Analysis of the chronic wound microbiota of 2,963 patients by 16S rDNA pyrosequencing

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ABSTRACT

The extent to which microorganisms impair wound healing is an ongoing controversy in the management of chronic wounds. Because the high diversity and extreme variability of the microbiota between individual chronic wounds lead to inconsistent findings in small cohort studies, evaluation of a large number of chronic wounds using identical sequencing and bioinformatics methods is necessary for clinicians to be able to select appropriate empiric therapies. In this study, we utilized 16S rDNA pyrosequencing to analyze the composition of the bacterial communities present in samples obtained from patients with chronic diabetic foot ulcers ($N = 910$), venous leg ulcers ($N = 916$), decubitus ulcers ($N = 767$), and nonhealing surgical wounds ($N = 370$). The wound samples contained a high proportion of *Staphylococcus* and *Pseudomonas* species in 63 and 25% of all wounds, respectively; however, a high prevalence of anaerobic bacteria and bacteria traditionally considered commensalistic was also observed. Our results suggest that neither patient demographics nor wound type influenced the bacterial composition of the chronic wound microbiome. Collectively, these findings indicate that empiric antibiotic selection need not be based on nor altered for wound type. Furthermore, the results provide a much clearer understanding of chronic wound microbiota in general; clinical application of this new knowledge over time may help in its translation to improved wound healing outcomes.

The minor negative effects on wound healing resulting from the minimal colonization of certain microorganisms are supported by a significant body of literature. Several host-related factors can negatively affect wound healing, including microcirculatory impairment, endothelial cell dysfunction, peripheral arterial disease, repetitive trauma, venous reflux, and poor nutrition. With regard to this "broken host" theory, it has been postulated that, once the breech occurs, the impaired host environment allows for bacterial surface colonization that does not impair healing. This inconsequential presence of microbes is seen in cases where a specific bacterial species is cultured from a chronic wound lacking any clear signs of infection, when treatment of the patient to eliminate the identified microorganism(s) does not improve wound healing.[1] Likewise, a Cochran study concluded that there was no evidence supporting the routine use of systemic antibiotics to promote healing in venous leg ulcers (VLUs),[2] while a separate study determined that antibiotics should only be used to treat wound infections in diabetic patients, but not for suppression of bacterial colonization to promote wound healing.[3] Moreover, it has been proposed that treatment of the wound microbiome with antibiotics may comprise a contributing factor driving the observed increase in bacterial antibiotic resistance.[4]

The SIDESTEP study highlights the confusing and often contradictory findings of randomized controlled trials utilizing cultivation methods.[5] The authors of this study found that many MRSA-positive patients exhibited positive responses to antibiotic treatments that were insufficient for this organism. Furthermore, this group demonstrated that chronic wounds "colonized" by *Pseudomonas* spp. healed as well when treated with ertapenem, which has little to no anti-pseudomonal activity, as those treated with piperacillin/tazobactam (anti-pseudomonal therapy). This and other studies have led to the conclusion that certain bacteria, including pathogens such as *Pseudomonas aeruginosa* or enterococci, can colonize wounds without impairing wound healing.[5] However, this position may fail to fully consider the polymicrobial nature of chronic wounds[6] as it is primarily based on the results of studies that have utilized culture-based approaches that are inadequate for assessing polymicrobial samples. It is, therefore, possible that wound care management, when based on incomplete diagnostics, may lead to suboptimal and confusing antimicrobial outcomes.

A second perspective is that the wound microbiota comprises a major barrier to healing in any chronic wound. According to this viewpoint, chronic wounds are, in essence, chronic infections of the skin and adjacent tissues whose

behaviors are in many instances directly related to the activities of a polymicrobial biofilm.[7] This view is predicated on the fact that microorganisms (bacteria and fungi) use two distinct infection strategies.[8] Planktonic (free-floating) microorganisms are associated with classic acute infections, such as cellulitis, acute urinary tract infection, pneumonia, and sepsis, which are characterized by rapid onset and a robust host response (rubor, dolor, color, and tumor) that can often be life-threatening. Typically, however, administration of low minimal inhibitory concentrations of appropriate antibiotics are required to eradicate the microorganism and, once cleared, the infection does not return. In contrast, the inflammation associated with chronic infections tends to wax and wane. Moreover, while chronic infections often require very high doses of antibiotics for long durations (6–12 weeks), they typically respond incompletely to treatment and reemerge once antibiotics are withdrawn. As such, infections are often clinically termed chronic once antibiotic therapy has failed.

The difficulty in treating chronic infections is primarily due to the ability of the infectious microorganism to produce biofilms,[7,9] which are polymicrobial communities (genetic diversity) in which each species exhibits quorum sensing control over gene expression (phenotypic diversity). Biofilm communities exhibit various characteristics that make them difficult to treat, including the slow penetration of antimicrobials, up-regulation of horizontal gene transfer in response to stress, anoxic cores, and the formation of persister cells.[10] Indeed, early studies showed that antibiotics were only marginally effective against microorganisms within biofilms,[11] and that biofilms are impervious to both antibodies[12] and white blood cells.[13]

As the initial model of biofilm infection, the subcellular mechanisms by which bacteria attach to host tissues,[14] utilize quorum sensing to control community-wide gene expression,[15] and induce inflammation to promote plasma leakage from local capillaries for sustainable nutrition[16] have been elucidated. However, one of the most interesting molecular strategies used by biofilm bacteria is the induction of host cell senescence.

There are various causes of wound bed cell senescence such as oxidative stress and host protease-mediated degradation of host cell receptors and/or cytokines. However, a more important and previously unknown cause for senescence occurs when biofilm bacteria use multiple small molecules to interfere with or commandeer the host cell processes, including rearrangement of the host cytoskeleton,[17,18] inhibition of mitosis,[19] and, most importantly, inhibition of apoptosis.[20–22] Due to the wide array of pathogenic effects exerted by distinct bacterial species, it may be necessary to fully characterize the entire bacterial population of each biofilm.

Biofilms often exhibit high levels of genetic diversity owing to the presence of multiple bacterial and/or fungal species, and this diversity provides numerous advantages to the biofilm community. For example, diverse biofilm environments comprise large gene pools that allow for more efficient sharing of DNA sequences via horizontal gene transfer.[23] Additionally, the microbial diversity of biofilms enables enhanced metabolic cooperation,[24] byproduct influences,[25] passive resistance,[26] and various other synergistic effects that provide the biofilm a competitive advantage against the host.

It is best to view biofilms as single entities that possess multiple genetic resources, which allow them to adapt and even thrive in the presence of various stresses. In general, increased genetic diversity imparts increased biofilm survival.[27] While individual biofilms almost always possess a dominate microbial species, species that are present in low abundance relative to the dominant organisms can have a significant impact on the microbial community and can even render the entire biofilm dysbiotic.[28] Indeed, normally nonpathogenic biofilms can cause disease in the host due to the activities of a minor constituent species. This fact adds great complexity to determination of the clinical importance of the microbes identified within wound biofilms.

Previous studies have shown that polymicrobial biofilms can result in more severe infections that are more recalcitrant to treatment than monoclonal biofilms. *Staphylococcus aureus* biofilms containing low levels of *P. aeruginosa* exhibited increased rates of infection in a rat model,[29] while *Prevotella* increased the pathogenicity of *S. aureus* biofilms in a mouse model of infection.[30] Furthermore, *P. aeruginosa* waste products were shown to protect *S. aureus* from aminoglycoside-mediated killing.[31] However, to understand whether the microbes found on the surface of chronic wounds are harmlessly propagating on or actively commandeering the wound bed requires further analysis.

Traditional bacterial culture methods are predicated on identifying a single dominant organism. The observation that certain biofilm bacteria are viable within the diverse wound microenvironment but remain unculturable suggests that these approaches are ineffective for analyzing biofilm populations.[33,34] Indeed, two separate studies showed that culture methods failed to detect the dominant organism in greater than 50% of the wound cultures analyzed, and detected only about 10% of the total microorganisms present.[31,32] As a result, molecular methods capable of identifying and quantifying a wide range of microorganisms would be much better suited to evaluate the microbial biofilm community.[35]

In this study, we utilized 16S rDNA pyrosequencing and identical bioinformatics methods throughout to analyze samples obtained from 2,963 chronic wound patients. Using this approach, we identified the predominant species present within four types of chronic wounds and assessed whether differences in patient demographics or wound type affected the composition of the microbiota in these samples.

MATERIALS AND METHODS

Study participants

The study protocol for this retrospective analysis was approved by Western Institutional Review Board (WIRB

DU	Decubitus ulcer
DFU	Diabetic foot ulcer
NHSW	Nonhealing surgical wound
VLU	Venous leg ulcer

PRO NUM: 20111320) and performed in accordance with the Declaration of Helsinki. All data were de-identified prior to analysis by a bioinformatician. For this study, only wound samples from patients treated for chronic wounds within four categories were included: decubitus ulcer (DU), diabetic foot ulcer (DFU), VLU, and nonhealing surgical wound (NHSW). The samples were obtained by sharp debridement of the chronic wound at the surface of the wound bed. During the course of care, chronic wounds were sampled for molecular analysis at the discretion of the treatment provider to identify and quantitate the microbes present. A pea size sample (~0.25 mg) of debrided material was placed in a 2 cc Eppendorf tube for transport to the laboratory on the same day.

The wounds sampled were primarily obtained from patients at high-risk for complications or from wounds that failed to resolve after previous therapy. As such, DFUs in patients at risk for limb loss and wounds tend to be of long duration and may be disproportionally represented in the study cohort.

Only data from microbial species comprising at least two orders of magnitude of the total bacterial population have been included. Thus, very minor microbial species representing less than 1% of the entire sample have not been reported in this study.

16S rDNA pyrosequencing analyses

Total genomic DNA was isolated from wound samples using TissueLyser (Qiagen, Valencia, CA) and High Pure PCR Template Preparation Kits (Roche, Pleasanton, CA). Samples were amplified for pyrosequencing using the 28F 16S rDNA forward primer constructed with a 5′-3′ Roche A linker and an 8–10 base pair barcode[36,37] and the 519R 16S rDNA reverse fusion primer constructed with (5′-3′) a biotin molecule and the Roche B linker.[37] Reactions were performed in 25 μL volumes containing 12.5 μL HotStar-Taq master mix (Qiagen), 9.5 μL water, 1 μL of each primer (diluted to 5 μM), and 1 μL of template DNA, and were amplified using an ABI Veriti thermocycler (Applied Biosystems, Carlsbad, CA) under the following conditions: 95 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, 54 °C for 40 seconds, and 72 °C for 1 minute; and a final extension at 72 °C for 10 minutes. Amplification products were visualized using eGels (Life Technologies, Grand Island, NY), pooled in equimolar amounts, and subjected to size selection using an Agencourt AMPure XP system (Beckman Coulter, Inc., Indianapolis, IN) according to Roche 454 protocols. Size-selected pools were then quantified with Nanodrop 1000 spectrophotometer, and 150 ng of each DNA sample was hybridized to Dynabeads M-270 (Life Technologies, Carlsbad, CA) to generate single-stranded DNA. Single-stranded DNA was diluted and subjected to emulsion-based PCR (emPCR), and the resulting amplification products were subsequently enriched and sequenced. All methods were performed according to the manufacturer's protocols (454 Life Sciences; Roche, Branford, CT).

Bioinformatic and biostatistical analyses

Sequences generated during 454 pyrosequencing have a per base accuracy rate of 99.5%.[38,39] Correction of these errors and removing chimeras from the sequencing was done by first trimming sequences back using a running average of Q25. Trimmed sequences were then run through USEARCH[40] to cluster the sequences at 4% divergence. Cluster selection, chimera depletion, and sequence mapping were completed using the USEARCH UPARSE OTU selection algorithm.[41] Mapped sequences were then grouped by OTU and quality scoring-based sequence correction was performed.

Corrected sequences were then run through the Research and Testing Laboratory Genomics taxonomic analysis pipeline to determine the taxonomic classifications and abundance for each sample. The first step of this pipeline was to perform quality analysis on each corrected sequence to check for and remove primers and ensure that each sequence is a minimum of 300-bp in length. OTU selection was then performed using the UPARSE OTU selection pipeline.[40,41] Selected OTUs were then aligned using MUSCLE[42,43] and a phylogenetic tree generated using FastTree.[44,45] The selected OTU sequences were then globally aligned using USEARCH[40] against a database of classified 16S sequences. Confidence values were assigned to each OTU classification and the lowest common ancestor was determined based on these confidence values. The top hit and lowest common ancestor was hen reported for each OTU.

Bar plots and pie charts were constructed to visualize the occurrence of most abundant bacteria across wound types. Relative abundance was determined by a percentage of amplicons for the species of interest vs. the total number of amplicons of the sample. Frequency histograms were constructed to characterize microbial diversity across wound types. Similarly, the frequency of occurrence and relative abundances of top 20 most dominant bacterial species was tabulated for each wound type. Similar summaries were calculated separately for *S. aureus* and all other coagulase negative *Staphylococcus*, and the frequency of detection of methicillin resistance marker within these groups was summarized. Bacterial diversity across wound types was also investigated at the generic level and reported for bacterial genera present at 10% and 50% or higher relative abundances. The prevalence of single species biofilms was summarized according to bacterial species. Effects of demographics and wound type on the top 20 most dominant bacterial species were assessed using a permutational multivariate analysis of variance with a Bray–Curtis dissimilarity matrix.[46] In addition, the effects of demographics and wound type on the individual frequencies of the top 20 bacterial species were assessed using analysis of variance with a Benjamini–Hochberg correction for multiple testing.[47] Similar analyses were conducted at the generic level considering bacterial genera observed to comprise 10% of wound samples.

RESULTS

The composition of the chronic wound microbiome is not wound type-dependent

Each of the wound types examined in this study exhibited similar levels of bacterial diversity and similar relative abundances of specific genera when an arbitrary threshold of two orders of magnitude (two $\log_{10}$ units) was used as a reporting cutoff (Figure 1). Table 1 summarizes the 20

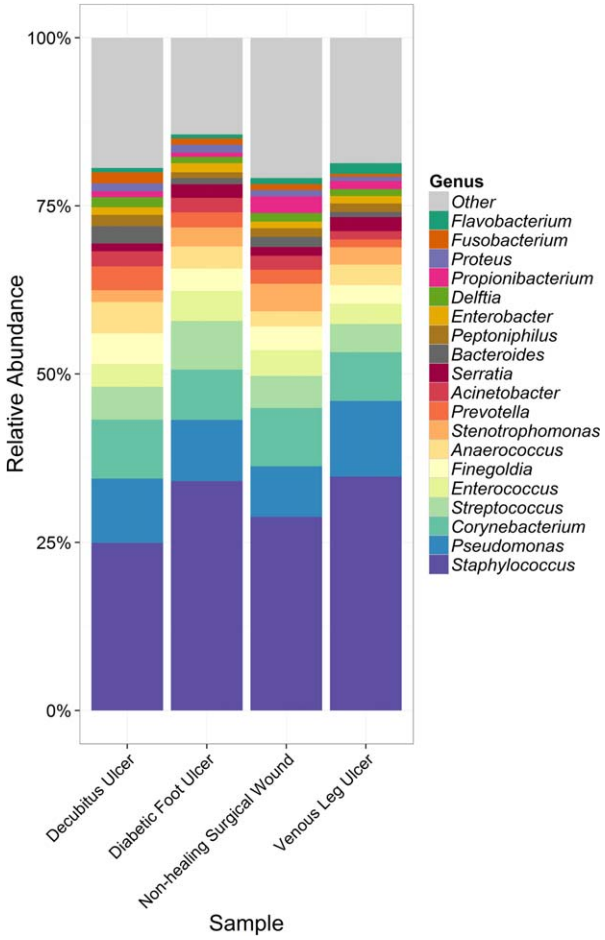

Figure 1. The relative abundance of the top 20 bacterial species by wound type. This figure shows for the top 20 species the percentage of amplicons assigned to a species vs. the total number of amplicons identified for each wound type.

most prevalent bacterial species, and the relative abundance of the species, for each wound type. A multivariate analysis of the 20 most abundant species across the four wound types determined that the percentage of total variation explained by wound type was less than 0.5% (data not shown). Likewise, wound type explained approximately 0.5% of the total variation among bacterial genera that comprised at least 10% (one $\log_{10}$ unit) of the total sample, and there were no significant differences in the abundance of these bacterial species across wound types (Table 2). Meanwhile, a univariate screen failed to detect significant effects of wound type on the abundance of each of the top 20 species after correction for multiple testing. Lastly, as depicted in Figure 2, there was a remarkable similarity in the number of distinct bacterial species across each wound type. The gestalt of the number of species per wound (Figure 2), along with the identification/abundance data (Tables 1 and 2), reveals the clinically significant diversity of the microbiota of chronic wounds. Moreover, the observed similarities in the diversity and relative abundance of the microorganisms present in varying wound types indicate that the selection pressure for these microorganisms may not be wound etiology-specific. That is, regardless of the underlying conditions that allow for bacterial attachment and biofilm formation, the mechanisms by which certain bacterial species occur and/or predominate are dictated by microbial factors and/or by the skin, skin structures, and other exposed host tissues. These findings, therefore, suggest that wound type-specific adjustment of antimicrobial therapies may be unnecessary.

The composition of the microbiota of chronic wounds is unaffected by differences in patient demographics

There were no obvious correlations between the demographic variables described in Table 3 and the microbes present in the four different types of chronic wounds (DFU, VLU, DU, and NHSW) examined in this study. While analysis of the relative abundance of *P. aeruginosa* across wound types failed to reveal a correlation between wound type and patient age (Figure 3), univariate analysis of the 20 most prevalent microbes in each wound type also revealed that gender, age, ethnicity, and the presence of diabetes explain only approximately 0.5% of the total variation in each dataset (data not shown). These findings indicate that demographic factors do not significantly affect the microbiota of chronic wounds.

Staphylococcus and *Pseudomonas* comprise the most prevalent genera present in the microbiota of chronic wounds

Staphylococcus was the most frequent bacterial genus present in the polymicrobial communities of the chronic wound samples tested (Figure 4). Indeed, approximately two-thirds of the wound samples contained greater than a 1% abundance of *Staphylococcus* spp. (Figure 4). Of these, *S. aureus* and *S. epidermidis* were the predominant species, each comprising approximately 25% of the *Staphylococcus* strains identified in the wound samples. Meanwhile, the *mecA* cassette was present in approximately 40% of all *Staphylococcus* species identified and was detected in both coagulase (coag)-positive and coag-negative strains (Table 4). As such, our analyses show that approximately one-quarter of the chronic wound samples (roughly 40% of the staphylococcal strains, which were present in 63% of all wounds, encoded *mecA*) examined were populated by a strain(s) of methicillin-resistant *Staphylococcus*, indicating that these organisms should be taken into consideration when selecting empiric therapies.

While *Pseudomonas* spp. were present in 25% of all wound samples analyzed, these organisms exhibited the propensity to constitute a high proportion of the biofilm communities in which they were present. For example, *S. epidermidis* (26% of wounds) was more prevalent in DU than *P. aeruginosa* (19%); however, *P. aeruginosa* exhibited a higher relative abundance (Table 1). Notably, *P. aeruginosa* was also the most common organism observed to produce "single species" biofilms (Table 5). Because *P. aeruginosa* commonly colonizes chronic wounds, is resistant to many extended spectrum beta lactamases, and is

Table 1. Occurrence and average relative abundancy* of top bacterial species in chronic wounds

	Nonhealing surgical wounds (370)			Diabetic foot ulcers (910)			Decubitus ulcers (767)			Venous leg ulcers (916)		
	# wnds	% wnds	Avg. abund.	# wnds	% wnds	Avg. abund.	# wnds	% wnds	Avg. abund.	# wnds	% wnds	Avg. abund.
Staphylococcus aureus	108	29%	13.39	297	33%	14.95	226	29%	12.67	316	34%	16.08
Staphylococcus epidermidis	119	32%	9.77	343	38%	10.72	218	28%	7.86	318	35%	10.94
Pseudomonas aeruginosa	54	15%	6.16	130	14%	7.97	144	19%	8.22	186	20%	10.46
Stenotrophomonas maltophilia	103	28%	4.09	142	16%	2.82	139	18%	1.74	170	19%	2.56
Finegoldia magna	74	20%	3.49	226	25%	3.32	259	34%	4.53	194	21%	2.77
Enterococcus faecalis	54	15%	3.15	159	17%	4.03	119	16%	2.42	103	11%	2.75
Corynebacterium striatum	42	11%	3.00	105	12%	2.82	120	16%	3.76	90	10%	2.26
Staphylococcus haemolyticus	62	17%	2.34	194	21%	2.41	88	11%	1.08	212	23%	2.75
Propionibacterium acnes	51	14%	2.25							103	11%	1.11
Corynebacterium tuberculostearicum	54	15%	2.23	121	13%	1.65	85	11%	1.26	145	16%	2.18
Anaerococcus vaginalis	40	11%	1.44	120	13%	1.60	159	21%	2.64	122	13%	1.45
Staphylococcus lugdunensis	53	14%	1.34							159	17%	1.49
Delftia acidovorans	20	5%	1.29	49	5%	0.93	59	8%	1.52	52	6%	1.03
Streptococcus agalactiae	19	5%	1.26	90	10%	3.97	57	7%	1.72	61	7%	2.26
Acinetobacter baumannii	13	4%	1.22	41	5%	1.92	51	7%	1.95	22	2%	0.76
Proteus mirabilis	11	3%	0.94	35	4%	1.10	67	9%	1.17			
Streptococcus salivarius	29	8%	0.93									
Serratia nematodiphila	16	4%	0.92	43	5%	1.63				44	5%	1.75
Ralstonia pickettii	16	4%	0.92									
Fusobacterium nucleatum	18	5%	0.84				50	7%	1.03			
Staphylococcus pettenkoferi				81	9%	1.58				35	4%	0.64
Staphylococcus lugdunensis				160	18%	1.20	111	14%	1.10			
Enterobacter hormaechei				78	9%	1.16	68	9%	1.00	81	9%	0.94
Prevotella bivia				27	3%	0.96	53	7%	1.66			
Corynebacterium jeikeium				46	5%	0.86	57	7%	1.58	38	4%	0.68
Bacteroides fragilis							47	6%	1.57			
Flavobacterium succinicans										74	8%	1.36

*Average relative abundance is calculated by dividing the total number of amplicons assigned to a specific species divided by the total number of amplicons identified within all the samples combined for each wound type.

sometimes associated with poor prognoses for wound healing, it is important to consider this organism when choosing empiric therapies.

Chronic wounds are frequently colonized by commensalistic and anaerobic bacteria

Notably, nearly half of the wound samples analyzed contained traditional commensal microorganisms, including coag-negative *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species. Indeed, *Corynebacterium* spp. comprised greater than 1% of the total bacterial population in more than one-third of the samples tested, while 75% of all *Staphylococcus* strains identified were coag-negative. Furthermore, despite the fact that chronic cutaneous wounds are exposed to relatively high levels of oxygenation, large numbers of anaerobic bacteria were detected in the wound samples. For example, strict anaerobes comprised four of the top 10 genera detected in the chronic wound samples (Figure 4). Specifically, *Finegoldia* spp. were present in 25% of wounds, while *Prevotella* spp., *Peptoniphilus* spp., and *Anaerococcus* spp. were detected in 12, 16, and 24% of the wounds, respectively, indicating that anaerobes comprise a significant proportion of the chronic wound microbiome.

DISCUSSION

Evaluation of the microbiota from 2,963 wounds revealed not only a large diversity in bacterial species but also a

Table 2. Frequency at which particular genera constituted greater than 10% of the bacterial population of individual samples when that specific genus was present

Genus	Decubitus ulcer	Diabetic foot ulcer	Nonhealing surgical wound	Venous leg ulcer
Staphylococcus	39%	51%	51%	51%
Pseudomonas	16%	14%	14%	14%
Corynebacterium	20%	17%	17%	17%
Streptococcus	13%	14%	14%	14%
Enterococcus	7%	8%	8%	8%
Finegoldia	13%	10%	10%	10%
Anaerococcus	15%	10%	10%	10%
Stenotrophomonas	5%	7%	7%	7%
Prevotella	9%	5%	5%	5%
Acinetobacter	4%	3%	3%	3%
Serratia	2%	3%	3%	3%
Bacteroides	6%	2%	2%	2%
Peptoniphilus	5%	2%	2%	2%
Enterobacter	2%	3%	3%	3%
Delftia	4%	2%	2%	2%
Propionibacterium	2%	2%	2%	2%
Proteus	3%	2%	2%	2%
Fusobacterium	4%	3%	3%	3%
Flavobacterium	2%	2%	2%	2%

wide dynamic range for each bacterial species. For example, *Pseudomonas* and *Staphylococcus* species were identified in a high percentage of wounds, but were present at very low abundance (near 1%) and very high abundance (greater than 90%) at equal frequencies. This significant variability may cause difficulties in selecting empiric antibiotics. This has been previously described in the literature, which suggests that DFUs are more polymicrobial[48,49] and contain more anaerobic bacteria[50] than other wound types and, therefore, require a different selection of empiric antibiotics.[51] Moreover, an early molecular survey with small cohorts (N = 40) for each wound type found that DUs harbored up to 62% anaerobic bacteria while anaerobes were present at less than 30% and 2% in DFUs and VLUs, respectively.[52] These findings differed dramatically from previous studies (VLU showed 49% anaerobic)[53] likely due to the small sample

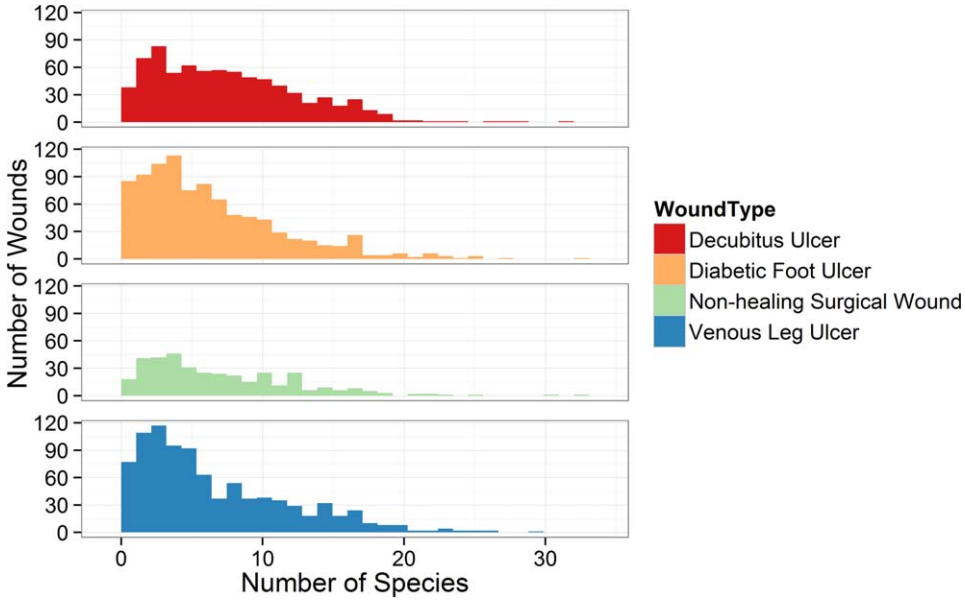

Figure 2. Number of reported species per wound for each wound type. Each wound type shows a Bell-shaped distribution for the number of microbes identified with the peak being from two to five species. Statistically, these graphs correlate quite closely.

Table 3. Demographic parameters of the subjects included in the study

Demographics*	All wounds ($N = 2,963$)	DFU ($N = 910$)	VLU ($N = 916$)	DU ($N = 767$)	NHSW ($N = 370$)
Gender (M/F)	**59%/41%**	**63%/37%**	**47%/53%**	**54%/46%**	**44%/56%**
Diabetes	**46%**	**100%**	**20%**	**25%**	**22%**
Age	**64 (17)**	**65 (13)**	**66 (18)**	**60 (19)**	**61 (17)**
Race					
White	**66%**	**49%**	**75%**	**71%**	**67%**
Black	**6%**	**9%**	**6%**	**6%**	**4%**
Hispanic	**28%**	**41%**	**19%**	**23%**	**28%**
Other	**<1%**	**1%**	**<1%**	**<1%**	**<1%**

*Data not included show that the top 20 most common species in terms of number of wounds and average relative abundance were not affected by age, gender, race, or the presence of diabetes.
DU, decubitus ulcers; DFU, diabetic foot ulcers; NHSW, nonhealing surgical wounds; VLU, venous leg ulcers.

size of these previous analyses. Because of the high diversity and variable abundance of wound microbiota for a specific chronic wound, this study surveyed a large numbers of patients for each wound type to overcome the statistical weakness of small cohorts to provide clinicians the best information for selecting the most appropriate empiric therapy.

DFUs have been described as a "polymicrobial soup," suggesting that this wound type is more microbiologically diverse than other wound etiologies. However, all wound types examined in this study exhibited similar levels of diversity and abundance at a genus level when an arbitrary threshold of two orders of magnitude was applied (Figure 1). Table 1 shows the top 20 bacterial species present along with relative abundance data for each wound type. In a multivariate analysis of top 20 species abundances across wound types, the percentage of total variation explained by wound type was less than 0.5% (data not shown). Similarly, a univariate screen for the effects of wound type on the abundance for each of the top 20 species was not significant after correction for multiple testing. Likewise, wound type explained approximately 0.5% of total variation among bacterial genera that comprised at least 10% of the total sample, and none of these individual bacterial species showed significant differences in abundance across wound types (Table 2). Last, as depicted in Figure 2, there was a remarkable similarity in the number of distinct bacterial species across each wound type. The gestalt of the number of species per wound (Figure 2) along with the identification/abundance (Tables 1 and 2) reveals the clinically significant diversity of the microbiota of chronic wounds. Moreover, the observed similarities in the diversity and relative abundance of the microorganisms present in varying wound types indicate that the selection pressure for these microorganisms may not be specific to wound etiology. That is, regardless of the underlying

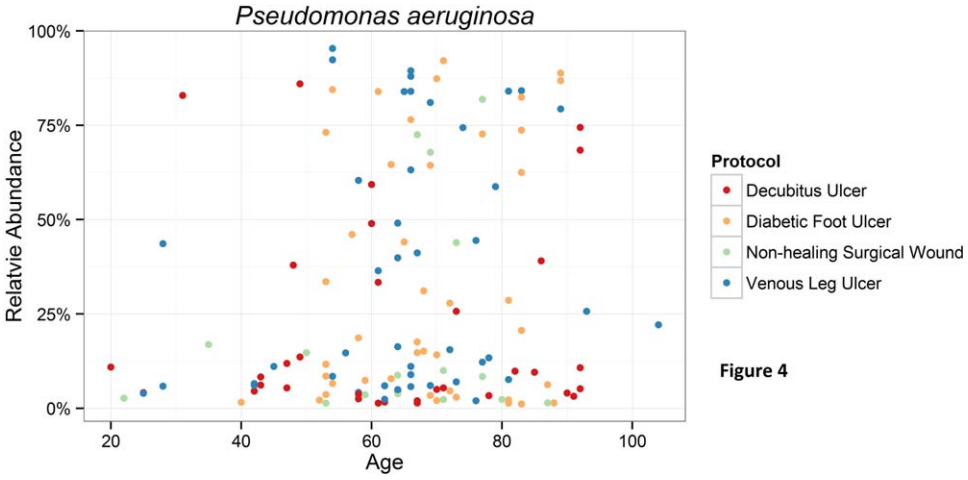

Figure 3. *Pseudomonas aeruginosa* vs. age for each wound type. No pattern emerges when the relative abundance of P. aeruginosa is plotted against age for each wound in each wound type. This analysis was performed for each of the top 20 bacterial species against each demographic variable with the same results (data not shown).

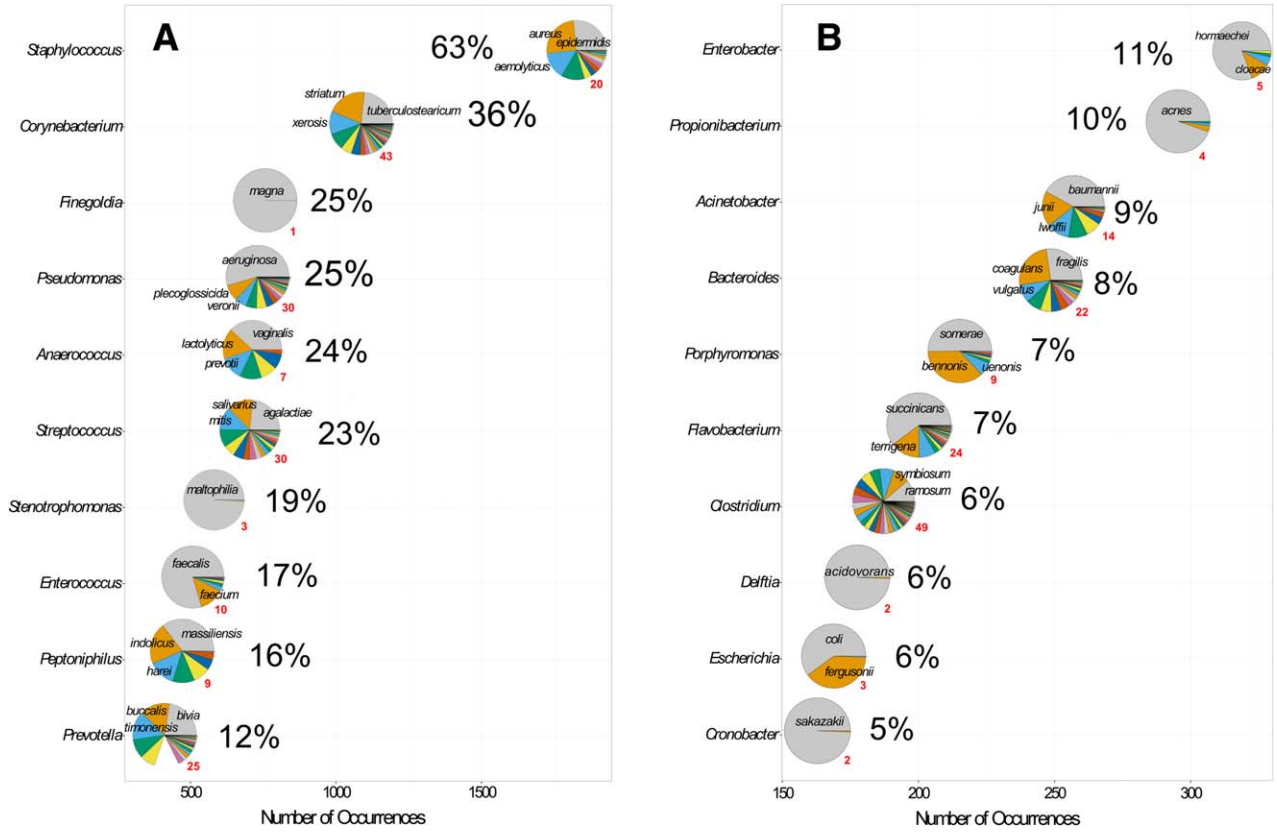

Figure 4. Bacterial species within chronic wounds. These data depict the presence of the 20 most prevalent bacterial genera identified in the 2,963 chronic wound samples evaluated. Numbers in large font black denote the prevalence (percentage of the total bacterial population) of a given genus in a sample. The small red number indicates the number of species identified within the genus. The pie chart represents the percentage of times that a specific species was present within samples that contained the given genus.

conditions that allow for attachment and biofilm formation, the mechanisms by which certain bacterial species occur and/or predominate are dictated microbial factors and/or by the skin, skin structures, and other exposed host tissues. Thus, these findings suggest that the wound type-specific adjustment of antimicrobial therapies may be unnecessary.

Composition of the microbiota of chronic wounds

There were no obvious correlation between the demographic variables described in Table 3 and the microbes present in the four different types of chronic wounds examined in this study: DFUs, VLUs, DUs, and NHSW. Likewise, analysis of the relative abundance of *P.*

Table 4. Percentage of each wound type that contained *Staphylococcus* spp., with or without PCR-based detection of the *mecA* cassette, and the prevalence of *Staphylococcus* isolates containing the *mecA* cassette

Methicillin resistance	All wounds	# wounds DFU	# wounds VLU	# wounds DU	# wounds NHSW
Staphylococcus aureus					
a) with *mecA* cassette	389 (13%)	158 (17%)	107 (12%)	89 (12%)	35 (10%)
b) without *mecA* cassette	558 (19%)	139 (15%)	209 (23%)	137 (18%)	73 (20%)
% *S. aureus* with cassette	41%	53%	34%	39%	32%
Coag-neg *Staphylococcus* (CNS)					
a) with *mecA* cassette	468 (16%)	191 (21%)	130 (14%)	105 (14%)	42 (11%)
b) without *mecA* cassette	832 (28%)	237 (26%)	297 (32%)	179 (23%)	119 (32%)
c) % CNS with cassette	36%	45%	30%	37%	26%

DU, decubitus ulcers; DFU, diabetic foot ulcers; NHSW, nonhealing surgical wounds; VLU, venous leg ulcers.

Table 5. Number of samples in which a single microorganism comprised at least 99% of the total bacteria detected

Species	# Monoclonal wound samples*
Pseudomonas aeruginosa	84
Staphylococcus epidermidis	43
Staphylococcus aureus	12
Enterococcus faecalis	12
Streptococcus agalactiae	12
Acinetobacter baumannii	10
Proteus mirabilis	6
Stenotrophomonas maltophilia	6
Corynebacterium jeikeium	5
Corynebacterium tuberculostearicum	4
Staphylococcus lugdunensis	4
Staphylococcus pseudintermedius	4
Ralstonia pickettii	4
Finegoldia magna	3
Enterococcus faecium	2
Corynebacterium striatum	1
Prevotella bivia	1
Streptococcus dysgalactiae	1
Streptococcus pyogenes	1
Parvimonas micra	1
Pseudomonas plecoglossicida	1
Mycoplasma hominis	1

*218/2,963 (7%) wound samples were monoclonal.

aeruginosa failed to reveal a correlation between wound type and patient age (Figure 3), while univariate analysis of the top 20 microbes for each wound type revealed that gender, age, ethnicity, and the presence of diabetes only explained ~0.5% of the total variation in each data set (data not shown). These findings indicated that demographic factors do not significantly affect the microbiota of chronic wounds.

Staphylococcus species were the most frequent bacterial genus present in the polymicrobial communities of chronic wounds (Figure 4). Additionally, there was high abundance of *Pseudomonas* species, including *P. aeruginosa*, in the chronic wound samples analyzed. However, *Corynebacterium*—a traditional commensal—comprised >1% of the total bacterial population in more than one-third of the samples. Furthermore, despite the fact that chronic cutaneous wounds are exposed to relatively high levels of oxygenation, large numbers of anaerobic bacteria were detected in the wound samples. Specifically, *Finegoldia* spp. were present in 25% of wounds, while *Prevotella* spp., *Peptoniphilus* spp., and *Anaerococcus* spp. were detected in 12, 16, and 24% of the wounds, respectively, indicating that anaerobes comprise a significant proportion of the chronic wound microbiome.

Approximately two-thirds of the wound samples had greater than 1% abundance of *Staphylococcus* (Figure 4). Of these, the predominant species were *S. aureus* and *S. epidermidis*. Indeed, each of these species comprised approximately 25% of the *Staphylococcus* strains identified in the wound samples. Meanwhile, approximately 75% of the *Staphylococcus* strains identified were coagulase (coag)-negative. Additionally, the *mecA* cassette was present in approximately 40% of all *Staphylococcus* species identified and was detected in both coag-positive and coag-negative strains (Table 4). As such, our analyses show that approximately one-quarter (about 40% *mecA* present in 63% Staphylococcus present in all wounds) of the chronic wound samples examined were populated by a strain(s) of methicillin-resistant *Staphylococcus*, indicating that these organisms should be taken into consideration when selecting empiric therapies.

While *Pseudomonas* spp. were present in 25% of all wound samples analyzed, these organisms exhibited the propensity to constitute a high proportion of the biofilm communities in which they were present. For example, *S. epidermidis* (26% of wounds) was more prevalent in DUs than *P. aeruginosa* (19%); however, *P. aeruginosa* exhibited a higher relative abundance (Table 1). Notably, *P. aeruginosa* was also the most common organism observed to produce "single species" biofilms (Table 5). Because *P. aeruginosa* commonly colonizes chronic wounds, is resistant to many extended spectrum beta lactamases, and is associated with poor prognoses for wound healing, it is important to consider this organism when choosing empiric therapies.

Commensals are considered microbes that provide benefits to the host organism, such as the "education" of the host adaptive immune response[54] or, as in the case of certain *Corynebacterium* and coag-negative *Staphylococcus* species, the inhibition of the growth of pathogenic organisms.[55] Notably, these interactions require redundant, complex host/microbe interactions that involve various host systems, including dendritic cells, keratinocytes, and antimicrobial peptides (defensins, alarmins, phenol soluble modulins, lipopeptides),[56] which do not exist in the wound bed. The lack of commensal signaling in the wound bed creates an environment that is permissive for commensal microbes to exert pathogenic behaviors. Coag-negative *Staphylococcus* strains have been shown to behave as pathogens when they are able to attach to implanted medical devices.[57] Meanwhile, the specific targeting of *Corynebacterium* with appropriate antibiotics was found to result in clinical wound improvements, indicating that these organisms can act as wound pathogens.[58] These findings demonstrate that, under certain conditions, commensals may produce or participate in chronic infections. Consistent with these findings, we detected a variety of traditional commensal microorganisms, including coag-negative *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, within the permissive chronic wound environment. However, while these commensals were present within nearly half of the chronic wounds samples tested, further analyses are required to assess whether the presence of these organisms affects the healing of chronic wounds.

Cultivation methods are often ineffective for detecting strict anaerobes. As a result, molecular methods, such as DNA sequencing analyses, may be necessary to accurately define and quantify anaerobic bacterial populations. Notably, four of the top 10 genera detected in the chronic wound samples analyzed in this study were strict

anaerobes (Figure 4). It should be pointed out that there are some indications that *Staphylococcus* may actually encourage colonization by strict anaerobes through colocalization and/or other mechanisms.[53] Therefore, the relative abundance of anaerobic bacteria observed in the chronic wound samples in this study may have been due, at least in part, to the high numbers of *Staphylococcus* spp. present. *Staphylococcus* spp. have the ability to produce energy via aerobic respiration, anaerobic respiration, and fermentation,[59] and thus may require different yet undefined treatment when cohabitating with anaerobic bacteria. Phenotypically, anaerobic *Staphylococcus* species are vastly different from their aerobic counterparts.[60,61] While it is currently unclear whether anaerobic bacteria inhibit chronic wound healing, the significant proportion of these organisms within the wound microbiota suggests that anaerobes must also be considered when deciding on empiric treatments. Indeed, it has been suggested that anaerobes produce more recalcitrant infections through undefined mechanisms.[62]

As documented above, chronic wounds were overwhelmingly polymicrobial, yet minor species were always identified (Table 5). Indeed, only 7% of wound microbiomes exhibited a 99% or greater predominance of a single species. While *Staphylococcus* and *Pseudomonas* were the genera most often associated with "single species" biofilms (Table 5), such biofilms were also formed by *Corynebacterium* and *Streptococcus* spp., as well as by several anaerobic bacterial species.

The microbial composition of the wound bioburden, although somewhat overwhelming, is of great clinical importance. Unfettered from the partial and often misleading results obtained from cultivation methods, medicine now has a reliable tool for understanding the effects of distinct microbes on wound healing. The development of DNA diagnostic techniques is likely still in its infancy. As a result, these methods will continue to yield improved levels of microbial identification, as well as detection of mobile genetic elements (for virulence and resistance) and host biomarkers, all of which will more accurately define the status of patient infection. The advantages of molecular methods, such as rapidity, sensitivity, specificity, and comprehensiveness, are well defined in the literature, and add up to a tool that can be used to face the challenge of diagnosing and characterizing biofilm infections. In this study, we utilized a Roche 454 platform capable of sequencing 600 million reads at an overall cost of about $13,000. However, in the time it took to write this document, the 454 platform has become obsolete with respect to cost and throughput. The current platforms are capable of sequencing over 6 million reads (PGM; Personal Genome Machine), 20 million reads (MiSeq), or 400 million reads (HiSeq) at 10–50% of the cost of the 454 platform. Meanwhile, new approaches are being developed to allow for sequencing of the entire metagenome (rather than just the 16S rDNA sequences), thereby enabling identification of pathogens down to the strain level. While the cost of this approach is still significantly higher than that of the 16S amplicon method utilized in this study, a time where this will no longer be true is likely not far off. Lastly, techniques are being developed and pilot studies performed to characterize the RNA expression profiles of distinct bacterial species that are associated with specific infection types. This knowledge will allow a thorough diagnosis of individual wounds, resulting in improved patient prognoses via the selection of optimal treatment strategies.

When any new technology is introduced into medicine, clinicians are confronted with information that can cause uncomfortable dilemmas. For example, the development of molecular diagnostic methods that enable rapid, inexpensive, comprehensive, and accurate identification of microorganisms has also resulted in a significant degree of uncertainty. Specifically, while these approaches are capable of identifying multiple organisms within patient samples, they are incapable of determining whether these microbes actively contribute to the infection or are simply comingling in an accommodating host environment. Therefore, until more information is available, it is important not to exclude microorganisms from therapeutic consideration on the basis of the incomplete and often inaccurate information obtained using previous technologies. Instead, it is essential that all microbial diversity be reported such that the information can be fully vetted by clinical experience over time.

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