MICROBIAL BIOBURDEN IN VENOUS LEG ULCERS

by

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Dedication

To Chris, Gwen, and Madeline June, who fill my cup with love and hope
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List of Abbreviations

Ankle brachial pressure index (ABPI)
Arterial insufficiency (AI)
Ultrasonography (US)
Photoplethysmography (PPG)
Shannon Diversity Index (SDI)
Venous leg ulcer (VLU)
Venous incompetence (VI)
Venous leg ulcers (VLUs) are susceptible to microbial invasion, and serious complications can result without timely control of infection. Diagnosis of wound infection is primarily based on subjective clinical characteristics and patient-reported symptoms, and treatment with antimicrobials has not consistently shown improvement in healing outcomes. Here we review studies using bacterial cultures and/or new molecular-based methods associating microbial bioburden with healing outcomes in VLU patients, with the goal of guiding future studies to better determine significant patterns of microbial involvement in chronic wounds. We then present a case that demonstrates the multi-dimensional changes in bacterial bioburden of a chronic venous leg ulcer over the course of healing using detailed sampling and multiple molecular techniques. Serial swabs of the ulcer center before debridement show qualitative and quantitative microbial dynamics that correlate with wound expansion, antibiotic therapy, and healing.
PART 1: CRITICAL REVIEW

Association between Microbial Bioburden and Healing Outcomes in Venous Leg Ulcers: A Review of the Evidence

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Abbreviated title: Microbial Bioburden in Venous Leg Ulcers
Abstract

**Significance:** Venous leg ulcers (VLUs) are susceptible to microbial invasion, and serious complications can result without timely control of infection. Diagnosis of wound infection is primarily based on subjective clinical characteristics and patient-reported symptoms, and treatment with antimicrobials has not consistently shown improvement in healing outcomes. This is a review of studies using bacterial cultures and/or new molecular-based methods associating microbial bioburden with healing outcomes in VLU patients, with the goal of guiding future studies to better determine significant patterns of microbial involvement in chronic wounds.

**Recent Advances:** Studies reviewed here use cultivation-based identification of bacteria and next-generation sequencing of the bacterial 16S rRNA gene to gain insight into microbial bioburden in VLUs. Further application of sophisticated DNA sequencing and bioinformatic analyses has potential to revolutionize our ability to further discern, with high resolution, complex microbial communities in chronic wounds.

**Critical Issues:** Few previous studies of microbial bioburden in VLUs have incorporated knowledge of clinical treatments, which includes close monitoring of patients’ symptoms and responses to therapy. Thus, wound care practitioners are currently without evidence-based guidance for diagnosis and treatment of wound infections.

**Future Directions:** Clinically relevant breakthroughs are possible by combining advanced microbial detection techniques with improved study designs that reflect clinical practices. Well-designed longitudinal studies have great potential to lead to better evidence-based diagnosis of chronic wounds. Greater understanding of microbial bioburden in chronic wounds is likely to lead to better therapies that speed healing and prevent wound infection without risking development of antimicrobial resistance.
Scope and Significance

Venous leg ulcers (VLUs) are the most common cause of chronic leg wounds with a distinct pathogenesis and unique susceptibilities to microbial invasion. Lack of timely control of microbial invasion can lead to serious complications including delayed healing, cellulitis, and sepsis. This is a review of evidence regarding the effect of microbial bioburden on healing outcomes in VLUs, including clinical studies using bacterial culture and advanced molecular methods. Combination of new microbial detection techniques with improved study designs learned from strengths and weaknesses of previous studies has great potential to lead to improved diagnostics and therapeutics for chronic wounds.

Translational Relevance

This review compares study designs and results from clinical investigations of microbial bioburden in VLUs with associated patient information including wound parameters and healing outcomes. Methods used for microbial detection in these studies range from cultivation-based methods to molecular-based methods including DNA sequencing of the bacterial 16S rRNA gene for microbiome analysis. Next-generation DNA sequencing has revolutionized our ability to discern complex microbial communities with high resolution. However, clinically relevant breakthroughs are only possible if further applications of these technologies are guided by studies that incorporate clinical practice to determine significant patterns of pathogenic microbial involvement in VLUs.

Clinical Relevance

Current clinical practice for wound infection includes use of subjective clinical signs and symptoms for diagnosis, then antimicrobial treatment with guidance by conventional bacterial culture. However, as reviewed here, there are very few studies of
wound microbial bioburden that include patients’ wound parameters and healing outcomes and no studies that examine changes in microbial communities with antimicrobial therapy. Additionally, there is little evidence that use of antimicrobials leads to improvements in clinical outcomes. We will discuss lessons learned from previous studies to guide future investigations to better reflect clinical practice and improve prevention and treatment of wound infections.

**Background**

Venous leg ulcers (VLUs) are a common and costly problem, accounting for up to 70% of all chronic leg ulcers.\(^1,2\) Among people aged 65 years and older, the annual prevalence of VLUs is 1.7%.\(^3\) Of all types of chronic ulcers, VLUs have the worst long-term healing prognosis and are more likely to be recurrent.\(^2,4\) In fact, half of patients diagnosed with VLUs have an ulcer duration greater than one year and recurrence has been estimated at >75% of those affected.\(^1,2\) With such long ulcer duration and high recurrence rate, patients presenting for wound care can be at any point along this continuum, and often have an extensive medical history with several co-morbidities and complex medication regimens. Thus, thorough patient assessment at initial and follow-up visits is essential to determine optimal treatment strategies.

An essential element of thorough assessment of VLU patients includes frequent and regular monitoring for infection. For evaluation and treatment of bacterial infection of chronic wounds, most wound care practitioners rely on subjective clinical signs and patient-reported symptoms.\(^5\) This reliance on subjective findings is necessitated by the lack of objective diagnostics that can guide clinical decisions to improve patient outcomes. Conventional bacterial culture is the most widely available tool for diagnosis of bacterial infection, but has been shown consistently to underestimate the complexity of wound bacterial burden as revealed by DNA-based molecular methods.\(^6-10\) Bacteria in
chronic wounds grow in biofilms which include many anaerobes and other species not identified by cultivation-based methods. This complexity of the microbial flora in chronic wounds may explain the lack of improved clinical outcomes observed in many studies of antimicrobial use targeting bacteria identified by cultivation-based methods. Thus, rather than aiming to eradicate microbes with an antimicrobial, the enhanced view of wound microbial bioburden revealed by molecular methods supports a more comprehensive approach, including further consideration of patient and wound factors that may contribute to increased susceptibility to microbial invasion.

VLUs occur in people with chronic venous insufficiency, which has many sequelae that can increase susceptibility to microbial invasion. These include edema, lipodermatosclerosis, iron overload and hemosiderin deposition, dermatitis, atrophie blanche, and persistent proinflammatory immune responses (PART 1 Figure 1). VLU patients also have propensities for neuropathy, limited ankle mobility, deep vein thrombosis, thrombophlebitis, and frequent occurrence of co-morbidities such as obesity, arterial insufficiency, diabetes, and autoimmune diseases. Most studies of VLU microbial bioburden either exclude or do not account for these characteristics, making deduction of significant patterns difficult. In this review, we will examine studies of microbial bioburden in VLUs that have included information on patient wound parameters and outcomes, with the goal of guiding future studies to improve objective assessment of bacterial infection and clarify the role of antimicrobial treatment in VLUs.

Discussion

1) Microbial Bioburden and VLU Healing Outcomes

The concept of increased wound microbial bioburden has been proposed as an important predictor of poor healing outcomes, including three dimensions of microbiology: 1) microbial load, 2) presence of pathogenic organisms, and 3) microbial
diversity. We will further examine the current evidence behind these dimensions of microbial bioburden in studies that have included VLU patient healing outcomes.

1.1 Microbial Load

Microbial load of $>10^5$ colony forming units (CFU)/gram is often held as the reference standard definition of clinical infection, although this figure is primarily based upon early studies of heterogeneous patient populations, including few VLU patients healing by secondary intention (healing without intervention with skin grafting, delayed surgical closure, or other surgical procedures). These studies included 50 skin graft patients with a mix of VLUs, traumatic, and burn wounds, 40 patients requiring delayed surgical closures (types of wounds not otherwise described), and 95 surgical incisions.
requiring delayed surgical closure.\textsuperscript{21} All of these studies demonstrated > 90% graft survival or successful delayed surgical closure only when bacterial colonization was ≤10\textsuperscript{5} CFU/gram and < 20% successful closure when bacterial colonization was > 10\textsuperscript{5} CFU/gram. Unfortunately, results have not been as clear in studies of wounds healing by secondary intention without further surgical intervention.\textsuperscript{23, 24}

Most studies of quantitative bacterial cultures in chronic wounds have not associated microbial load with healing outcomes.\textsuperscript{25-27} Two exceptions include Davies et al.,\textsuperscript{23} and Lantis et al.\textsuperscript{24} Davies et al. prospectively performed quantitative tissue biopsies and surface swabs of 66 patients with clinically non-infected chronic VLUs at baseline and monitored healing outcomes. They showed that neither quantitative tissue biopsy (CFU/gram) nor surface swabs (CFU/mL) of the wound at baseline predicted healing at six months in a logistic regression model.\textsuperscript{23} The investigators state that the 7-log fold variation in bacterial numbers suggests that the microbial load is not directly related to healing outcome in clinically non-infected VLUs. However, they did report that increased bacterial load in the tissue biopsies was significantly correlated with longer ulcer duration at the time of sampling. There was also a significant relationship between healing at four weeks (as measured in percent reduction in surface area) and bacterial load in the biopsies and swabs, suggesting that microbial load may be applicable to healing rates within a month of sampling.

Lantis et al.\textsuperscript{24} analyzed the impact of microbial load and other measured variables in 227 patients in a prospective randomized VLU trial of HP802-247, an allogeneic bioformulation of neonatal keratinocytes and fibroblasts administered via pump spray.\textsuperscript{28} Similar to Davies et al.,\textsuperscript{23} they found that increased bacterial load in quantitative tissue biopsies was significantly greater in wounds of longer duration, though there was no association between bacterial load and healing. However, an overall negative effect on healing was seen by regression analysis for wounds harboring
≥ 1 x 10⁴ CFU/g of select bacterial species termed “inhibitory bacteria” (species associated with a lower than average proportion of wounds healed), supporting the multidimensional concept of microbial bioburden requiring integration of the both the load and species of bacterial involvement.

Both of these studies of VLU patients suggest that increased bacterial load in tissue biopsies is associated with ulcers of longer duration, but cannot be used to predict overall healing outcomes. Further associations of bacterial load and healing outcomes may be difficult to determine due to the underestimation of CFU by cultivation-based quantitation compared to molecular methods such as quantitative PCR as demonstrated in diabetic foot ulcers.⁷ Thus, application of molecular methods in future studies of VLUs may help illuminate the potential correlation between bacterial load and healing rates further.

1.2 Presence of Pathogenic Organisms

Although there have been many investigations aimed at identifying the effect of different bacteria on the size, duration, and healing outcome of VLUs, there are few clear or consistent associations for any single species implicated (summarized in Tables 1-3). As summarized in Table 1, several studies have shown no association between any specific bacterial species and healing outcome.¹⁶, ²⁹-³³ Among studies that show associations, there are conflicting conclusions on the relevance of many of the commonly isolated species such as Staphylococcus aureus and Pseudomonas species as well as anaerobes (Table 2). Most studies of S. aureus show no association with healing delay or increased ulcer size.²⁴, ³⁴, ³⁵ However, there are studies that associate S. aureus both with delayed healing¹⁴, ³⁶ and smaller, healing ulcers.²³, ³⁷ Similarly, Pseudomonas species have been associated both with larger ulcers and delayed healing,²⁴, ³⁴-³⁶, ³⁸ while other (though fewer) studies have found that ulcers harboring Pseudomonas were more likely to heal normally.¹⁰, ¹⁴ Fewer studies comment on
Table 1: Studies that conclude bacteria are unlikely associated with healing outcomes of VLUs

<table>
<thead>
<tr>
<th>Source</th>
<th># of Patients (Ulcers)</th>
<th>Patient Details</th>
<th>Sampling Time Points</th>
<th>Sampling Method</th>
<th>Report of Healing Outcome</th>
<th>Summary of Study Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriksson et al. (Stockholm, Sweden) 1984</td>
<td>53</td>
<td>• VI by clinical judgement</td>
<td>Baseline, then every 1-2 weeks</td>
<td>2 swabs of ulcer (for aerobic and anaerobic culture)</td>
<td>10 weeks</td>
<td>No difference between occurrence of various bacterial species or semiquantitative levels initially or throughout the study irrespective of treatment or outcome</td>
</tr>
<tr>
<td>Gilchrist et al. (London, UK) 1984</td>
<td>18 (20)</td>
<td>• VI by clinical judgement</td>
<td>Baseline, then weekly</td>
<td>Swab</td>
<td>12 weeks</td>
<td>Ulcers treated with hydrocolloid dressing maintain a fairly stable microbial flora, except for appearance of differences in 10/20 ulcers, and consistent decline and loss of Pseudomonas</td>
</tr>
<tr>
<td>Skene et al. (Harrow, UK) 1992</td>
<td>140</td>
<td>• VI confirmed by US and PPG</td>
<td>Baseline, then every month</td>
<td>Swab</td>
<td>16 weeks</td>
<td>No association between bacteria present at baseline and healing</td>
</tr>
<tr>
<td>Hansson et al. (Goteborg, Sweden) 1995</td>
<td>58</td>
<td>• VI by clinical judgement</td>
<td>Baseline, then monthly</td>
<td>10 mm &quot;sampling disc&quot; pressed against ulcer surface for 1 min</td>
<td>16 weeks</td>
<td>No single microbial strain was correlated to ulcer size changes, with a multiple linear regression analysis showing presence of different species could not explain 1/5th of variability of ulcer size changes</td>
</tr>
<tr>
<td>Moore et al. (Cardiff, UK) 2010</td>
<td>176</td>
<td>• VI by clinical judgement</td>
<td>Weekly</td>
<td>Swab in a zig-zag pattern over the whole ulcer surface</td>
<td>12 weeks</td>
<td>No significant association with any strains identified by aerobic and anaerobic culture, total # of anaerobes, # of species identified, or 4 or more taxonomic groups</td>
</tr>
</tbody>
</table>

**Summary of Study Conclusions**

- No significant association between bacterial populations and healing were more likely to exclude patients with clinical signs of infection in the study design (Table 1 vs. Table 2).

**Abbreviations:** Ankle brachial pressure index (ABPI), Arterial insufficiency (AI), Ultrasonography (US), Photoplethysmography (PPG), Venous incompetence (VI)

anaerobes and the rigour of anaerobic cultivation methods vary widely, but even these studies disagree on the association of anaerobes with healing. Results for *Streptococcus* species are most consistent, with most studies showing association with ulcers of increased size, longer duration, and healing delay.

Interestingly, studies that did not find associations between bacterial populations and healing were more likely to exclude patients with clinical signs of infection in the study design (Table 1 vs. Table 2). In all of the studies examined, clinical signs of infection were not defined except for the report by Moffatt et al., which listed pain, odor, swelling, erythema, and confirmation by microbiological results without further definition.
### Table 2: Studies that conclude bacteria are likely associated with healing outcomes of VLUs

<table>
<thead>
<tr>
<th>Source</th>
<th># of Patients (Ucers)</th>
<th>Patient Details</th>
<th>Sampling Time Points</th>
<th>Sampling Method</th>
<th>Report of Healing Outcome</th>
<th>Summary of Study Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lookingbill et al. (PA, USA) 1978</td>
<td>9 (13)</td>
<td>• Vi by clinical judgement  • No signs of infection noted throughout study  • Concurrent study of benzoyl peroxide 10% lotion</td>
<td>All ulcers: Baseline and 2 weeks  Only 3 ulcers: 6 weeks</td>
<td>• Swab 1 cm distal ulcer  • Biopsy 3mm of same area  • Good correlation noted between methods, though swabs had higher quantitative and qualitative recovery rate than biopsies</td>
<td>16 weeks</td>
<td>8 ulcers with ≥75% did not heal  Of 5 ulcers with &lt;100 healed, 1 almost healed, 1 had light healing  Qualitative differences between ulcers described, no differences noted</td>
</tr>
<tr>
<td>Halbert et al. (Fremantle, Australia) 1992</td>
<td>82 (180)</td>
<td>• Vi confirmed by PPG  • Excluded patients with other causes ulceration by performing testing for blood glucose, urea and electrolytes, rheumatoid serology, autoantibody screen, and AI with ABPI &lt; 0.9  • Did not exclude patients with clinical signs of infection  • Concurrent dressing trial for a zinc oxide impregnated paste bandage an calcium alginate fiber dressing</td>
<td>Baseline  • Swab not otherwise specified</td>
<td>24 weeks</td>
<td>• Compared to ulcers with no bacterial growth, ulcers were significantly larger and of longer duration if colonized with anerobes. Beta hemolytic streptococci, aerobioana alone, and &quot;coliforms&quot; not otherwise specified  • Ulcer healing time was prolonged when colonized with mixed flora. Though not significantly prolonged  • P. aeruginosa, P. aerobioana alone, and normal skin flora  • 2 patients developed cellulitis and were treated with antibiotics. Cultures showed aureus and mixed coliforms from one and anaerobes and mixed coliforms from the other</td>
<td></td>
</tr>
<tr>
<td>Midson et al. (Copenhagen, Denmark) 1996</td>
<td>59</td>
<td>• Vi confirmed by US  • Excluded patients with AI with toe pressures &gt;60 mmHg and diabetes  • Did not exclude patients with clinical signs of infection  • Concurrent study of patients undergoing ligaton of incompetent perforators and wide debridement</td>
<td>Weekly x 4 weeks, then monthly  • Swab samples from proximal ulcer edge</td>
<td>24 weeks</td>
<td>• P. aeruginosa, S. aureus, and hemolytic streptococci were associated with healing delay  • P. aeruginosa was associated with ulcer enlargement  • 41% of patients had colonization of cellulitis on 1-3 occasions; cultures showed aeruginosa in 7, hemolytic streptococci in 4, and others not noted  • Ulcers with P. aeruginosa and cellulitus were not significant larger than without cellulitis  • Healing delay occurred despite antibiotic treatment when cellulitis occurred  • No mention of changes in bacterial species throughout healing</td>
<td></td>
</tr>
<tr>
<td>Trengove et al. (Fremantle, Australia) 1996</td>
<td>52</td>
<td>• Vi confirmed by PPG  • Included patients with AI, though all had ABPI &gt; 0.7  • Did not exclude patients with clinical signs of infection  • Concurrent study of different compression therapies</td>
<td>Weekly  • 2 swabs of ulcer for aerobic and anaerobic cultures</td>
<td>24 weeks</td>
<td>• A significantly greater number of ulcers with four or more bacteria failed to heal  • The presence of any one specific bacterial group was not associated with healing  • The bacterial flora changes as the ulcers heal, though the changes were not related to healing  • No mention of clinical signs of infection or antibiotic use</td>
<td></td>
</tr>
<tr>
<td>Gjodsbol et al. (Copenhagen, Denmark) 2009</td>
<td>36</td>
<td>• Vi confirmed by US  • Patient had ABPI &gt; 0.6, no diabetes or other diseases that could influence the ulcer  • No patients had antibiotics within 14 days of inclusion, or antibiotics during the study</td>
<td>Swab: Baseline only  Biopsy: Baseline and after 4 weeks  Filter paper: Baseline and every 2 weeks</td>
<td>• Swab  • Biopsy 4mm from ulcer center  • Sterile 10 mm filter paper pad placed in ulcer until saturated  • Combined findings from methods for conclusions</td>
<td>8 weeks</td>
<td>• All of the ulcers had resident flora (defined as one or more species isolated from all or at least one of the 8 occasions; cultures showed aeruginosa in 7, hemolytic streptococci in 4, and others not noted  • P. aeruginosa was present as resident bacteria in all ulcers that increased in size but nonresident in all ulcers that did not increase in size  • Ucers with S. aureus were significantly smaller  • No significant difference in ulcer size was associated with presence of anaerobic bacteria</td>
</tr>
<tr>
<td>Davies et al. (Cardiff, UK) 2007</td>
<td>66</td>
<td>• Vi confirmed with US  • Excluded patients with AI with ABPI &gt;0.8, diabetes, immunosuppression, or antimicrobials within 1 mo</td>
<td>Baseline  • Swab of 1 cm distal ulcer  • Biopsy 3mm of same area of ulcers  • Differentiated swabs and biopsies in conclusions</td>
<td>24 weeks</td>
<td>• No association between individual bacteria or bacterial groups present at baseline and overall healing  • The presence of ≥4 bacterial genera in the ulcer was associated with delayed healing  • Ulcer duration correlated with bacterial load in the biopsy and not the swab  • Significant relationship between reduction in surface area four weeks and bacterial load in the biopsies and swabs</td>
<td></td>
</tr>
<tr>
<td>Moffat et al. (Glasgow, UK) 2010</td>
<td>113</td>
<td>• Vi confirmed with US and PPG  • AI assessed with ABPI, but not excluded  • Did not exclude patients with clinical signs of infection, clinically characterizing cellulitis by pain, odor, swelling, and erythema  • Extensive characterization of patient for evaluation of clinical predictors of healing</td>
<td>Not stated  • Swabs not otherwise specified</td>
<td>Up to 48 weeks</td>
<td>• Clinical cellulitis  • aureus, hemolytic streptococci was associated with reduced healing, but did not approach statistical significance  • The presence of Pseudomonas was associated with an increase in healing  • The number of species in an ulcer did not show a direct relationship with healing, although the presence of all least bacterium reduced the chance of healing</td>
<td></td>
</tr>
<tr>
<td>Lants et al. (USA) 2013</td>
<td>227</td>
<td>• Vi confirmed by US  • Excluded patients with clinical signs of infection, elevated HbA1c or prebiannia, thyroid disease, systemic lupus or elevated anti-DNA  • Concurrent prospective randomized trial of HPR02-247, an allogeneic bioformulation of neonatal keratinocytes and fibroblasts</td>
<td>Baseline  • Biopsy 4 mm from ulcer</td>
<td>12 weeks</td>
<td>• Bacteria divided into inhibitory (lower than average 80%) proportion healed vs noninhibitory bacteria  • Inhibitory bacteria all facultative anaerobes, with the excep of Pseudomonas species  • Regression analysis showed a significantly negative effect healing associated with the inhibitory bioburden index, who was the number of inhibitory bacteria present  • No significant association between bacterial load and healing</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** Ankle brachial pressure index (ABPI), Arterial insufficiency (AI), Ultrasoundography (US), Photoplethysmography (PPG), Venous incompetence (VI)
As shown in Table 3, there have been two studies using molecular-based methods for identification of bacteria that also include VLU healing outcome measures, Davies et al.\(^6\) and Tuttle et al.\(^10\) To compare and contrast these studies, both of these studies compared aerobic and anaerobic bacterial cultures to sequencing of the bacterial 16S rRNA gene, which can be used for identification of bacteria by matching the unique 16S rRNA gene sequence to the taxonomic classification based on large reference databases such as Ribosomal Database Project (RDP), SILVA, and Greengenes. As Davies et al. was prior to the dissemination of next-generation sequencing technology, they selected 16S rRNA genes for sequencing that were unique from culture isolates on the basis of profiling by denaturing gradient gel electrophoresis (DGGE).\(^6\) In contrast, Tuttle et al. utilized next-generation (454 FLX titanium series) 16S rRNA sequencing, as well as the Ibis T5000 universal biosensor, which uses microbial DNA for taxonomic identification based on the molecular sizes of amplicons generated by using multiple PCR primer pairs.\(^10\)

<table>
<thead>
<tr>
<th>Source</th>
<th># of Patients (Ulcers)</th>
<th>Patient Details</th>
<th>Sampling Time Points</th>
<th>Sampling Method</th>
<th>Report of Healing Outcome</th>
<th>Summary of Study Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davies et al. (Cardiff, UK)</td>
<td>18</td>
<td>VI confirmed by US • Excluded patients with AI • ABPI ≥ 0.8, diabetes, all antibiotic use 1 mo</td>
<td>Baseline</td>
<td>Swab of 1 cm² of central ulcer for aerobic and anaerobic culture • Biopsy 6 mm of same area of ulcer bisection for culture and molecular analysis</td>
<td>24 weeks</td>
<td>• More micrococcus and streptococcus in nonhealers, though not significant due to small numbers • No difference between anaerobes between healers and nonhealers</td>
</tr>
<tr>
<td>Tuttle et al. (USA)</td>
<td>10</td>
<td>VI by clinical judgement • Did not exclude patients with clinical signs of infection or use of antibiotics</td>
<td>Baseline</td>
<td>Curette 3 mm from proximal and distal ulcer edge and center • Swab of ulcer base in areas curetted • Combined findings from methods for conclusions</td>
<td>24 weeks</td>
<td>• More diverse bacterial populations in nonhealed ulcers • Differences between non-healed and healed wounds showed 16S sequencing methods, including more Actinomycetales in non-healed wounds, and more Pseudomonadaeae in healed wounds • No differences revealed by culture or other molecular diagnostic method studied (Ibis)</td>
</tr>
</tbody>
</table>

Abbreviations: Ankle brachial pressure index (ABPI), Arterial insufficiency (AI), Ultrasonography (US), Photoplethysmography (PPG), Venous incompetence (VI)

Overall, the reports from both Davies et al. and Tuttle et al. demonstrate that sequencing methods identify significantly more bacteria than cultivation methods, with
increased detection of even known culturable bacteria. Increased detection of culturable microbes could be in part due to the ability of molecular methods to detect DNA from non-viable or “viable but unculturable” microbes in the ulcer. While Davies et al. did not identify significant differences between microbial communities from ulcers that were either healed or unhealed at 6 month follow-up, Tuttle et al. showed significantly greater bacterial diversity in unhealed ulcers, which incorporates information on the type and relative proportion of the bacteria, as discussed further in section 5.1.3.6.10

1.3 Microbial Diversity

Another component of microbial bioburden of chronic ulcers that has been implicated in delayed healing is bacterial “diversity,” which is defined differently depending on the data used for analysis. For the cultivation-based studies reviewed here, diversity is defined as the number of different species of bacteria cultured from an ulcer sample. As summarized in Tables 1 and 2, some studies have concluded that non-healing ulcers were more likely to have higher microbial diversity,23, 35 but others have shown that diversity could not be used to predict healing.14, 32 For studies using DNA sequencing data, more specific ecological concepts of diversity can be used. Tuttle et al.10 used the Shannon diversity index, which takes into account the relative proportions of each species, showing that diversity was significantly higher in VLUs that were not healed by six month follow-up. This diversity within each sample is referred to as “alpha diversity,” or “species richness.”

The concept of diversity also includes variation in bacterial communities between different samples, which is termed “beta diversity.” Evaluation of beta diversity could include comparison of: 1) different sampling time points of the same ulcer, 2) different sampling methods (e.g. swab vs. curette/biopsy), 3) different geographic regions within the same ulcer (intra-wound variation), 4) similar time points in different ulcers (inter-wound variation), and 5) between wounds and intact skin. Of the studies reviewed here,
only the cultivation-based studies included sampling schemes that could be used to
determine beta diversity. Unfortunately, few conclusions can be drawn from the mostly
descriptive culture data due to insufficient details in the results. For example, of the
studies with multiple sampling time points, some concluded that the bacterial
communities remained relatively the same,29-31,38 while others reported wide variations
with time,16,32,35 however, there is no objective measure of the relative extent of variation
in the different studies, making these conclusions subjective. Also, these studies
included minimal information on changes in patient/ulcer characteristics (such as clinical
signs and symptoms of infection) that could aid in further interpretation of differences
between samples at various time points.

There have not yet been studies using molecular methods to further determine
different aspects of beta diversity in VLUs that also incorporated patient/ulcer
characteristics or healing outcome data. Therefore, this is an important future direction
for research. To guide this research, we can learn from studies that have been done
examining beta diversity in chronic wounds of mixed etiology using cultivation and/or
molecular based methods. These studies have aimed to determine variation in microbial
composition in 1) different sampling methods (e.g. biopsy vs. swab) and 2) intra- vs.
inter-wound variation (using similar sampling methods), as detailed in sections 5.1.3.1
and 5.1.3.2. Although these studies do not directly contribute to determining the
influence of microbes on healing, they can help understand how differences in sampling
method or location may obscure patterns in investigations of microbial bioburden.

1.3.1 Differences Between Sampling Methods

Studies comparing different wound sampling methods—including tissue biopsies
vs. less invasive techniques such as swabbing and wound fluid collection—have
primarily used cultivation-based methods in chronic wounds arising from multiple
etioologies (as reviewed by Reddy et al.39). Overall, cultivation methods have been shown
to be comparable between biopsies and swabs for aerobes, though there are more discrepancies for anaerobes and overall quantities of bacteria, suggesting uneven vertical distribution of at least some species as discussed in section 5.1.3.2.\textsuperscript{23, 25, 37, 40} Molecular based studies performed to date have predominantly used sharp debridement samples without comparison of different sampling methods or indication of the depth of the wound sampled.

1.3.2 Intra- and Inter-wound Variation

Though cultivation-based methods are unlikely to be sensitive enough to determine intra-wound variation in bacterial composition of ulcers, quantitative culture studies have suggested that bacterial concentration is increased superficially in wounds.\textsuperscript{35, 40} Sophisticated imaging techniques such as fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) have confirmed this result, further displaying wound bacteria in both mixed and single species biofilms as well as scattered individual cells.\textsuperscript{41-43} In general, \textit{S. aureus} has been visualized more towards the surface of the ulcers and biofilms, particularly compared to \textit{Pseudomonas aeruginosa}, potentially explaining why \textit{S. aureus} is most commonly isolated from swab samples of chronic wounds and \textit{P. aeruginosa} is likely frequently underestimated.\textsuperscript{41, 43, 44}

Studies that have used bacterial 16S rRNA sequencing to investigate intra-wound variation in chronic wounds include Price et al.,\textsuperscript{45} and Wolcott et al.\textsuperscript{46} In both studies, curette samples were collected from different geographic regions of chronic wounds, including the leading edge, opposing leading edge, and/or center of chronic wounds. Wolcott et al. concluded there was substantial variation across the wound surface without further statistical analysis.\textsuperscript{46} Price et al. further compared intra-wound variation to bacterial communities from wounds on different individuals (inter-wound variation), showing that the intra-wound variation is significantly smaller than the inter-wound variation.\textsuperscript{45} They therefore argue that studies that do not control for sampling site
within the wound can still be valid for comparing inter-wound variation, though controlling for sampling site likely improves the overall quality of the study. It is important to note that these were cross-sectional studies of patients without further characterization of the healing trajectory. To date, no other studies have compared different time points, patient characteristics, wound parameters, or healing outcomes.

2) Clinical Signs and Symptoms of VLU Infection

Studies to date have regarded patient clinical signs and symptoms as subjective and secondary to more objective measures, most often using >10^5 CFU/gram tissue as the reference standard for diagnosis of infection. However, as discussed above, this one-dimensional reference standard does not fully capture the extent of microbial bioburden, of which microbial pathogenicity and diversity are also important aspects. Regardless of whether quantitative tissue cultures are an adequate reference standard of infection, they are generally considered impractical for routine use in clinical practice due to variability in cultivation methods in different clinical laboratories and frequency of refusal by patients to undergo biopsy. Therefore, in reality, most wound care practitioners rely solely on clinical signs and symptoms to identify wound infection.5 Once infection is suspected, topical antiseptics (for superficial infection) and/or systemic antibiotics (for deep infection) are recommended, and conventional culture may or may not be performed to guide antimicrobial therapy.47

Because studies on the accuracy of clinical signs and symptoms of infection use >10^5 CFU/gram as a reference standard and include minimal information on healing outcomes, there is currently no evidence to assess how well clinical signs and symptoms predict infection-related complications. As reviewed by Reddy et al.,39 no sign or symptom was consistently evaluated in more than two studies, with the likelihood of infection being increased when the ulcer causes increased pain, and being decreased in wounds that had no serous exudates or were healing rapidly.48 Other clinical signs and
symptoms evaluated include non-healing status, increase in wound exudate (more than 50% of the dressing stained with exudate), red tissue at the wound base that bleeds easily, presence of slough or non-viable tissue at the wound base, and unpleasant odor for a superficial infection for which a topical antiseptic would be recommended.49 Wounds with a deeper tissue infection for which a systemic antibiotic would be recommended have additional signs and symptoms, including increased size, surrounding areas of tissue breakdown, and increased peri-wound temperature, redness, and swelling.49 Of note, these studies have included leg and foot ulcers of various etiologies, not only VLUs; therefore some signs and symptoms may be more or less relevant for VLUs.

Summary

Thorough assessment of VLU patients includes regular monitoring for signs of infection. Failure to do so may lead to serious complications, including further wound breakdown, cellulitis, and sepsis. As this review demonstrates, attempts to define wound infection using a one-dimensional measure have been largely unsuccessful; rather, full evaluation of microbial bioburden—including microbial load, pathogenicity, and diversity—as well as a defined set of clinical parameters and patient-reported symptoms is likely required, though studies have not been designed to support this practice. Previous studies linking microbial bioburden to wound parameters and healing outcomes have focused primarily on clinically non-infected wounds. Few cultivation-based studies and no molecular-based studies have analyzed changes in wound microbial bioburden over time. Even more rare are studies that link results on microbial bioburden with the clinical status of the patient or use of antimicrobials or other therapies.

Therefore, recommendations for future studies to better support clinical practice include the following:
1) Further use of molecular methods for more comprehensive evaluation of microbial burden;

2) Prospective determination of patients’ signs and symptoms of infection and wound parameters to better link changes in microbial bioburden with patients’ stages of healing and responses to treatments, including wound exacerbation, stalled healing, and resolution; and

3) Inclusion of different sampling methods as well as serial sampling to compare intra-wound variation throughout the course of healing.

Implementation of these study design changes would enhance and corroborate with the clinical decision making process in which modifications to therapy are made at frequent and regular intervals based on changes in patients’ clinical signs and symptoms⁴⁷. These studies would better elucidate the responses of microbial bioburden to therapeutics, clarifying the impact of antimicrobials versus other treatments such as compression therapy. Results from these studies would likely support a more comprehensive approach to infection control in chronic wounds that would include controlling patient factors that increase susceptibility to microbial invasion (as suggested in Figure 1).

Overall, a combination of improved study design and use of higher resolution molecular methods to assess wound microbial bioburden has great potential to lead to better evidence-based diagnosis of chronic wounds. In addition to taxonomic information, the future of molecular methods also includes further discernment of functional differences between bacterial communities, potentially leading to more targeted therapies that speed healing and prevent wound infection without risking development of antimicrobial resistance.
Take-Home Messages

- There is no established reference standard for diagnosis of wound infection in VLUs.
- To better support clinical practice, further studies are needed that include evaluation of all aspects of microbial bioburden—including microbial load, pathogenicity, and diversity—correlated with patient signs and symptoms of infection and healing outcomes.
- Molecular methods identify significantly more bacteria than cultivation methods, with increased detection of even culturable bacteria.
- Therefore, a combination of improved study design and use of molecular methods has potential to lead to improved diagnostics and therapeutics to speed healing of chronic wounds.
PART 2: BRIEF REPORT

Chronic Wound Microbial Bioburden Dynamics Correlate with Wound Expansion, Antibiotic Therapy, and Healing

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Abbreviated title: Bacterial Bioburden of a Chronic Venous Leg Ulcer
Abstract

We use 16S rRNA profiling and qPCR to show changes in bacterial community pathogenicity, load, and diversity in a chronic venous leg ulcer over the course of healing. Our detailed sampling and molecular analysis reveal that the bacterial bioburden of the wound center before debridement correlates with phases of healing.
Introduction

Venous leg ulcers (VLUs) account for 70% of all chronic leg wounds and have an annual prevalence of 1.7% among people aged 65 years and older. VLUs have high bacterial bioburden at baseline, leading to frequent prescriptions for antibiotics in this patient population, even though antibiotic treatment has not been shown to consistently improve healing outcomes. A major barrier to determining appropriate antibiotic treatment is differentiating between benign bacterial colonization versus pathogenic infection in chronic wounds. Diagnosis of wound infection is predominantly based on subjective clinical characteristics and patient-reported symptoms according to a survey of wound care professionals, with only 12% routinely performing bacterial culture prior to antibiotic treatment. Bacterial culture, the current standard for bacterial identification in routine clinical settings, requires several days for bacterial cultivation and identification, and thus cannot be relied upon for acute decision making when signs and symptoms of wound infection develop. Additionally, cultivation-based methods are known to be biased toward microbes that thrive in standard growth conditions, often underestimating the complexity of wound bacterial burden as revealed by DNA-based bacterial identification methods.

DNA-based bacterial identification methods, including increasingly available and reliable DNA sequencing for microbial community analysis, have many promising advantages over cultivation-based methods, including improving both the speed and accuracy of infection diagnosis. Initial clinical integration of these technologies has also spurred a paradigm shift in microbial pathogenesis of chronic wounds, incorporating ecological concepts such as the multidimensional nature of microbial bioburden. In this sense, bioburden integrates not only the identification of microbial pathogens, but also the total abundance and diversity of the host-associated microbial community. Collectively, these three aspects—microbial pathogenicity, load, and diversity— give a
more complete accounting of the host-microbe interactions present in a chronic wound, and more completely identify microbial-associated barriers to healing. Unfortunately, few studies of chronic wounds have considered this expanded definition of microbial bioburden, and no studies have associated changes in bioburden with clinical factors such as changing wound size, clinical signs of infection, antimicrobial therapy, or healing outcomes. Here, we provide a detailed molecular characterization of the bacterial bioburden in a VLU over the course of treatment and healing, thus laying the groundwork for more evidence-based guidance for the diagnosis and treatment of infections in chronic wounds.

**Case Presentation**

The patient is a 40 year old African American man with a history of obesity and previous VLU. He developed a new wound on the right medial calf within two weeks prior to presenting to our wound clinic. The wound had not received other treatments. He was started on standard therapy, including debridement, wound dressings, compression bandages, and weekly follow-up visits. At each follow-up visit, wound size was documented by planimetry and clinical signs of infection were prospectively documented and treated according to a previously described scoring system specific to chronic wounds. Further details on the patient’s medical history are available in the Supplementary Information.

Over the first two weeks, he had worsening signs and symptoms of infection, including increasing pain, foul odor, erythema and edema of the periwound skin, expanding wound size, and friable, yellow-brown wound bed tissue that bled with gentle manipulation. At his second visit, conventional bacterial culture of the central wound was performed, and the patient was started empirically on antibiotic treatment with doxycycline 100 mg by mouth twice daily. The wound bacterial culture was positive for
Enterobacter cloacae, and the antibiotic was changed according to culture sensitivities to trimethoprim/sulfamethoxazole (TMP/SMX) 160/800 mg by mouth twice daily on week 3, and continued for two more weeks. After antibiotic treatment concluded at week 5, wound healing stalled for two weeks, and then progressed to heal after 15 weeks without further antimicrobial treatment. Thus, this patient's wound evolved through different phases of healing, including wound expansion, antibiotic treatment, stalled healing, and resolution, as shown in Figure 1.

Prospective Wound Sampling of Bacterial Bioburden

Prior to each weekly treatment, several wound samples were obtained to assess longitudinal changes in bacterial bioburden. Samples included two wound regions (center and distal edge) at three depths (swab before debridement, debridement curettings, and swab after debridement) for the first 8 weeks of healing. After week 8, the wound did not require further debridement, so swab samples were taken from the two wound regions from week 10 until healing completed at week 15. All samples were stored in -80°C until bacterial bioburden analyses were completed following healing; therefore, results were not available until after healing, and thus did not influence clinical decision-making. In order to characterize the bacterial bioburden present in the wound, we performed quantitative PCR (qPCR) for analysis of bacterial load (Figure 1B) and 16S rRNA profiling, which identifies bacterial taxa and quantifies their relative abundances in each sample (Figure 1C).52,53 Further details on methods of sampling, sequencing, and qPCR are available in the Supplementary Information.

Qualitative and Quantitative Bacterial Dynamics Throughout Healing

As detailed in Figure 1, bacterial bioburden changed with the different phases of healing. The increase of wound area through week 3 was associated with both
PART 2 Figure 1
1A) Wound Area

1B) Bacterial Load

1C) Microbiome

PART 2 Figure 1: Weekly change in wound area and microbial bioburden
1A) Wound area (mm$^2$) at each weekly time point, showing phases of wound expansion, antibiotic treatment (abx tx), stalled healing, and resolution.
1B) Bacterial load (copies per µL extracted DNA sample) at each weekly time point from swab samples collected from the wound center before debridement.
1C) Stacked graphs showing the relative abundance of the bacterial taxa at each weekly time point. Other low abundance bacteria representing <5% and >5% relative abundance of any sample are grouped together and labeled accordingly. As with 1B, samples shown here are from the wound center before debridement. The bacterial load and stacked graphs of other sampling regions and depths are shown in Figure S2.
increased bacterial load and relative abundance of *Enterobacteriaceae* (Figure 1 depicts bacterial burden of the central wound before debridement; other samples are shown in Supplemental Figures 1 and 2). This dominance of *Enterobacteriaceae* decreased only minimally with doxycycline treatment (weeks 2-3). However, switching to TMP/SMX was correlated with a decrease in wound size (Figure 1A), bacterial load (Figure 1B), and relative abundance of *Enterobacteriaceae* (Figure 1C). Bacterial diversity transiently increased immediately after starting TMP/SMX treatment, and then again during the 2-week stalled healing phase, but was low during the resolution phase (Supplemental Figure 3). The decrease in bacterial diversity during the resolution phase correlated with dominance of *Staphylococcus*, even as the wound area steadily declined without clinical indications warranting further antibiotic treatment.

**Biogeography of Bacterial Bioburden**

In addition to longitudinal characterization of wound healing, we also performed detailed sampling of the different wound regions (wound center vs. edge) and depths (before debridement, debrided tissue, and after debridement) to examine intra-wound variation in bacterial burden, including bacterial load and community structure. The bacterial load was significantly higher in samples from the wound center than the wound edge (p=0.049); however, the load at different wound depths was not significantly different (Supplemental Figure 1). In order to quantify differences in bacterial community structure between the wound regions and depths, we calculated the UniFrac distance, a metric used in microbial ecology to account for phylogenetic relatedness between bacterial communities (Supplemental Figure 4). A lower UniFrac distance indicates more phylogenetic relatedness between communities. The average weighted UniFrac distance between samples was significantly lower between wound regions than between wound depths or consecutive time points (at same region and depth). This indicates a higher
level of similarity in bacterial community structure between different wound regions than between different wound depths sampled on the same day. Interestingly, changes in the before-debridement samples most strongly correlate with wound expansion, antibiotic therapy, and healing. Conversely, deeper samples (debrided tissue and post-debridement) were overall dominated by *Acinetobacter*, and did not experience such large shifts in microbial community structure.

**Discussion and Conclusions**

Molecular techniques including next-generation DNA sequencing and qPCR have revolutionized our ability to discern complex microbial communities with high resolution. However, few cultivation-based studies and no molecular-based studies have analyzed changes in wound microbial bioburden over time, and most studies include limited information on the clinical status of the patient or use of antimicrobials or other therapies. Rather, most studies focus primarily on clinically non-infected wounds on cross-sectional patient populations using one-dimensional definitions of bioburden.51

In addition, most molecular-based studies have only used sharp debridement samples without comparison of different sampling methods or indication of depth of the wound sampled. One previous study, Price et al.45, used 16S DNA sequencing to investigate intra-wound bacterial communities in debridement samples from different geographic regions of chronic wounds, including the leading edge, the opposing leading edge, and/or center of chronic wounds. As in the current study, samples from the different wound regions had similar community structure. Bacterial communities from wounds of different individuals were also compared, showing that the intra-wound variation was significantly smaller than the inter-wound variation. However, this was a cross-sectional study of patients with different types of chronic wounds without further
characterization of the healing trajectory, and different wound depths or quantitation of bacterial load were not examined.

Though no other studies have examined sampling at different wound depths, other studies comparing different sampling methods—including tissue biopsies vs. more superficial sampling techniques such as swabbing and wound fluid collection—have been done using cultivation-based methods in chronic wounds arising from multiple etiologies (as recently reviewed by Reddy et al.39). Overall, biopsies and swabs have been shown to be comparable for aerobes, and there are more discrepancies for anaerobes and overall quantities of bacteria, suggesting uneven vertical distribution of at least some species, though results are likely biased by the cultivation methods used.

The current case study demonstrates differences in the vertical distribution of bacterial community structure, though not in bacterial load. Compared to the deeper samples, the superficial, before debridement samples showed more pronounced changes in Enterobacteriaceae, including predominance during wound expansion and precipitous fall with antibacterial treatment. The superficial swabs could be collected even when debridement was not required from week 10 through 15, demonstrating the predominance of Staphylococcus during healing. This finding that microbial bioburden from swabs before debridement can correlate with healing is novel and clinically meaningful because these superficial samples are more feasible to collect serially and less costly than debridement or biopsy samples in many ways, including time and tools required as well as the risk of patient pain and blood loss.

The DNA-based methods utilized in this case study have the potential to increase the speed and sensitivity of wound infection diagnosis, particularly for fastidious microbes that require specialized culture conditions and extended growth times. The conventional bacterial culture utilized at one time point in accordance with current clinical standards required several days for bacterial cultivation and sensitivities. In contrast,
molecular-based microbial identification techniques can rapidly incorporate the multi-dimensional aspects of microbial bioburden—including microbial pathogenicity, load, and diversity—increasing the potential for clinically-relevant breakthroughs in the diagnosis and treatment of chronic wound infections. However, these advances are only possible if guided by studies that include detailed clinical indicators to determine the pathogenic significance of patterns of microbial dynamics. Thus, a combination of improved study design and use of molecular methods has potential to lead to more targeted therapeutics, thereby decreasing wound infection rates while reducing the risk of antimicrobial resistance.
Supplementary Information

Patient’s Medical History

The patient was a 40 year old African American man with known venous insufficiency. Previous venous insufficiency scans showed venous reflux in the right greater saphenous vein at the level of the mid calf as well as in the left small saphenous vein. Varicosities were noted from the right knee to distal calf and the left proximal thigh to distal calf. Arterial studies including ankle brachial index and pulse volume returns revealed no significant arterial occlusive disease. He had a past medical history of other VLUs as well as other comorbidities including obesity, diverticulosis, pulmonary embolism (two years prior), and cardiomyopathy (NYHA Class I). His medications included warfarin, furosemide, lisinopril, carvedilol, pentoxifylline and vitamin D supplementation throughout the course of healing. Wound treatments included debridement followed by application of absorptive dressing (hydrofiber or collagen and oxidized regenerated cellulose) and multilayered compression wraps changed at least weekly (Unna boot or Coflex boot).

Sample Collection

Samples were collected in accordance with the University Hospitals Case Medical Center IRB (protocol number 05-11-30) with informed consent from the patient. Samples were obtained after his dressing was removed and his wound was wiped with sterile gauze to remove excess drainage and loosely adherent slough. An Epicentre Catch-All Sample Collection Swab (Epicentre Cat # QEC89100) soaked in sterile collection buffer (0.1% Tween 20 in PBS) was then rolled over a 1 cm² area in the center of the wound for 10 seconds using sufficient pressure to extract wound tissue fluid (Levine technique). The same technique was used to collect samples from a 1 cm² area at the most distal wound edge. Debrided tissue was collected from same 1 cm² regions...
using a 4 mm curette after anesthesia was obtained with 2% lidocaine gel to the wound under occlusion for approximately 5 minutes. After debridement, swabs were collected from the same 1 cm$^2$ regions using the same type of swab, collection buffer, and technique as the before debridement swab. Samples were stored in 1.5 mL microfuge tubes at -80°C until further processing.

**Bacterial Culture**

Samples for bacterial culture were obtained when patient developed three or more clinical signs and symptoms of infection according to a previously described scoring system.$^{49}$ Samples were collected for aerobic and anaerobic bacterial culture of the wound center after debridement by rotating a Copan Diagnostics ESwab (#480C) over a 1 cm$^2$ area in the center of the wound for 5-15 seconds using sufficient pressure to extract wound tissue fluid. The swab was placed in the ESwab transport media and immediately delivered for aerobic and anaerobic bacterial culture in the Clinical Laboratory Improvement Amendments (CLIA) certified clinical microbiology laboratory of University Hospitals Case Medical Center. Cultures were prepared by plating on BBL Trypticase Soy Agar with 5% Sheep Blood, MacConkey II, Chocolate II, and CDC Anaerobe 5% Sheep Blood Agar. Cultures were incubated at 35°C aerobically and anaerobically (Remel AnaeroPack System) and deemed negative after 48 hours of aerobic growth and 72 hours of anaerobic growth.

**DNA Extraction**

Swabs and accompanying tissue were transferred to 0.7 mm garnet bead tubes (MoBio # 13123-50) using sterile forceps, and total DNA was extracted using a modified protocol for the MoBioPowerMag Soil DNA Isolation Kit, optimized for use with the
Eppendorf epMotion (MoBio # 27100-4-EP). Prior to bead beating, 750 μl of the RNase A/Bead Solution and 60 μl of the lysis solution was added to tubes, which were then incubated at 70°C for 10-30 minutes. Cells were then lysed using a MP FastPrep-24 at 6.5 m/s for 60 seconds. Tubes were pelleted twice for 10 minutes at room temperature and 21×10³ xg to ensure complete removal of inorganic swab material. The standard protocol utilizing an Eppendorf epMotion 5075 was then followed from the IRT removal step onward. DNA was eluted in MagnaClear Elution Buffer and stored at -20°C until library preparation.

Quantitative PCR

Bacterial load was determined by qPCR to determine the overall copy number of 16S rRNA per extracted DNA sample. Standards were developed by transforming Stellar Competent cells (Clontech #636763) with pGBT9 plasmid (Clontech #K1605-A) digested with BamH1-HF (New England Biolabs #R3136S) and ligated with the Ion Plus Fragment Library Kit DNA Ligase and Buffer (Life Technologies #602-1152-01, #602-1150-01) to include an insert of 16S rRNA (23F/806R). Transformed cells were grown in Miller LB Broth (Fisher #BP1426) with 100 μg/mL Ampicillin (Roche #10-825-242-001) for 18 hours prior to plasmid harvest with the Qiagen Plasmid Midi Kit (Qiagen #12143). Standardized plasmid DNA was confirmed to be free of genomic contamination by gel electrophoresis, and then diluted from 1.4x10¹⁰ copies/µL to 1.4x10⁷ copies/µL, the lower detection limit of the assay. Quantification of the DNA extract from samples was performed by qPCR. Each 20 µL reaction contained 10 µL 2X Bullseye TaqProbe qPCR Master Mix (Midsci #BEQPCR-P), 2 µL template DNA diluted to 0.8ng/µL, 6 µL DNA grade filter sterilized water (Fisher #BP2470-1) and 2 µL 10X primer/probe assay mix (Nadkarni, 2002). DNA was denatured 10 minutes at 95°C followed by 40 cycles of 95°C
for 15 seconds and 60°C for 60 seconds in a StepOne Plus RT PCR System (Applied Biosystems). Bacterial load was reported as copies/µL of extracted DNA sample. Results were compared by Student’s paired t-test between different wound regions (wound center vs. edge) and depths (swab before debridement and after debridement).

**Library Preparation and High-Throughput Sequencing**

PCR targeting the V4 region of the 16S rRNA (F515/R806) was performed on each sample in triplicate, according to established protocols. Briefly, the forward primer was composed of a region targeting 515F, primer linker and pad regions, and the 5’ Illumina adapter. The reverse primers were made of a region targeting 806R, primer linker and pad regions, and the reverse complement of the 3’ Illumina adapter. Each reverse primer also contained a unique 12-base error-correcting Golay barcode, enabling all of the samples to be multiplexed on a single MiSeq run.

PCR reactions contained 16.25 µL Molecular Biology Grade Water (Fisher BP2819-1), 0.25 µL TaKaRa Hot Start DNA Polymerase (ClontechTaKaRa R007A) (5 units/µl), 2.5 µL 10X Buffer with MgCl₂ (100 mM Tris-HCl (pH8.3), 500 mM KCl, 15 mM MgCl₂), 2.0 µL dNTP’s (2.5 mM each), 1.0 µL Primers 515F and 806R working solutions (0.5 µM final concentration), and 2.0 µL of total genomic DNA. The DNA was denatured for at 98°C for 3 minutes, followed by 35 amplification cycles of 98°C for 10 seconds, 56°C for 30 seconds, 72°C for 60 seconds, and a final extension step of 72°C for 10 minutes. Triplicate samples were pooled and purified using the MoBio UltraClean-htp 96 Well PCR Clean-Up Kit (MoBio Cat# 12596-4). Purified PCR products were then quantified using a Qubit and normalized to 1 µg/mL. Normalized samples were then pooled to create a sequencing library, and quantified via qPCR. A 50% spike of PhiX control was then added to the library during sample preparation prior to sequencing. All
93 samples were sequenced in single Paired-End 2×151 bp run on an Illumina MiSeq (Illumina #MS-102-2002).

**Data Quality Filtering**

A total of 15,996,809 raw sequencing reads were produced on the MiSeq with a range of 12,391–445,616 sequences per sample. The QIIME software package version 1.6.0 was used to split the forward read into libraries for each sample, followed by length and quality filtering. Reads that mapped to the PhiX control genome commonly used on the MiSeq platform were removed. The UPARSE Pipeline was used to dereplicate, sort by abundance and discard singletons. Chimeras were also removed using USEARCH64. Quality filtered sequences are then clustered into operational taxonomic units (OTU’s) at 97% identity, which is normally considered an appropriate proxy for species level identification. A total of 1,196 OTUs were identified from 10,279,341 high-quality sequences from 93 samples. OTU’s were then identified using the Ribosomal Database Project (RDP) Classifier, as implemented in QIIME. The Shannon Diversity Index of each sample was also computed using the alpha diversity function in QIIME after samples were rarefacted to 10,000 sequences/sample. Bacterial community differences were quantified using weighted UniFrac, a distance metric for comparison of complex communities.
PART 2 Supplemental Figure 1: Bacterial load each week at different sampling locations
Bacterial load as determined by qPCR of 16S rRNA (copies per µL extracted DNA sample) at each weekly time point at the different sampling regions (central wound and distal wound edge) and depths (before debridement, debrided tissue, and after debridement).

PART 2 Supplemental Figure 2: Wound microbiome each week at different sampling locations
Stacked graphs show the relative abundance of the bacterial taxa in the wound at each weekly time point at the different sampling regions (central wound and distal wound edge) and depths (before debridement, debrided tissue, and after debridement). As with Figure 1C, low abundance bacteria representing <5% and >5% relative abundance of any sample are grouped together and labeled accordingly.
PART 2 Supplemental Figure 3: Bacterial diversity at weekly sampling time points and locations
Shannon diversity index (SDI) at each weekly sampling time point and location. The SDI accounts for the relative abundance of each species within a sample. The SDI in this patient was bimodal in the superficial and debridement samples, with a peak on week 4 (after starting antibiotic treatment with TMP/SMX), and week 7-8 (during the 2 week phase of stalled healing after antibiotic treatment). Otherwise sample diversity was low during both wound expansion and resolution phases, and thus not clearly correlated with healing.

PART 2 Supplemental Figure 4: Bacterial community average weighted UniFrac distances
Bacterial community differences quantified using weighted UniFrac, a distance metric used in ecology for comparison of microbial communities. A UniFrac distance value of 0 describes identical communities, while a value of 1 means that no community members are shared. By this metric, samples taken from different regions (wound center vs. distal wound edge at the same depth) have microbial communities that are more similar than samples of different depths (before debridement, debridement, and after debridement) or consecutive time points.
References


