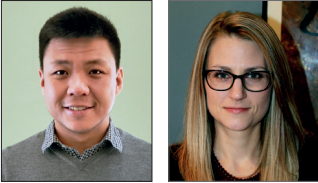


Wound microbiome update: sequencing and behaviour



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It has been over a decade since the first culture-independent, DNA-sequence-based methods were introduced to study the microbiome in a wound environment. This article provides a brief overview of the most commonly used methods of sequencing and what has been learned from studying the microbiology of chronic wounds at a community level.

Medical microbiology has traditionally been successful at identifying and targeting single causative pathogenic agents, especially for acute infections. While this may be useful for classical overt infections on the wound infection spectrum (Haesler and Ousey, 2018), many chronic wounds fall outside the single pathogen paradigm. Current evidence suggests that all wounds are contaminated to some extent by microbes and this may eventually result in the assembly of a diverse community of microbes (a microbiome) that may or may not display classical signs of infection.

Both culture-based and culture-independent methods of surveying microbial diversity in wounds support this conclusion. In general, microbiomes in a wound environment are comprised of anywhere from one to dozens of bacterial species encompassing aerobic, facultatively anaerobic and obligate anaerobic Gram-positive and Gram-negative bacteria, and often fungal species (Dowd et al, 2008; 2011; Chellan et al, 2010; Wolcott et al, 2016; Kalan et al, 2016; Loesche et al, 2017).

In this article, we discuss current evidence and the latest research on wound microbiomes, focusing on chronic non-healing wounds. We highlight the two major approaches to the study of wound microbiomes using culture-independent techniques, as well as the importance of study design incorporating longitudinal sampling compared to cross-sectional design. Definitions of technical terms used in this article are given in [Table 1](#).

Culture-independent microbiology

There are two main methods for the identification of microbes from clinical specimens using culture-independent approaches.

Amplicon sequencing

The most common and cost-effective technique is referred to as amplicon sequencing, which involves high-throughput DNA sequencing of a targeted bacterial or fungal barcode gene (Dowd et al, 2008; Rhoads et al, 2012; Wolcott et al, 2016; Kalan et al, 2016; Loesche et al, 2017; Tipton et al, 2017; Malone, 2017; Choi et al, 2019). The availability of commercial services providing amplicon sequencing make this method perhaps one of the most accessible. However, this method does have limitations:

- Many studies that sequence the 16S rRNA gene amplicon are constrained, in that this gene exclusively profiles bacteria and does not account for fungi or viruses that may be present in the community.
- The common sequencing primers used for different regions of the 16S rRNA gene are biased against certain microbes. For example, *Cutibacterium* spp (formerly *Propionibacterium* spp) — common skin commensals — are severely underestimated when hypervariable region 4 of the 16S rRNA gene is sequenced (Meisel et al, 2016).
- The sequenced 'barcode' has to be assigned a taxonomy based on, and limited by, reference databases of known species. Amplicon

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Table 1. Definitions of technical terms relevant to study of the microbiome.

Term	Description
16S rRNA gene	Gene encoding 16S ribosomal RNA (rRNA), a component of the 30S sub-unit of the prokaryotic ribosome; gene targeted as bacterial barcode during amplicon sequencing
Amplicon	DNA sequence that is amplified by polymerase chain reaction to be used for DNA sequencing
Biofilm	Aggregates of microbial cells, encased in a matrix, that are often more resistant to antimicrobials
Diversity	Referring to ecological descriptions of the wound microbiome; includes richness (number of species present) and evenness (dominated by few species or present in equal abundance)
ITS1 region	Internal (or intergenic) transcribed spacer 1 region of the eukaryotic rRNA cistron; DNA sequence targeted as fungal barcode during amplicon sequencing
Microbiome	Community of microbes associated with a host
Mycobiome	Community of fungi associated with a host
Shotgun metagenomic sequencing	Sequencing of all DNA within a sample; also referred to as whole-genome shotgun (WGS) sequencing, shotgun sequencing or metagenomic sequencing
Strain	A variant or subtype of an organism classified at a higher resolution than a species
Taxonomy	Classification of living organisms into groups within a hierarchical taxonomic rank; from highest to lowest: domain, kingdom, phylum, class, order, family, genus and species

sequencing of the fungal internal transcribed spacer (ITS) barcode from a cohort of diabetic foot ulcer (DFU) specimens found that 32% of samples contained ITS1 sequences could not be classified beyond the kingdom level, ie fungi (Kalan et al, 2016).

- The resolution of successful taxonomic assignments is only to the genus level. Species- and strain-level identification is not usually possible with 16S rRNA gene sequencing because only a portion of the gene is sequenced.

Shotgun metagenomic sequencing

Many of the limitations of amplicon sequencing are circumvented with shotgun metagenomic sequencing. The shotgun technique sequences all DNA within the sample, thereby capturing entire genomes from all organisms present. This allows for simultaneous analysis of bacteria, fungi and viruses to permit species- and even strain-level identification.

While shotgun sequencing is powerful, it costs much more than amplicon sequencing. The high cost of the technique represents a significant barrier to its widespread use.

What has been learnt?

Although 16S rRNA gene sequencing has some limitations, it still has many advantages over traditional culture-based identification of wound

microbes. Studies over the past decade have revolutionised the way in which we view chronic wound microbiology.

Grice and colleagues enrolled a cohort of 100 subjects with neuropathic DFUs and sampled deep wound fluid post-debridement every 2 weeks for up to 26 weeks, obtaining a total of 384 samples (Kalan et al, 2016; Loesche et al, 2017). To describe the diversity and composition of bacterial and fungal communities within the DFUs over time, researchers used amplicon sequencing of the 16S rRNA gene for bacterial community analysis and the ITS1 region for fungal community analysis (ie mycobiome). Each specimen was also submitted to the microbiology lab for standard culture-based microbial isolation and identification. Researchers found that 71.6% (275/384) of samples — corresponding to 79% (79/100) of patients — were positive for fungi, while only five samples were culture-positive for yeast isolates (Kalan et al, 2016). This discrepancy between culture- and sequencing-based identification has previously been reported for bacteria within chronic wounds (Rhoads et al, 2012). Notably, the DFU mycobiome varied significantly between patients and over time, with no distinct 'core' of shared fungal species (Kalan et al, 2016). This finding suggests the microbiome in DFUs is also highly personalised, given that 87% of patients were offloaded with a total contact cast between visits, minimising wound exposure to external contaminants. However, even though there was variability between individuals, consistent patterns emerged. For example, pathogenic fungi (such as *Candida* spp and *Trichosporon* spp) as opposed to allergenic fungal moulds often found in the environment (such as *Penicillium* spp and *Aspergillus* spp) were significantly associated with wound necrosis, suggesting that fungal pathogens may contribute to tissue necrosis and poorer wound-healing outcomes.

Bacterial communities from these same wounds were grouped into four subtypes based on the abundances of different bacteria present. The transitions between each subtype were followed over time. Interestingly, fewer transitions between community subtypes over time (ie a more stable microbiome) were significantly associated with poorer wound healing outcomes, such as amputation (Loesche et al, 2017). This finding was also reflected in the fungal communities, along with experimental evidence that fungal and bacterial isolates from DFUs were able to form mixed polymicrobial biofilms *in vitro*. These findings led to the hypothesis that chronic wound microbiome stability may be due to the formation of interkingdom fungal–bacterial biofilms (Kalan

et al, 2016). Follow-up studies from independent groups have reported similar trends (Tipton et al, 2017).

Fungi such as *Candida albicans* form robust biofilms and they can grow in close association with a wide variety of bacteria relevant to human health (Shirliff et al, 2009; Harriott and Noverr, 2011; Morales and Hogan, 2010; Peters et al, 2012; Arvanitis and Mylonakis, 2015; Allison et al, 2016). Close interactions between fungi and bacteria have also been shown to protect against antibiotics, increase virulence, and may have implications for immune evasion (Dühring et al, 2015; Kong et al, 2016; Todd et al, 2019). For example, an *in vitro* wound model of mixed fungal–bacterial biofilm within a three-dimensional cellulose matrix demonstrated that the use of both antibacterial and antifungal drugs was required to decrease overall bioburden; treatment with a single antibacterial or antifungal drug only disrupted the polymicrobial biofilm to a small extent (Townsend et al, 2017).

Although this study was performed *in vitro*, there is clinical evidence to suggest that control of fungal colonisation leads to better outcomes. A randomised clinical study assigned DFU patients with deep tissue fungal- and bacterial-positive culture to standard care (surgical debridement, bacterial culture-specific antibiotics, off-loading and glycaemic control) or standard care plus daily fluconazole to assess the impact of these regimens on healing rates (Chellan et al, 2012). Researchers found the addition of fluconazole to standard care resulted in decreased healing times and a smaller wound surface area when compared to standard care (Chellan et al, 2012).

A convergent conclusion among chronic wound microbiome studies is that we, as a scientific community, are unable to point to specific microbial taxa that are causative of wound healing outcomes. Ecological properties of the microbial community as a whole may be more informative about microbial strategies within chronic wounds. For example, the stability of the wound microbiome over time may be a pattern that is shared regardless of the specific species present; such stability may be achieved through lifestyle strategies, such as biofilm formation, which is highly common within chronic wounds (Bjarnsholt et al, 2008; Bjarnsholt, 2013; Percival et al, 2010; 2012; 2015; Isabelle et al, 2018). This proposal is in line with the hypothesis of ‘functional equivalence’ put forth by Dowd et al (2008) more than a decade ago. Perhaps it is the microbial strategies and lifestyles of many species in concert, rather than the single pathogenic microbe alone, that contribute to impaired healing in chronic wounds.

How does the microbiome respond to intervention?

If microbial community structure and stability are important for delayed wound healing, how do microbial communities respond to common perturbations introduced through standardised care? Sharp debridement is considered the ‘gold standard’ for wound bed preparation (Lipsky et al, 2012). It is thought that debridement can revitalise chronic wounds by returning them into an ‘acute’ state (Ashrafi et al, 2016) and may disrupt microbial biofilms, providing a therapeutic window before the biofilm reforms (Wolcott et al, 2010).

The 100-patient DFU cohort studied by Grice and colleagues investigated the effects of sharp debridement on wound microbiome composition. No changes in aerobic bacteria and fungi were detected. However, debridement significantly reduced the microbial diversity within wounds that healed within 12 weeks and was driven by a reduction in the abundance of anaerobes (Kalan et al, 2019). These results suggest a role for anaerobes in chronic wound pathogenesis and may offer a metric for evaluating the effectiveness of debridement as a predictor of wound-healing outcomes.

Anaerobes have long been implicated in chronic wounds (Stephens et al, 2003; Wolcott et al, 2016; Percival et al, 2018; Choi et al, 2019). Polymicrobial bacterial biofilms allow anaerobes to proliferate in aerobic conditions within an *in vitro* chronic wound biofilm model (Sun et al, 2009), presumably because aerobic microbes reduce oxygen tension deep within the biofilm environment (Fox et al, 2014). Choi et al (2019) recently identified a subset of wounds dominated by a co-occurring group of obligate anaerobes within their cohort of 60 chronic wounds of mixed aetiology.

Interestingly, a number of studies have reported that neither topical nor systemic antibiotic therapy significantly alter the wound microbiome (Lipsky and Hoey, 2009; Loesche et al, 2017; Kalan et al, 2019). This suggests systemic antibiotic treatment may not effectively reach microbes within chronic wounds, perhaps due to complicating host factors.

The microbial perspective

Amplicon-based sequencing methods have transformed the way we think about wound microbiology. However, in order to understand the roles of specific species or strains and their potential to influence DFU pathogenesis, all genomic content must be sequenced to include entire bacterial genomes. This was recently reported for a cohort of patients with DFUs. Metagenomic shotgun sequencing was used to investigate the microbiome of DFUs over time (Kalan et al,

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2019). Similar to 16S rRNA gene profiling studies, *Staphylococcus* spp were the most abundant genera, comprising 18.95% of all bacteria detected. *S aureus* was the dominant species (found in 94% of specimens) and increases in *S aureus* abundance were significantly associated with longer healing times (Kalan et al, 2019). Furthermore, strain-level distribution was unequal among healing categories. Specifically, *S aureus* 7372 — a ‘generalist’ strain — was detected in 28.7% (56/195) of specimens with different healing outcomes whereas *S aureus* 10757 — a ‘specialist’ strain — was found exclusively in wounds that remained unhealed after 12 weeks. While both strains were closely related to the USA400 lineage of *S aureus*, the authors found that bacteriophage-associated virulence factors, such as the production of enterotoxins, differed between the strains and thus could be driving differences in virulence and, consequently, clinical outcome (Kalan et al, 2019).

Although all of these *S aureus* strains resulted in delayed wound healing in a diabetic mouse model of impaired wound healing, wounds infected with specialist strain 10757 were significantly larger — as measured by cross-sectional epithelial gap — at 28 days post-infection compared to wounds infected with generalist strain 7372 (Kalan et al, 2019). While these results provide functional evidence for the role of microbes in delayed healing in chronic wounds, more work is needed to understand the nuances of strain-level variation in relation to clinical outcomes.

Interestingly, infection with *Corynebacterium striatum* — which is commonly categorised as a skin contaminant — also elicited an impaired wound healing response. This finding suggests *Corynebacterium* spp may have a more significant role than originally thought (Kalan et al, 2019). *Alcaligenes faecalis* is another organism considered an environmental contaminant that is found in a large proportion of DFUs. In the murine model, infection with *A faecalis* showed accelerated early wound healing while also stimulating the production of cytokines — granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin-6, interleukin-8, interferon gamma-induced protein 10, transforming growth factor alpha and tumour necrosis factor alpha — from keratinocytes *in vitro* (Kalan et al, 2019). While these organisms are traditionally thought of as ‘non-pathogenic’, these results demonstrate the complexities of a microbial community–host interaction and allow us to question how we define pathogenesis.

Metagenomic sequencing permits annotation of metabolic functions of the microbiome. For example, antibiotic resistance genes were found

to be widely distributed in wounds, regardless of whether antibiotic therapy was prescribed or not, again raising questions about the viability of targeting DFUs with systemic antibiotic therapy (Kalan et al, 2019). The presence of metabolic genes related to virulence was strongly associated with increased wound depth and decreased tissue oxygenation while biofilm-related genes were enriched in later-healing wounds (Kalan et al, 2019). Together, these results support the hypothesis that the wound microbiome exists in a biofilm state, although functional data are required to validate such findings.

Overall, high-throughput sequencing has allowed a precise and high-resolution characterisation of wound microbiome members, generating new hypotheses to be tested using models of wound healing. Functional characterisation of these microbes is required to gain insight into the genes and pathway interactions underlying microbe–microbe and host–microbe interactions, in order to explore targeted diagnostic, prognostic and treatment opportunities.

Conclusions and recommendations

While initially heralded as a method of molecular diagnosis for chronic wounds, evidence from culture-independent sequencing suggests no single pathogen is likely to be the cause of delayed wound healing in the absence of spreading systemic infection. Rather, chronic wounds should be considered a diverse, polymicrobial environment. At this time, our understanding of the basic biology underlying microbial community interactions within wounds is still lacking, but — unlike antibiotics and other antimicrobials — debridement appears to effectively disrupt the wound microbiome.

As we move forward, new tools and wound infection models are being developed to study the interactions between microbes within communities of increasing complexity and with host factors. Ultimately the collective goal of this research is to better understand the underlying biological mechanisms driving microbial community assembly in the wound environment and their effects on chronic wound pathogenesis. WINT

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