Temporal Stability in Chronic Wound Microbiota Is Associated With Poor Healing

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Microbial burden of chronic wounds is believed to play an important role in impaired healing and the development of infection-related complications. However, clinical cultures have little predictive value of wound outcomes, and culture-independent studies have been limited by cross-sectional design and small cohort size. We systematically evaluated the temporal dynamics of the microbiota colonizing diabetic foot ulcers, a common and costly complication of diabetes, and its association with healing and clinical complications. Dirichlet multinomial mixture modeling, Markov chain analysis, and mixed-effect models were used to investigate shifts in the microbiota over time and their associations with healing. Here we show, to our knowledge, previously unreported temporal dynamics of the chronic wound microbiome. Microbiota community instability was associated with faster healing and improved outcomes. Diabetic foot ulcer microbiota were found to exist in one of four community types that experienced frequent and nonrandom transitions. Transition patterns and frequencies were associated with healing time. Exposure to systemic antibiotics destabilized the wound microbiota, rather than altering overall diversity or relative abundance of specific taxa. This study provides evidence that the dynamic wound microbiome is indicative of clinical outcomes and may be a valuable guide for personalized management and treatment of chronic wounds.

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INTRODUCTION

Chronic, nonhealing wounds affect 6.5 million patients annually in the United States and are an increasing public health and economic threat, exceeding estimated annual treatment costs of $9.7 billion (Bickers et al., 2006). Chronic wounds almost always affect individuals with an underlying predisposition (e.g., obesity, advanced age, diabetes) and are often disguised as a comorbid condition. A major type of chronic wound is the diabetic foot ulcer (DFU), a common complication of diabetes that results from neuropathy coupled with mechanical stress and tissue breakdown. Those with diabetes have a 15–25% lifetime incidence of DFU (Valensi et al., 2005), which results in amputation in 15.6% of patients (Ramsey et al., 1999). Projections estimate that diabetes will continue to increase in prevalence (Guariguata et al., 2014); thus, addressing management and treatment strategies for this complication is critical.

Microbial bioburden is believed to contribute to impaired healing of chronic wounds, and it is estimated that over 50% of DFUs are infected upon presentation (Prompers et al., 2007); however, infections are difficult to diagnose because of the diminished or absent clinical signs in DFUs resulting from peripheral neuropathy and/or vascular disease (Glaudemans et al., 2015). Without clinical suspicion, wound cultures provide little diagnostic value, because bacteria colonize all open wounds. Our previous work showed that clinical cultures underestimate bacterial diversity and load when compared with culture-independent techniques, based on the prokaryote-specific 16S ribosomal RNA gene. Multiple dimensions of the microbiota may be important, including microbial diversity, microbial load, and abundance of potential pathogens (Gardner and Frantz, 2008). Although other studies have used culture-independent methods to examine DFUs and other chronic wound microbiomes, these studies used cross-sectional designs (Dowd et al., 2008; Gardner et al., 2013; Gontcharova et al., 2010; Price et al., 2009; Wittebole et al., 2014; Wolcott et al., 2015), and the relationship between the wound microbiome and outcomes has not been rigorously examined.

Microbial communities exhibit a wide range of stabilities across the human body (Ding and Schloss, 2014; Flores et al., 2014); however, what these differing stabilities mean for the

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Abbreviations: CT, community type; DFU, diabetic foot ulcer; OTU, operational taxonomic unit

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health of the community or the host remain poorly understood. Very little is known about the dynamics of the wound microbiota during healing, deterioration, or exposure to antibiotics. To our knowledge, no study has investigated the microbial dynamics of chronic wounds. These dynamics may contain information about the vulnerability of the wound to opportunistic infections or provide insight as to the origin of stalled wound healing. It is critical to study these dynamics to enhance our understanding of chronic wounds and improve our ability to effectively treat them.

We addressed several important limitations of previous studies by performing a study designed to capture the longitudinal dynamics of microbiota colonizing DFUs and examining the association between the DFU microbiome and clinical outcomes. Microbiota were sampled from DFUs every 2 weeks for 26 weeks or until healed. We used high-throughput sequencing of the 16S ribosomal RNA gene to define multiple metrics of the microbiome, including diversity, stability, and relative abundance of potential pathogens and identified microbiomic features associated with DFU clinical outcomes. Although our study was focused on the microbiota in DFUs, many of these findings may be true of other chronic wounds and should be considered in future studies and treatments of chronic wounds.

RESULTS

We enrolled 100 subjects into a prospective, longitudinal cohort study to analyze the temporal dynamics of DFU microbiota and association with outcomes using culture-independent approaches. DFU microbiota was collected at initial presentation (baseline) and resampled every two weeks until (i) the DFU healed, (ii) lower extremity amputation, or (iii) the conclusion of 26 weeks of follow-up. All subjects received standardized treatment of surgical debridement and offloading. Of the 100 enrolled subjects, 31 experienced an infection-related complication, defined as (i) amputation, (ii) wound deterioration, or (iii) development of osteomyelitis. Supplementary Table S1 online summarizes clinical factors by complication status.

Characterization of the DFU microbiota at baseline

DFU microbiomes were determined by sequencing of hypervariable regions V1−V3 of the 16S ribosomal RNA gene. The most abundant genus identified was *Staphylococcus*, present in 345 of the 349 samples, with an average relative abundance of 22.77%. The second, third, and fourth most abundant genera were *Streptococcus* (11.98%, 318 of 349 samples), *Corynebacterium* (11.46%, 346 of 349 samples), and *Anaerococcus* (7%, 300 of 349 samples), respectively. All other genera represented less than 5% of bacterial relative abundance in this dataset. A more detailed characterization can be found in Supplementary Table S2 online. We further classified *Staphylococcus* operational taxonomic units (OTUs) to the species level for 79.5% of the OTUs. Of the 22.77% attributed to *Staphylococcus*, 13.3% were classified as *Staphylococcus aureus*, 5.3% were *Staphylococcus pettenkoferi*, and 4% were not further classified. Although *S. aureus* is a common DFU isolate, the high abundance of *S. pettenkoferi* was surprising, because this species was only recently characterized in 2007 (Trülzsch et al., 2007), although it was identified as the cause of osteomyelitis in a patient with a chronic DFU in France (Loïez et al., 2007).

DFU microbiota can be partitioned into four community types

We assigned DFUs to community types with the Dirichlet multinomial mixture model-based approach (Holmes et al., 2012). The Dirichlet multinomial mixture model supposes a more biologically relevant distribution of data, which overcomes limitations of alternative methods such as *k*-means (Holmes et al., 2012) and partitioning around medoids (PAM) clustering (Ding and Schloss, 2014). The DFU microbiomes were clustered into four groups, or community types (CTs), by minimizing the Laplace approximation (see Supplementary Figure S1 online). The top five differentiating taxa contributed 48.9% of the total difference between a one- and four-component model, although the major distinguishing taxa were *Streptococcus* (25.6%) species and *S. aureus* (11.8%) (Figure 1a). CT3 DFUs were characterized by high relative abundances of *Streptococcus* species (median = 64.0%). CT4 DFUs were composed of relatively high levels of *S. aureus* (median = 23.8%). CT1 and CT2 were highly heterogeneous, with no dominant taxa contributing more than a median of 5% of total relative abundance. This was also reflected by theta values—a measure of cluster variability for which smaller values correspond to highly variable communities—which were 3.7 and 6.9 for CT1 and CT2 compared with 16.4 and 10.5 for CT3 and CT4, respectively. CT summaries are described in greater detail in Supplementary Table S3 online.

To better visualize how CTs were associated with microbiota composition and clinical features, we generated a biplot depicting these relationships (Figure 1b). As would be expected, the taxa vectors for *Streptococcus* species and *S. aureus* are closely associated with the CT3 and CT4, respectively. The samples with the highest proportion of *S. aureus* are not included in CT4, showing the importance of the whole community in distinguishing clusters. *Streptococcus* species were closely associated with HbA1C levels and anaerobe levels with ulcer depth. Serum C-reactive protein levels and white blood cell counts, both measures of inflammation used to inform the diagnosis of infections, localized separately with CT4 and CT3, respectively. Subject outcomes also contributed to data separation, with amputation localizing with CT1 and CT2 and unhealed subjects localizing with CT4. Further quantification of the correlation between clinical factors and DFU microbiota is provided in Supplementary Table S4 online.

The frequency of CT transitions in DFU are associated with clinical outcomes

We next investigated the stability of the CTs by exploring the frequency and type of CT transitions. The DFU microbiota was highly dynamic, with CT transitions occurring every 1.76 study visits (approximately 3.52 weeks) on average (Figure 2a). Transition frequencies were significantly associated with subject outcomes (healed = 1.60 study visits/CT transition, unhealed = 2.04 study visits/CT transition, amputation = 3.08 study visits/CT transition). We further subdivided healed subjects into those whose ulcers closed in less than 12 weeks and those that closed in more than 12 weeks. Consistent with our analysis, the faster-healing

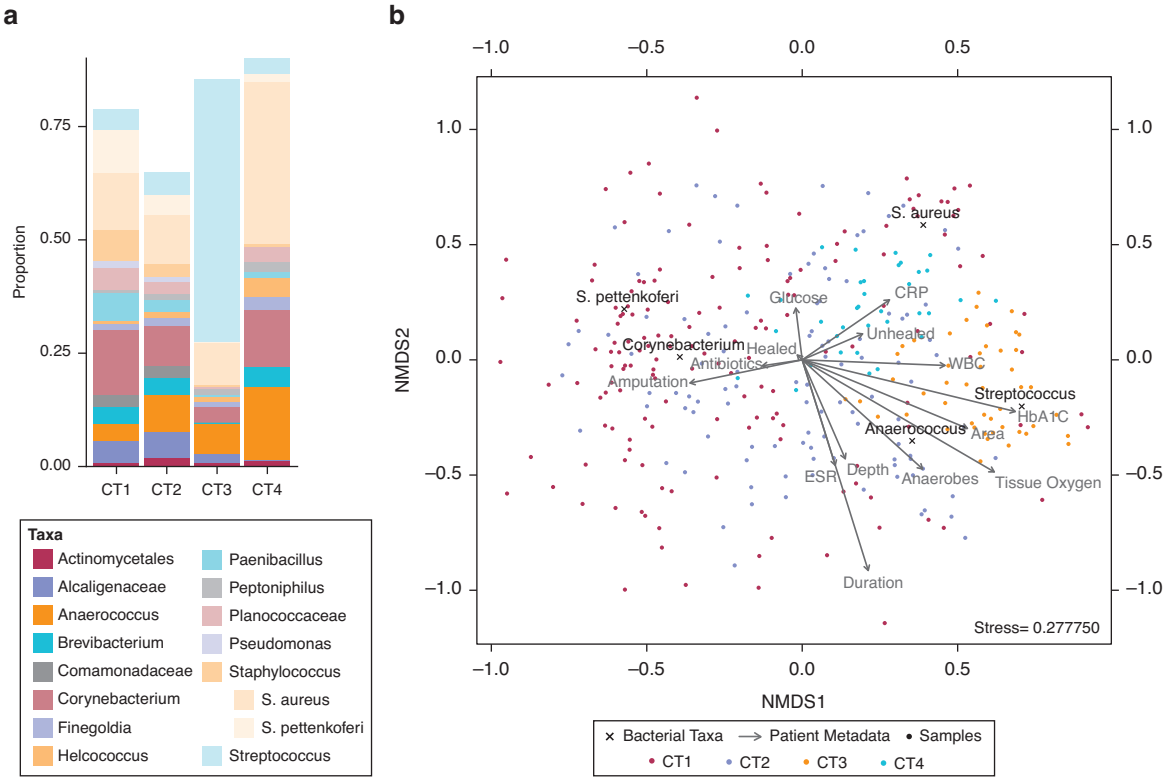

Figure 1. The DFU microbiome clusters into four CTs. (**a**) DFU samples partitioned into four clusters by Dirichlet multinomial mixture model. Mean relative abundances of bacterial taxa in DFU samples assigned to each CT. Relative abundance is shown on the y-axis. Taxa are filtered to those with a mean abundance greater than 1%. (**b**) Sample similarity between DFU microbial communities was calculated using the Bray-Curtis distance, and these distances were ordinated and visualized via NMDS. Each taxonomic contribution to community differentiation is overlaid with black text and "x" indicating the exact location. The impacts of various metadata are depicted as vectors labeled with gray text. Success of NMDS ordination is represented by the stress score, which measures the agreement between the two-dimensional and multidimensional representations. Stress scores range from 0 to 1, and scores below 0.3 are considered good approximations. Samples, taxa, and metadata that are closer together are more related. Samples are color-coded based on CT. CRP, C-reactive protein level; CT, community type; DFU, diabetic foot ulcer; ESR, erythrocyte sedimentation rate; NMDS, nonmetric multidimensional scaling; WBC, white blood cell count.

subjects experienced greater transition frequencies (<12 weeks = 1.45 study visits/CT transition, >12 weeks = 2.11 study visits/CT-transition; Wilcoxon *P*-value = 0.011).

We then questioned whether transition patterns between CTs were related to ulcer outcomes. By quantifying transitions between CTs we could represent the data as a Markov chain, with nodes representing CTs and edges representing transition frequencies by their weight (Figure 2b). The transition patterns between those that healed in less than 12 weeks and those that healed in more than 12 weeks were significantly different ($P < 0.0001$). In those who healed in less than 12 weeks, CT1 and CT2 dominated the transitions and were noted to have high self-transition rates of 0.74 and 0.53, respectively. In contrast, CT3 and CT4 experienced lower self-transition rates of 0.23 and 0.29, respectively, and had a predilection for transitioning to CT2. For subjects whose DFUs took longer than 12 weeks to heal, there was a marked increase in self-transitions, with ulcers stalling in CT3 and CT4 at rates of 0.45 and 0.84, respectively, indicating that the stability of these CTs may be detrimental to wound healing. Analysis of the stationary distribution and expected recurrence time showed similar trends (see Supplementary Table S5 online). The presence or absence of transitions between CT3 and CT4 also differentiated the two groups, with no recorded instances in wounds healing in less than 12 weeks. Together, these findings suggest that community stability reflects a delayed healing phenotype.

DFUs with more dynamic microbiota heal faster than those with less dynamic microbiota

To address more subtle patterns of variation, which may not be apparent when examining broad CTs, we used the intervisit weighted UniFrac distance as a proxy of stability. The weighted UniFrac metric measures the proportion of shared OTUs, their phylogenetic relationships, and their relative distributions on a scale of 0−1, with higher values indicating greater instability. We generated mixed-effect linear regressions to model the relationship between microbiota instability and time required to heal in those whose DFUs healed within 24 weeks. This model suggests that all ulcers are slowly stabilizing at a rate of −0.024/visit; however, slow-healing ulcers begin in a more stable state (−0.036/visit required to heal; units are weighted UniFrac distance/visit) (Figure 3a). Because mixed-effect models do not allow generation of a traditional R^2 value, we calculated marginal and conditional pseudo-R^2 values, which show an estimate of the variance due to the fixed effects alone and the combined model of fixed and random effects, respectively. The marginal R^2 was estimated to be 0.201 and the conditional to be 0.280, indicating that our model explains a moderate amount of the variation.

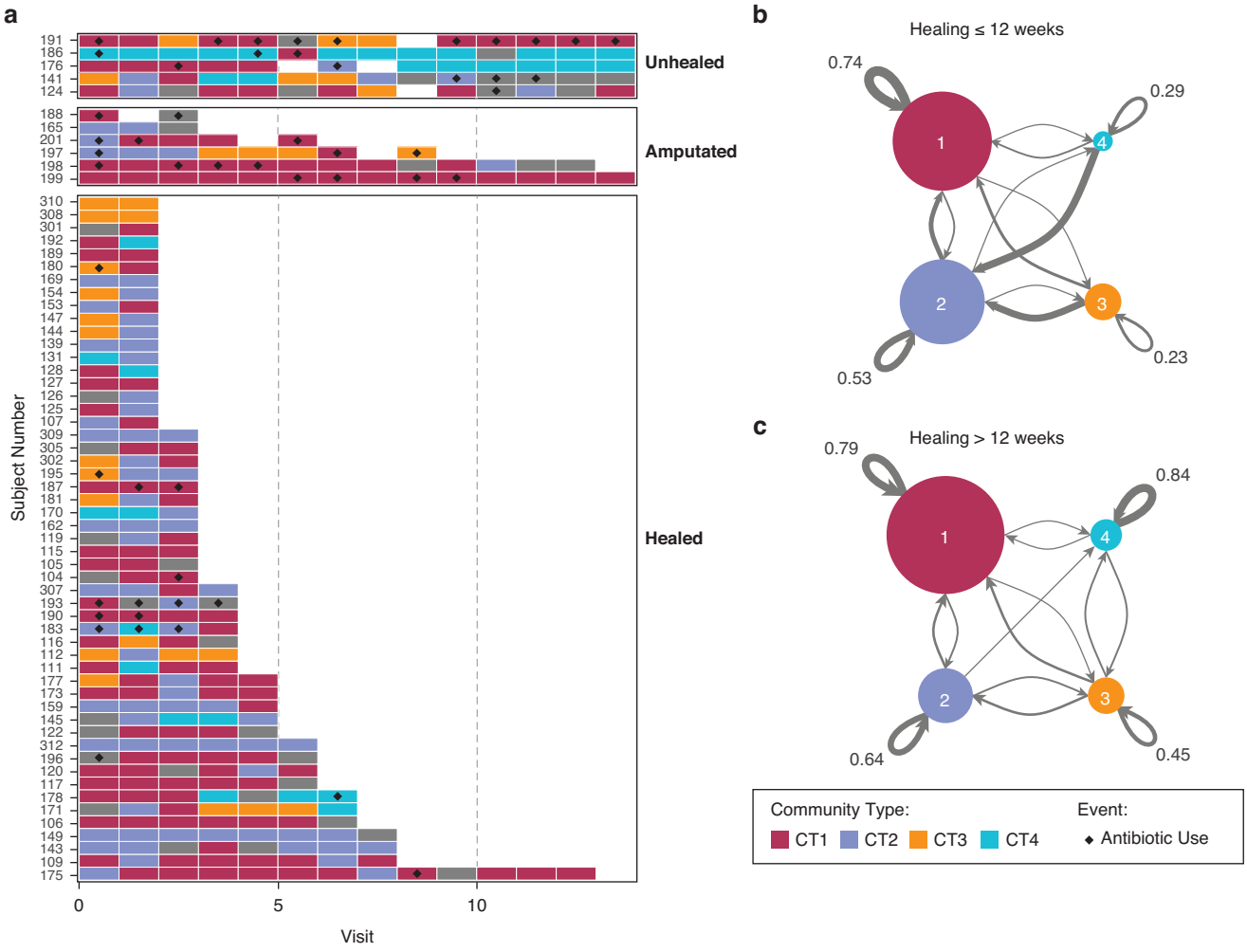

Figure 2. DFU CTs are dynamic. (**a**) Per-patient illustration of CT switching grouped by outcome. Depicted on the x-axis is visit number. Each row on the y-axis represents a subject with a DFU. Colored boxes illustrate which CT was colonizing the DFU at the indicated visit number. Empty tiles represent a missed visit, and gray tiles indicate that a sample was not collected or available for analysis at that time point. The black diamonds indicate that the patient received antibiotics since the last visit. Only subjects who participated in more than 1 study visit are shown. (**b** and **c**) Markov chain visualization depicting the differential transition probabilities between CTs of DFUs that healed in 12 weeks or did not. Each node represents a CT, arrows indicate the transition direction and probability (thickness), and node size represents number of samples. Annotated are the self-transition probabilities. CT, community type; DFU, diabetic foot ulcer.

The first intervisit distance, between the baseline study visit and following visit, includes the effect of the initial surgical debridement. Thus, it was possible that the high instability in faster-healing wounds was an artifact of the first study visit being weighted more. To address this concern, we investigated the relationship between healing time and the amount of change between baseline and the following visit (2 weeks' time) using a traditional linear model. We found the same negative association between healing time and the intervisit distance ($R^2 = 0.16$, $P < 0.0001$) (Figure 3b), suggesting the effect is independent of debridement.

Effect of antibiotics on temporal stability in DFU microbiota

During the course of the study, 32 subjects required the administration of antibiotics, which afforded us the opportunity to glean the effects of antibiotics on ulcer microbiomes. Antibiotic exposure did not drive microbiota variation in our samples (Figure 1b). Furthermore, we did not detect any significant changes in community diversity as measured by the Shannon index or OTU richness, perhaps because of unique interactions between specific antibiotic classes and personal microbial communities. We binned antibiotics into categories based on their class and mechanism of action and assessed their potential to disrupt microbial communities using the intervisit weighted UniFrac distances, as before. We did not detect significant differences in microbial stability due to antibiotic class. However, in half of the subjects, the antibiotics were prescribed to treat infections not involving the studied ulcer (e.g., other ulcers, urinary tract infection, upper respiratory infection, sinus infection). When we examined the subjects treated specifically for the study ulcer, we found that antibiotics administered produced significantly higher community disruption than if the antibiotic was given for a different indication (Figure 4a).

In some patients, during the same time period when antibiotics were administered, the ulcer was designated as having a complication (wound deterioration or osteomyelitis).

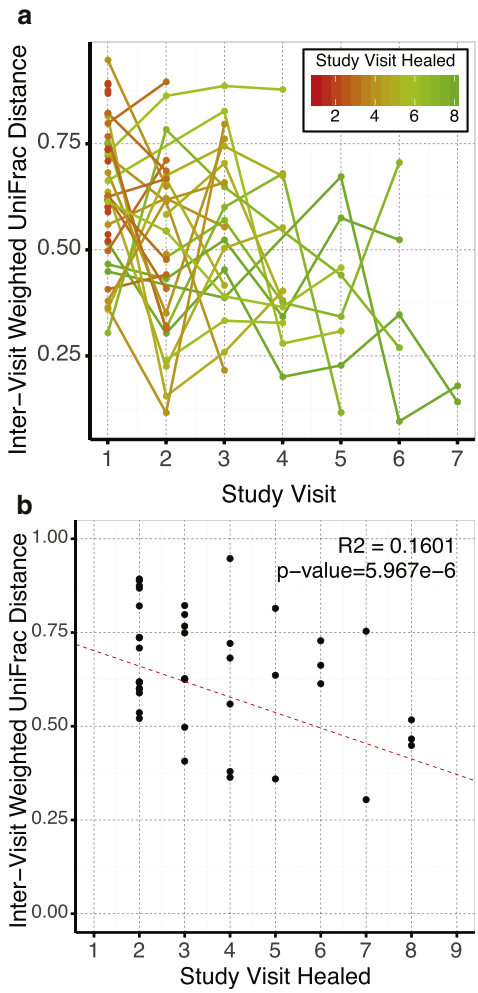

Figure 3. Intervisit weighted UniFrac distance associations with healing time for subjects whose DFUs healed during the study. The x-axes represent the study visit; study visits were 2 weeks apart. (**a**) Intervisit distances are shown for each subject and depict a negative trend over time. Line and point colors represent the number of study visits during which the ulcer persisted (red = 1, green = 8). (**b**) Intervisit distances between baseline and first study visit as a function of number of visits until healing. A negative correlation was found even within this initial comparison ($R^2 = 0.1601$, $P < 0.0001$). DFU, diabetic foot ulcer.

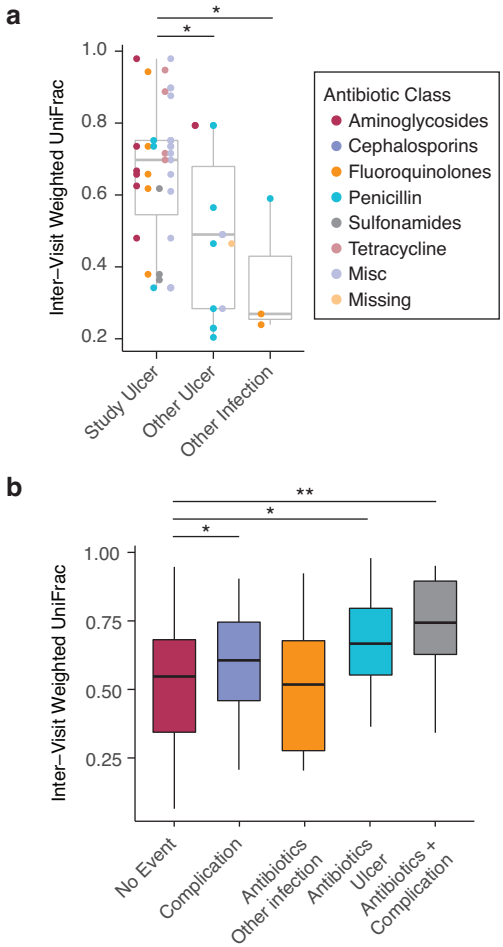

Figure 4. Effects of antibiotics on microbial communities in DFUs. (**a**) Boxplot showing the intervisit Weighted UniFrac distances of subjects during exposure to antibiotics split by indication. Antibiotics given for the ulcer being studied produces greater community disruption than antibiotics given for other ulcers or other infections. Antibiotic class did not yield more information. (**b**) Boxplot showing the intervisit distances of all samples binned by event type (complication, antibiotics, both, or none). Antibiotics and ulcer complications disrupt the microbiota, and their combined effect is additive. DFU, diabetic foot ulcer. *$P < 0.05$; **$P < 0.01$.

We modeled how these complications interacted with the antibiotics using mixed-effect linear regressions, as before (Figure 4b). We found that both complications and antibiotics contributed to community disruption, although the larger effect was noted for antibiotics (weighted UniFrac = 0.084 and 0.140, respectively). Furthermore, targeted antibiotics and complications had an additive effect on the amount of community disruption (weighted UniFrac = 0.201).

DISCUSSION

Here, we explore the temporal dynamics of the human chronic wound microbiota. Microbiome studies in other body sites have shown that disease states are associated with less stability (DiGiulio et al., 2015; Jenq et al., 2012; Martinez et al., 2008). Surprisingly, DFUs that experienced delayed healing or resulted in amputation were associated with increased stability, whereas the inverse was true for faster-healing wounds. One way of interpreting these findings is to conclude that there is no "normal" DFU community. A wound is by definition an abnormal and transient state in physiology. As such, colonizing bacteria should be considered opportunistic and unlikely to have evolved harmonious methods of existing with the host. From this perspective, instability in the microbiome is a reflection of effective control of wound bacteria, which prevents any community structure from stabilizing. In contrast, a DFU with a stable outgrowth of certain bacteria reflects a stalled healing state in which the colonizing bacteria have overridden the host's defenses.

We found that the DFU microbiome can be partitioned into four CTs. Increased CT transitions were associated with improved healing rates; however, these CT transitions were not random. In quickly healing ulcers, CT1 and CT2 were substantially more likely to remain unchanged, whereas CT3 and CT4 were more likely to transition to CT2. In slow or

unhealing wounds, we found that CT3 and CT4 became much more resilient. These findings suggest that the prognostic capacity of transition frequencies would be augmented by information about community structure. Further studies are needed to delineate cause-and-effect relationships between the microbiota and the wound environment.

Despite the regular use of antibiotics to treat infections, little is known about their impact on microbial communities in chronic wounds. We did not detect any differences in community diversity or composition due to antibiotic exposure, unlike in the gut, where exposure to certain antibiotics is known to decrease diversity levels, predisposing to infection by *Clostridium difficile* (Dethlefsen and Relman, 2011; Stein et al., 2013). Instead, as in other body sites (Keeney et al., 2014; Mayer et al., 2015; Modi et al., 2014; Zhang et al., 2014), antibiotics disrupted the microbiota. The extent of community disruption was not dependent on the class of antibiotic but rather on whether the antibiotic was targeted toward the ulcer being studied. However, our analysis is limited by the biweekly sampling frequency, which limited detection of short-lived changes.

Another limitation of this study is that relatively few subjects required amputations or did not heal during the study, perhaps a reflection of the regular care the subjects received for their DFUs at 2-week intervals. Therefore, we could not robustly analyze these specific outcomes with respect to the microbiota. To circumvent this obstacle, we relied on alternative endpoints, including rate of healing and aggregate infection-related complications (i.e., wound deterioration, osteomyelitis, amputation). The cohort was also disproportionately white and male, a reflection of the demographic composition at the study site. Although a homogeneous cohort is advantageous from a study design standpoint, limiting potential variability due to race and sex, the findings should be interpreted with caution. Studies in more diverse cohorts should be conducted to determine if the findings presented here are broadly applicable across race and sex.

In some reports, over half of DFUs are infected at the time of presentation (Prompers et al., 2007); however, identifying reliable criteria to diagnose an infection is complicated by the attenuated response to infections in diabetic persons (Brem and Tomic-Canic, 2007). Although our results would benefit from validation in larger cohorts, and their applicability to other types of chronic wounds needs to be tested, we provide evidence that the temporal dynamics of the wound microbiome may be useful for identifying stalled wounds requiring antibiotic treatment. We envision that these findings will ultimately guide clinicians in the management of chronic wounds in a personalized manner.

MATERIALS AND METHODS

Study design

A prospective, longitudinal cohort design was used to examine DFU microbiota and outcomes in 100 subjects. DFU microbiota was collected at initial presentation (baseline) and resampled every 2 weeks until (i) the DFU healed, (ii) lower extremity amputation, or (iii) the conclusion of 26 weeks of follow up. The institutional review boards at the University of Iowa and the University of Pennsylvania approved all study procedures.

Setting and sample

Subjects were enrolled from September 2008 through October 2012 at the University of Iowa Hospitals and Clinics and the Iowa City Veteran's Affairs Medical Center. Subjects were recruited through local media advertisements and from outpatient clinics at University of Iowa Hospitals and Clinics and the Iowa City Veteran's Affairs Medical Center. The target population was diabetic adults (i.e., 18 years of age or older) with a DFU on the plantar surface of the foot and ankle/brachial or toe/brachial indexes greater than 0.5 to ensure that the sample was a homogenous group of neuropathic DFUs. Individuals meeting these criteria were enrolled after providing informed written consent.

We standardized the management of the study DFUs after enrollment, including ulcer dressings (i.e., Lyofoam, Molnlycke Health Care, Gothenburg, Sweden), devices used for offloading (i.e., total contact casts were used for 87 subjects; offloading boots for 13 subjects), and ulcer debridement (i.e., aggressive sharp debridement of necrotic tissue in the wound bed was completed at baseline, and callus on the wound edge was removed every 2 weeks), to minimize the number of factors unrelated to ulcer bioburden that could affect DFU outcomes. DFU management did not include antimicrobial dressings, topical antimicrobials, and/or systemic antibiotics unless an infection-related complication was present at enrollment or occurred during follow-up. Baseline data were collected immediately after enrollment. Study data were collected every 2 weeks until one of the study endpoints was reached.

Study variables

Clinical factors. The research team measured a set of clinical factors to identify pertinent covariates for the analyses and to comprehensively describe the study sample. At baseline, demographic data, diabetes type and duration, and duration of study ulcer were collected using subject self-report and medical records. Standard laboratory tests were used to measure baseline glycemic control (hemoglobin A1c levels) and immune (white blood cell count) and inflammatory markers (C-reactive protein). The research team assessed each subject for ischemia using the toe-brachial index and for neuropathy using 5.07 Semmes-Weinstein monofilament. Transcutaneous oxygen pressure was measured at baseline and at each follow-up visit using a transcutaneous oxygen monitor (Novametrix 840, Novametrix Medical Systems, Wallingford, CT). Ulcer location was categorized as forefoot, midfoot, or heel.

Microbiome. Ulcer specimens were collected using the Levine technique. After cleansing with nonbacteriostatic saline, an Amies swab (Copan, Murrieta, CA) was rotated over a 1-cm^2 area of viable wound tissue in the center of the wound bed for 5 seconds, using sufficient pressure to extract wound-tissue fluid, DNA was isolated from swab specimens as previously described (Gardner et al., 2013). Levine's swab technique was used because it samples the viable, deep wound tissue in a noninvasive manner, allowing for serial sampling of the wound over time. Levine's swab produces comparable results to tissue specimens for microbial load and diversity (Gardner et al., 2006). Amplification of the 16S ribosomal RNA gene V1−V3 region was performed as described previously (Meisel et al., 2016), using the Illumina (San Diego, CA) MiSeq platform with 300-base pairs paired-end V3 chemistry. This resulted in a dataset of 7,702,607 high-quality, classifiable sequences used in the final analysis, with a mean of 22,070 (range = 1,206−69,167) sequences per sample. Sequence preprocessing followed methods described previously (Meisel et al., 2016), modified by performing de novo

OTU clustering via UCLUST, assigning taxonomy with BLAST, and subsampling at 1,200 sequences per sample. Sequences corresponding to the taxa *Geobacillus, Bacillus,* and *Lactococcus* were removed, because these were identified as contaminants in the negative controls. QIIME 1.9.0 (Caporaso et al., 2010) was used for initial stages of sequence analysis. Sequences were clustered into OTUs (a proxy for species) using UCLUST (Edgar, 2010) at 97% sequence similarity. Microbial diversity was calculated using the following alpha diversity indices: (i) Shannon diversity index, (ii) Faith's phylogenetic distance, and (iii) number of observed OTUs. Taxonomic classification of sequences were made using BLAST, as implemented in QIIME.

Outcomes. Members of the research team, who were blinded to the microbiota status, assessed healing and infection-related complications every 2 weeks. Ulcer closure was assessed using the Wound Healing Society's definition of *an acceptably healed wound,* a valid and reliable definition (Margolis et al., 1996). The outcome *healed by 12 weeks* was defined as wound closure before or at 12 weeks of follow-up. *Development of infection-related complications* was defined as wound deterioration, new osteomyelitis, and/or amputations due to DFU infections.

Wound deterioration was defined as the new development of frank erythema and heat and an increase in size of greater than 50% over baseline. Two members of the research team independently assessed each DFU for erythema and heat. Two members of the research team independently assessed size using the VeVMD digital software system (Vista Medical, Winnipeg, Manitoba, Canada), which was loaded on a Dell Latitude D630 laptop computer (Dell, Round Rock, TX). Digital images were taken that contained the ulcer, a 3 × 3-cm^2 image orientation card, and a single-point wound-depth indicator (i.e., a cotton-tipped swab that had been placed in the deepest aspect of the DFU and marked where the swab intersected with the plane of the periwound skin) and uploaded into the VeVMD program. VeVMD tools were used to trace the ulcer outline and a line along the wound depth indicator to generate measures of depth and surface area.

Osteomyelitis was assessed using radiographs and magnetic resonance imaging at baseline and during follow-up visits when subjects presented with new tracts to bone, wound deterioration, elevated temperature, elevated white blood cell count, elevated erythrocyte sedimentation rate, or elevated C-reactive protein level. If these indicators were absent at follow-up, radiographs were not retaken. Subjects experiencing lower-limb amputations had their medical records reviewed by the research team to ensure that amputations were due to DFU infection and not some other cause.

Data analyses

The R Statistical Package (R Core Team, 2016) was used for all computations. Nonparametric Wilcoxon rank-sum tests were used to compare differences between groups. Spearman correlations were used to correlate continuous variables. Kruskal-Wallis tests, followed by Wilcoxon rank-sum post hoc tests, were used for categorical variables. Linear models were calculated in base R; mixed-effect regressions were generated using the Linear and Non-linear Mixed Effect Models (NLME) package (Pinheiro et al., 2007). Partial and conditional pseudo-R2 values were calculated using the piecewiseSEM package (Lefcheck, 2016). Sample biplot was generated using the Breadcrumbs package as described previously (Morgan et al., 2015). Differences in Markov chain transition frequencies were tested with a Fisher's test and simulated *P*-value. Dirichlet multinomial mixture modeling was performed using the R package Dirichlet Multinomial (version 1.10.0). Counts were calculated at the highest level of taxonomic classification. The number of CTs was determined by selecting the number of Dirichlet components that minimized the Laplace approximation of the model evidence (Holmes et al., 2012). Each sample was assigned to the community type that had the largest posterior probability. Intervisit distances were calculated using the weighted UniFrac distance between consecutive visits. If visits were discontinuous (i.e., missing sample) no distances were reported.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

SEG and EAG oversaw conceptualization, supervision, and funding acquisition. MAL, SEG, DJM, and EAG were responsible for methodology. MAL, LK, SLH, and DJM contributed to formal analysis. MAL, LK, JH, AST, and BPH conducted the investigation. QZ and CLF oversaw data curation. SEG, SM, DJM, and EAG were responsible for resources. MAL was responsible for visualization. MAL, SEG, and EAG wrote the original draft. All authors participated in review and editing.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.08.009.

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