are exceedingly biodiverse, and the roster of pathogens changes over time. Different microbes can cohabitate and interact within the same biofilm, including members from different kingdoms (e.g., bacteria and fungi).[88] Individual pathogens can temporally shift their phenotypes, which influences virulence. These characteristics are likely to impact the aggressiveness and tenacity of an infection. It should not be surprising, therefore, that the effect of an antibiotic on this complex ecosystem is difficult to predict. Treatment guidelines recommend reserving antibiotics for wounds that are complicated by overt infections, such as cellulitis, deep space abscesses, and osteomyelitis.[20] These recommendations stem from (1) a lack of evidence that antibiotics improve ulcer outcomes prior to these stages and (2) concerns that injudicious use of antibiotics will promote resistance. The evidence that does exist supports both points.

Recently, Loesche *et al.* determined that antibiotic treatment of DFUs failed to significantly perturb the microbiome composition.[52] They examined the effects of antibiotic use on DFU microbiomes followed for 26 weeks by sequencing the V1-3 hypervariable region of the 16S rRNA gene. The longitudinal design of this study permitted a snapshot of the microbiome before, during, and after systemic antibiotic administration, while comparing patients receiving antibiotics ($n = 23$) to those who did not ($n = 68$) within a single cohort. Discernable changes of wound microbiomes during the course of antibiotic treatment were not observed, as measured by changes in community composition, richness, or stability over time. Stability was calculated by the intervisit weighted UniFrac distances within a single patient's timeline. This held true for all antibiotic classes,

regardless of their mechanism of action. However, when taking into consideration the reason an antibiotic was administered, antibiotic use specifically for the study ulcer resulted in significantly greater community disruption. This effect was further amplified when systemic antibiotics were administered due to a progressing infection (overt signs of infection) complicating the ulcer, such as osteomyelitis, rather than colonization or covert infection.[52] While this study concluded that systemic antibiotics can mildly disrupt the microbiome during overt infection, characterization of the fungal mycobiome in the same wound specimens found that those patients also had significantly higher fungal diversity in their wounds than those who had not been administered antibiotics. This suggests that antibiotics may have minimal effectiveness at disrupting the DFU microbiome while small changes in the bacteriome may be permitting fungal colonization and expansion, for which treatment options are more limited.[86] Another study examined the effects of a topical antimicrobial agent on chronic wound microbiome diversity.[102] Topical agents are ubiquitous in wound care practice and most commonly used in DFUs without evidence of overt infection.[104,105] In this study, the use of such a topical antimicrobial did not result in long-term, significant changes in microbiome composition.[86] Within the first 24 h of application, there was a shift in the community structure, but the microbial community composition reverted to baseline within the first week of use. A third study used a murine model to determine that vancomycin administration was associated with delayed wound healing.[106] While vancomycin did reduce skin colonization by *Staphylococcus*, it was also associated with downregulation of IL-17-induced RegIIIγ and delayed wound healing. Reducing bacterial colonization did not promote the ultimate goal of epithelialization. These three studies suggest the use of antimicrobials, either systemic or topical, does not significantly alter microbiome/biofilm composition when clinically overt infection is absent and may actually negatively impact the overarching goal of wound healing. Antibiotics should therefore be reserved for clinically overt infections.[107,108]

Antibiotic pressure may select for drug resistance in the microbiome of chronic wounds. Indeed, the first strain of vancomycin-resistant *S. aureus* was isolated from a DFU.[109] Price *et al.*[66] applied community ecology concepts to model the associations of chronic wound microbiomes (mixed wound types) with outcomes related to diabetes status and antibiotic use. By applying nondimensional scaling to reduce the complexity of ecological community data, they found that antibiotic-treated and nontreated microbiomes were significantly different from each other. Further indicator analysis revealed that antibiotic use selected for bacterial families that either harbor intrinsic resistance genes (*Corynebacteriaceae* and *Pseudomonadaceae*) or are opportunists (*Oxalobacteraceae*), although they also noted large within-group variation.[66] Our team has found that DFU microbiomes inherently possess a library of antibiotic resistance genes, regardless of host factors or clinical outcomes; within a single wound, we found resistance genes to more than 11 antibiotic classes (unpublished data). Antibiotics had no effect on the underlying presence of these genes, and further studies are needed to determine whether antibiotic pressure drives expression of genes conferring resistance to that agent. Antibiotic exposure can select for resistant pathogens in the DFU wound bed, such as *Pseudomonas* and fungi.[52,86] Additionally, the presence of multiple resistance genes in different species, the close proximity of those species in the biofilm, and the selective pressure of antibiotics is the perfect storm to promote multidrug resistance.

While antibiotic resistance is a concern, we do want to be clear that systemic antibiotics are indicated for clinically overt infection. However, we would argue that many patients receive antibiotics in the absence of overt infection or as part of an incomplete treatment plan that may jeopardize their utility. In some cohorts, more than 60% of patients with chronic wounds are prescribed an antibiotic.[107,108,110] In others, nonantibiotic therapeutics, such as off-loading shoes, are vastly underutilized.[111] We also recognize that robust, well-powered studies evaluating the effectiveness of both systemic and topical antibiotics for chronic wound therapy are lacking, particularly for diabetic wounds.[112] Wound progression and wound closure are the most common metric to define endpoints for clinical studies evaluating the effectiveness of wound therapies. However, complete wound closure is not always achievable for patients living with a chronic wound. Other outcomes, such as limb salvage, infection reduction, and improved quality of life, are more important to these patients.[113]

Furthermore, wound closure is not the sole metric to evaluate eradication of infection.

Conclusions and future directions

As the population ages and the number of people with diabetes grows, so does the number of chronic nonhealing wounds, placing a significant burden on the healthcare system. Even though antibiotic use is not linked to better outcomes for chronic wounds, they are still prescribed for the majority of patients and antibiotic resistance is ever present. There is an acute need for innovative treatment strategies that target wound infection and biofilm. In the meantime, we emphasize that there is one modality that disrupts the biofilm: debridement. Either sharp or enzymatic debridement should be included in almost any therapeutic plan. This should be coupled with diagnostic and therapeutic measures to address underlying host factors that promote biofilm formation and persistence: glycemic control, medical and surgical management of underlying vascular disease, off-loading, and antibiotics driven by the presence of overt infection.[20]

High-throughput sequencing technologies hold promise to continue advancing the field. They have already allowed researchers to more precisely characterize the microbial communities that assemble in chronic wounds, providing new insights into the diversity and make-up of wound microbiomes associated with impaired healing. But there is still much to learn. Advances in computational and statistical approaches will result in better models of microbiome–host dynamics. In the future, we can continue to use genomic approaches, such as transcriptome analysis (RNAseq), to further delineate which genes and pathways are involved in biofilm assembly in wound tissue, resistance to antimicrobials, and responses to host factors (e.g., glucose levels, inflammatory cytokines). Ultimately, discovering the microbe–microbe and microbe–host interactions important to the establishment of a stable microbiome/biofilm in a chronic wound will lead to development of new therapies. For example, small molecules that successfully manipulate and dismantle the polymicrobial biofilm of chronic wounds are a tantalizing and promising avenue of research. Targets may include novel microbial interactions between skin commensals and pathogens, bacteria, and fungi, or quorum sensing pathways yet to be discovered. Finally, continued development of advanced sequenced-based technology could change the field as we move toward better diagnostic capabilities and real-time monitoring of antibiotic resistance and/or markers of biofilm formation in wound tissue.

Competing interests

The authors declare no competing interests.

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