Cultures of Diabetic Foot Ulcers Without Clinical Signs of Infection Do Not Predict Outcomes

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OBJECTIVE
We examined associations between ulcer bioburden and ulcer outcomes in neuropathic diabetic foot ulcers (DFUs) that lacked clinical signs of infection.

RESEARCH DESIGN AND METHODS
Three dimensions of bioburden (i.e., microbial load, microbial diversity, and the presence of likely pathogens) were measured at baseline using swab cultures obtained by Levine’s technique. Subjects were assessed every 2 weeks for 26 weeks to determine the rate of healing and development of infection-related complications. Foot ulcers were offloaded using total-contact casts and routinely debrided. To establish associations between bioburden and rate of healing, Cox proportional hazards and least-squares regression were used after adjusting for ulcer depth, surface area, and duration.

RESULTS
A total of 77 subjects completed the study. Sixty-five (84.4%) had ulcers that healed during follow-up; weeks-to-closure ranged from 2 to 26 (median 4.0). Mean (± SD) percent reduction in surface area/week was 25.0% (± 23.33). Five (6.5%) of the DFUs developed an infection-related complication. None of the bioburden dimensions (i.e., microbial load, microbial diversity, or presence of likely pathogens) was significantly associated with weeks-to-closure or percent reduction in surface area per week. Weeks-to-closure was best predicted by ulcer duration, depth, and surface area (c-statistic = 0.75).

CONCLUSIONS
Culturing DFUs that showed no clinical signs of infection had no predictive value for outcomes of DFUs managed with total-contact casts and routine debridement. These findings support recommendations of the Infectious Disease Society of America that culturing and antibiotics should be avoided in treating DFUs that show no clinical signs of infection.

Early identification of diabetic foot ulcers (DFUs) destined for poor healing and/or infection-related complications remains problematic, often delaying and compromising treatment. Although current guidelines recommend antibiotic treatment be initiated when obvious clinical signs of infection develop (1), these signs may not appear until destruction of underlying tissue and bone triggers a systemic inflammatory response. Patients with diabetes, however, may not express clinical signs of infection, despite high levels of bacteria in local DFU tissue (1,2) because peripheral vascular disease, poor metabolic control, and neuropathy dampen first-line inflammatory
responses (3). Still, for patients with uninfected DFUs, indiscriminate use of antibiotics likely contributes to the growing problem of antibiotic resistance (4). Theoretically, in DFUs that show no clinical signs of infection, the judicious and time-limited use of antimicrobial treatment might be guided by first assessing bioburden. In reality, however, it is unclear which dimension(s) of bioburden are actually associated with poor healing or the development of a DFU infection.

Although bioburden has traditionally been used to refer to the number of microorganisms contaminating a surface (5), wound bioburden is often used to refer to three dimensions or aspects of the microbial community that may contribute to poor healing and/or development of infection-related complications (6). These dimensions include: 1) microbial load (i.e., the number of organisms per gram of tissue); 2) microbial diversity (i.e., the number of different species); and 3) the presence of pathogenic organisms. Research suggests, in DFUs and other types of chronic wounds, a high microbial load (7,8) and microbe diversity (9) result in poor healing and infection. Common DFU pathogens include *Staphylococcus aureus* (10), methicillin-resistant *S. aureus* (MRSA) (11,12), Gram-negative bacilli (13), β-hemolytic *Streptococcus* (10), and obligate anaerobes (10); these microbes are targeted for antibiotic treatment in DFUs with moderate to severe clinical signs of infection (1). Unfortunately, studies of DFU bioburden typically have focused on only one or two dimensions of bioburden; however, to determine which, if any, of the dimensions of bioburden are important in predicting poor healing or the development of infection-related complications, it is necessary to examine all three dimensions of bioburden in a single cohort of subjects.

Accordingly, the purpose of this study was to determine whether the three dimensions of wound bioburden can be used to predict outcomes among persons who have neuropathic DFUs but no clinical signs of DFU infection. Our specific aims were to examine the associations between baseline measures of each dimension of bioburden (listed below): and 1) DFU rate of healing (i.e., weeks-to-closure and percent reduction of ulcer surface area per week); and 2) the development of DFU infection-related complications (i.e., new wound deterioration, new osteomyelitis, or new amputation due to DFU infection). Three bioburden dimensions were examined as predictors: 1) microbial load; 2) microbial diversity; and 3) presence of *S. aureus* (including MRSA), obligate anaerobes, proteobacteria (i.e., Gram-negative rods), and β-hemolytic *Streptococcus*.

### RESEARCH DESIGN AND METHODS

#### Design

A prospective-cohort design was used. Each dimension of ulcer bioburden was measured at baseline; and the research team assessed the rate of healing and development of infection-related complications every 2 weeks, until: 1) the ulcer healed; 2) the DFU foot was amputated; 3) the subject was lost to follow-up; or 4) after 26 weeks of follow-up. The Institutional Review Board at the University of Iowa approved study procedures. Subjects were enrolled from September 2008 through February 2012.

#### Setting and Sample

Subjects were recruited through local media advertisements (newspaper and television advertisements) and from outpatient clinics at a large, academic-affiliated medical center and a Veterans Affairs medical center. The research team screened diabetic adults (i.e., ≥18 years of age) with a DFU on the foot. The research team discontinued the study after recruiting 100 subjects. Inclusion/exclusion criteria: 1) significant ischemia (i.e., toe-brachial index or ankle-brachial pressure index <0.5); 2) signs or symptoms of clinical DFU infection (i.e., increasing pain, erythema, heat, edema, or purulent exudate) or osteomyelitis (i.e., positive radiograph); and 3) treated with systemic antibiotics in the prior 2 weeks. Presence of osteomyelitis was assessed using X-rays of the ulcer foot at the time of screening. Excluding individuals on systemic antibiotics minimized any influence of antibiotics on baseline ulcer bioburden. Some individuals met all inclusion and none of the exclusion criteria, except having been administered systemic antibiotics in the 2 weeks prior despite the lack of clinical signs of DFU infection. In these cases, the research team discontinued antibiotics and enrolled the subjects 2 weeks later. Those on long-term systemic antibiotics for chronic non-DFU infections, such as chronic urinary tract infections, were excluded from the study. Individuals meeting inclusion/exclusion criteria were enrolled after providing informed written consent. Baseline data were collected immediately after enrollment.

Ulcer dressings (i.e., Lyofoam; Molnlycke Health Care), offloading devices (i.e., total-contact casts were used for 72 10% of subjects and DH boots for 5 subjects), and debridement (i.e., sharp debridement of necrotic tissue in the wound bed and callus on the wound edge) was standardized for all study subjects as a way to limit factors unrelated to ulcer bioburden and minimize variability in DFU outcomes. DFU management did not include antimicrobial dressings, topical antimicrobials, and/or systemic antibiotics, unless an infection-related complication occurred during follow-up.

#### Study Variables

**Predictors: Three Dimensions of DFU Bioburden**

Baseline measures of 1) microbial load; 2) microbial diversity; and 3) presence of likely pathogens were obtained to determine ulcer bioburden. After cleansing with nonbacteriostatic saline, ulcer specimens were collected using Levine’s technique: An Amies swab (Copan, Brescia, Italy) was rotated over a 1-cm² area of viable wound tissue for 5 s, using sufficient pressure to extract wound-tissue fluid, and immediately transported in a charcoal transport to a research microbiology laboratory. The swab was vortexed in 1 mL of tryptic soy broth, and the resulting suspension was processed to measure three dimensions of bioburden using the procedures described below.

**Microbial Load**

Each suspension was serially diluted in tryptic soy broth and each dilution plated on Columbia blood agar (Remel, Lenexa, KS), eosin-methylene blue agar (EMB; Remel), and CHROMagar MRSA (Becton Dickinson, Sparks, MD) plates. Columbia and EMB plates were incubated in 5% CO₂ at 37°C for 48 h, and MRSA plates were incubated aerobically at 37°C for 48 h. Each dilution was also plated onto *Brucella* Agar supplemented with blood, hemin, and vitamin K (Remel).
and incubated in an anaerobe jar at 37°C for 48 h. The species of infecting organisms was identified via standard microbiological procedures (14). Because dilutions are based on a single swab, the plate count of each species was multiplied by the dilution factor to yield total number of colony-forming units (CFUs) for that species. Microbial load was defined as the sum CFUs of all species or total CFUs per swab.

Microbial Diversity
Microbial diversity was defined as the number of different species identified from both aerobic and anaerobic plates.

Presence of Likely Pathogens
*S. aureus* was identified on Columbia blood agar, based on the appearance of characteristic yellow β-hemolytic colonies, which appeared as Gram-positive cocci organized into grapelike clusters on stain and tested catalase positive as well as *Staphylococcus* latex-agglutination positive. To identify MRSA strains, all *S. aureus* isolates were screened by PCR for the mecA gene, according to previously published methods (15,16). Organisms that grew anaerobically on supplemented *Brucella* agar, but not aerobically, were identified as anaerobes. Organisms that grew on EMB plates and stained Gram-negative were identified as proteobacteria (Vitek Legacy; Biomerieux, Durham, NC). β-hemolytic, catalase-negative, Gram-positive cocci were classified to Lancefield group (A, B, C, F, and G) using the PathoDX Strep Grouping Kit (reference 62076; Remel).

Outcomes: DFU Rate of Healing and Development of Infection-Related Complications
Outcomes were measured every 2 weeks during follow-up. The members of the research team who assessed the rate of healing and development of infection-related complications were blind to DFU bioburden status at all follow-up visits.

Rate of Healing
The rate of healing was defined as: 1) weeks-to-closure (complete healing); and 2) percent reduction in ulcer surface area per week. Two of several members of the research team independently assessed ulcer closure at each study visit using the Wound Healing Society’s definition of “an acceptably healed wound,” a valid and reliable definition (17). Agreement between the assessments was 98.7%. The principal investigator resolved any discrepancies between the raters.

To compute changes in ulcer size, ulcers were measured at baseline and, if the ulcer was not healed, at each follow-up visit. Two members of the research team independently assessed size using the VeVMD digital software system (Vista Medical, Winnipeg, Manitoba, Canada) and procedures previously described (18). Inter- and intrarater reliability of VeVMD was 0.76 and 0.87 (Pearson *r*), respectively (18). A cotton-tipped swab, placed in the deepest aspect of the DFU, was marked where the swab intersected with the plane of the peri-wound skin. The distance between the tip of the swab and the mark was measured as ulcer depth using a centimeter ruler.

Development of Infection-Related Complications
Development of infection-related complications was defined as wound deterioration, new osteomyelitis, and/or a new amputation due to DFU infection. Subjects whose wounds deteriorated and/or developed new osteomyelitis were treated for infection and monitored until either their ulcer healed, they were lost to follow-up, or at the end of the 26-week follow-up period. Study participation ended after amputation.

Wound deterioration was defined as the new development of erythema and frank heat and an increase in size >50% over baseline. Ulcer size was measured as described above. Two members of the research team independently assessed each DFU for erythema and frank heat, and agreement between raters was 92.5 and 95.5%, respectively. The principal investigator resolved any discrepancies. Members of the research team assessed other clinical signs of infection, including increasing pain, edema, and purulent drainage. These signs and symptoms were not used to define wound deterioration because they only occurred in one subject who also had erythema and heat. Development of new osteomyelitis was assessed using radiographs and magnetic resonance imaging when subjects presented with new tracts to bone, wound deterioration, elevated temperature, elevated white count, elevated erythrocyte sedimentation rate, or elevated C-reactive protein at follow-up visits. If these indicators were absent at follow-up, radiographs were not retaken. Subjects experiencing new amputations had their medical records reviewed by the research team (J.E.F. and P.P.) to ensure amputations were due to DFU infection and not some other reason.

Demographic and Secondary Variables
At baseline, the research team collected demographic variables (age, sex, and race), smoking history (packs per day and years of smoking), diabetes type and duration, and duration of the study ulcer using subject self-report and medical records. Standard laboratory tests were used to measured baseline glycemic control (HbA1c) and nutritional status (albumin and prealbumin levels). At baseline, trained members of the research team assessed neuropathy (5.07 Semmes-Weinstein monofilament). Microcirculation (transcutaneous oxygen pressure) was measured at baseline and at each follow-up visit using a transcutaneous oxygen monitor (Novametrix 840; Novametrix Medical Systems Inc.).

Data Analysis
Rate of Healing
DFU rate of healing was defined using two metrics: 1) weeks to complete wound closure; and 2) percent reduction in ulcer surface area per week. The association between each dimension of bioburden (i.e., microbial load, microbial diversity, and presence of each pathogen) and the number of weeks to complete wound closure were examined using Cox proportional hazards regression, with weeks-to-closure treated as censored when subjects were lost to follow-up before healing. We regressed weeks-to-closure separately on each dimension of bioburden. Both unadjusted and adjusted models were fit. For adjusted models, baseline measures of ulcer duration, depth, and surface area were used as covariates because they are associated with rate-of-healing outcomes (19). To assess the additional usefulness provided by each bioburden dimension, we also fit a model containing only covariates (i.e., ulcer duration, depth, and surface area) as independent variables. We described effects of each dimension of
bioburden with relative risk (RR). We described the discrimination ability of the proportional hazards models using the c-statistic, as discussed by Harrell et al. (20,21). The c-statistic estimates the probability that the model correctly discriminates between two subjects having different weeks to wound closure.

The association between bioburden and percent reduction in surface area per week was determined as in the weeks-to-wound-closure analyses, except that a least-squares regression was used. Adjusted models included the covariates described above. We described effects of each dimension of bioburden with regression coefficients. We described the discrimination ability of the model with $R^2$.

These analyses were considered exploratory, so we did not control for type I error. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC) using PROC LOGISTIC and PROC PHREG. Because PROC PHREG does not compute c-statistic for Cox proportional hazards regression, it was computed using added SAS statements. An $\alpha = 0.05$ was used for all analyses.

**Development of Infection-Related Complications**

Only five subjects developed infection-related complications, making the power too low for testing associations between each dimension of bioburden and the development of infection-related complications. Alternatively, we described the bioburden associated with ulcers that developed an infection-related complication.

### RESULTS

#### Recruitment and Enrollment

Ninety-six individuals met the inclusion criteria and were screened for eligibility. Twelve of these were subsequently excluded due to osteomyelitis ($n = 6$), long-term antibiotics for chronic infections (e.g., chronic urinary tract infection; $n = 3$), ischemia (toe-brachial index or ankle-brachial pressure index $\leq 0.5$; $n = 1$), clinical signs of DFU infection ($n = 1$), and inability to use the off-loading device ($n = 1$). The remaining 84 persons were enrolled in the study, including 17 (20.0%) who were initially on systemic antibiotics for a clinically uninfected DFU. These 17 subjects were enrolled 2 weeks after discontinuing antibiotics. Of the 84 enrolled subjects, 7 were excluded from analyses due to missing baseline or follow-up data. Seventy-seven subjects were included in final analyses.

**Descriptive Statistics of Sample**

Table 1 contains descriptive statistics for study subjects and ulcers. The mean age of subjects was 54.9 (SD ± 11.6) years, and they were predominantly white ($n = 70$; 90.9%), male ($n = 62$; 80.5%), and type 2 diabetic ($n = 66$; 85.7%). All (100%) subjects had neuropathy by monofilament testing. Sixty (77.9%) ulcers were located on the forefoot, 12 (15.6%) on the midfoot, and 5 (6.5%) on the heel.

Table 2 summarizes baseline measures for each dimension of bioburden. Six (7.8%) DFU specimens produced no growth following incubation. These ulcers had microbial load and diversity values of 0 and none of the likely pathogens.

Subject follow-up ranged from 2 to 26 weeks, with a mean of 7.2 (SD ± 6.3) weeks. During follow-up, 65 (84.4%) ulcers healed, 1 (1.3%) resulted in an amputation, 8 (10.4%) were unhealed when lost to follow-up, and 3 (3.9%) were unhealed at the end of 26 weeks. DFU outcomes are summarized in Table 2.

### Bioburden and Rate of Healing

Results from analyses of the association between each dimension of bioburden and weeks-to-closure are displayed in Table 3. Unadjusted results are in section 1, adjusted results in section 2, and results for the model with only covariates in section 3.

Microbial load and microbial diversity were not significant predictors of the number of weeks to wound closure. Similarly, presence of *S. aureus*, MRSA, anaerobes, proteobacteria, or β-hemolytic *Streptococcus* did not predict the number of weeks to wound closure. Interestingly, when growth of any organism versus no growth was examined, the presence of any organism was significantly associated with more weeks-to-closure in unadjusted analyses (RR
Table 2—Baseline ulcer bioburden and ulcer outcomes after follow-up (N = 77)

<table>
<thead>
<tr>
<th>Bioburden dimensions</th>
<th>Mean (± SD)</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial load (total CFU/swab)</td>
<td>$1.0 \times 10^6$ (± $3.75 \times 10^6$)</td>
<td>$5.1 \times 10^6$ (0–2.7 × $10^7$)</td>
</tr>
<tr>
<td>Microbial diversity (number of different species/swab)</td>
<td>3.6 (± 2.37)</td>
<td>3.0 (0–9.0)</td>
</tr>
</tbody>
</table>

Potential pathogens
- Number (% of ulcers with S. aureus: 28 (36.4)
- Number (% of ulcers with MRSA: 8 (10.4)
- Number (% of ulcers with Proteobacteria: 27 (35.1)
- Number (% of ulcers with β-hemolytic Streptococcus: 19 (24.7)
- Number (% of ulcers with anaerobes: 15 (19.5)

Ulcer outcomes
- Rate of healing
  - Ulcers achieving complete closure/healing [n (%)]: 65 (84.4)
  - Weeks to wound closure [mean (± SD)/median (range)] (n = 65): $6.0 ± 4.81)/4.0$ (1.9–25.9)
  - Percent reduction in surface area/week [mean (± SD)/median (range)]: $25.0 ± 19.46)/23.3$ (−14.5 to 53.9)
  - Developed infection-related complication [n (%)]: 5 (6.5)
  - Wound deterioration [n (%)]: 4 (5.2)
  - Osteomyelitis [n (%)]: 0 (0.0)
  - Amputation [n (%)]: 1 (1.3)

Six of the 77 subjects had no growth on culture plates. Therefore, the microbial load and microbial diversity for these subjects was 0. The mean and median for microbial load and microbial diversity were computed for the entire sample, including those with no growth. Therefore, the range includes 0 as the lower level.

0.34; $P = 0.02$; c-statistic = 0.54) and nearly significantly associated with more weeks-to-closure in the adjusted analyses (RR 0.42; $P = 0.06$; c-statistic = 0.74). Nevertheless, the model with only covariates showed slightly better discrimination in predicting weeks-to-closure (c-statistic = 0.75) than the adjusted model containing any organism versus no growth and all three covariates. In the covariate-only model, ulcer depth and surface area had a significant ($P = 0.02$ and 0.006, respectively) positive association with more weeks to wound closure (RR 0.30 and 0.81, respectively). Ulcer duration had a close to significant ($P = 0.05$) but slightly negative association with more weeks to wound closure (RR 1.01).

Results from analyses of the association between each dimension of bioburden and percent reduction in surface area per week are displayed in Table 4. Unadjusted results are presented in section 1, adjusted results in section 2, and results for the model that includes only covariates in section 3.

None of the dimensions of bioburden (i.e., microbial load, microbial diversity, or presence of potential pathogens) was significant in predicting percent reduction in surface area per week in either the unadjusted or adjusted analyses. The three covariates had significant or close to significant associations with percent reduction in surface area per week in all of the adjusted models. In the covariate-only model ($R^2 = 0.27$), ulcer depth and surface area had significant ($P = 0.001$ and 0.004, respectively) negative associations (regression coefficient $= −24.29$ and $−2.33$, respectively) with percent reduction in surface area per week. Ulcer duration had a close to significant ($P = 0.05$) and slightly positive association with percent reduction in ulcer surface area per week (regression coefficient 0.10).

Bioburden and Development of Infection-Related Complications
Five (6.5%) subjects developed an infection-related complication. The type of complication and bioburden data for these subjects are provided in Table 5.

CONCLUSIONS
None of the three dimensions of bioburden (i.e., microbial load, microbial diversity, and presence of potential pathogens) predicted the number of weeks to ulcer closure (before or after adjusting for ulcer duration, depth, and surface area). Sixty-five (84.4%) of DFUs in this study healed during the 6-month follow-up period, 50% of which healed in ≤4 weeks. This is considerably shorter than the 9 weeks (63 days) reported by Ince et al. (19), even though the DFUs in their retrospective study were similar in terms of inclusion/exclusion criteria, baseline ulcer size, ulcer management, and proportion achieving complete closure. However, Ince et al. (19) included some nonplanter DFUs below the malleolus in their sample, which may explain the longer time-to-heal reported in their study.

Similarly, in our study, none of the dimensions of bioburden predicted the percent reduction in ulcer surface area per week. In contrast, Xu et al. (8) found high microbial load was inversely related to the percent change in ulcer area per day in a sample of neuropathic DFUs; however, their analyses did not control for ulcer duration, depth, and surface area. In our study, we found ulcer duration, depth, and surface area did indeed predict weeks-to-closure and percent reduction in surface area per week. In fact, the predictive power of these three variables was substantial in modeling the number of weeks to DFU closure (c-statistic = 0.75). In addition, although Xu et al. (8) reported that subjects in their sample were provided with regular care, including debridement, it is unclear if any, or which, offloading techniques were used. All subjects in our study were off-loaded using a total-contact cast, and DFUs were sharp-debrided on a regular basis. Total-contact casting (22) and routine debridement (23) likely contributed to the high number of subjects who healed, the rapid rates of healing, and the low number of infection-related complications observed in this study. In the absence of these management techniques, the time-to-complete closure would likely be increased.

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Bioburden and DFU Outcomes

Diabetes Care

A wider range than the often-cited threshold of $10^6$ organisms/g of tissue (25) believed to be indicative of infection. Microbial diversity also varied, ranging from one to nine microbial species. Of the nine species detected in one DFU, four were common pathogens, including β-hemolytic Streptococcus. Despite treatment with antibiotics, that DFU remained unhealed at the end of the 6-month follow-up period.

In our study, of the 28 ulcers that harbored S. aureus, including MRSA, only 4 developed an infection-related complication. (The DFU that harbored MRSA resulted in an amputation.) A previous study demonstrated that species isolated from ischemic DFUs were likely to contain antibiotic resistant S. epidermidis (90%) (26). Of 27 study ulcers that harbored proteobacteria (Gram-negative bacteria), the condition of only three deteriorated. Gram-negative bacteria, such as Pseudomonas aeruginosa, are believed to be common pathogens of chronic wounds (10), and indeed, we frequently found that species in our study subjects’ infected ulcers. Of the 15 ulcers that harbored anaerobes, the condition of 1 deteriorated during the study. Finally, of the 19 DFUs harboring β-hemolytic Streptococcus, only 2 developed an infection-related complication despite widespread concern that β-hemolytic Streptococcus is a common pathogen in DFUs (25). Together, these findings indicate that DFUs harboring the pathogens for which we assayed need not be treated with antibiotics unless the abscess displays clinical signs of a pathogenic infection. An antibiotic-free approach is particularly recommended when rigorous offloading and debridement are part of the wound-care regimen.

This is the first study to prospectively examine whether any of the three dimensions of bioburden predict outcomes among neuropathic DFUs that show no clinical signs of infection. Our data suggest bioburden does not correlate with DFU outcomes. Nonischemic, neuropathic ulcers represent 70% (27) of all DFUs and therefore are most frequently encountered by clinicians. Typically, persons with DFUs present to their care provider for treatment when they are free from clinical infection, and the findings of this study suggest that culturing their wounds to assess bioburden (including microbial load) is unnecessary, further supporting the Infectious Diseases Society of America (IDSA) guideline that DFUs without clinical signs of infection should not be treated with antibiotics (1). Nevertheless, before our subjects were enrolled in our study, 20% were being treated with antibiotics with no clear clinical rationale. Such practices likely contribute to the growing problem of antibiotic resistance (4). The findings of this study stress the need to translate the IDSA guideline into practice.

Many published classification systems are used for diagnosing DFU infections. All are based on clinical symptoms and signs of inflammation, the extent of the ulcer, and the host response to the inflammation. Of the variety of classification systems, the IDSA system is easiest to use and has been prospectively validated as predicting the need for hospitalization (28–31).

### Table 3—Proportional hazards regression results for weeks to wound closure ($N = 77$)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>P value</th>
<th>RR</th>
<th>LCL</th>
<th>UCL</th>
<th>c-Stat</th>
<th>Duration</th>
<th>Depth</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial load</td>
<td>0.23</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.51</td>
<td>0.77</td>
<td>0.36</td>
<td>0.009</td>
</tr>
<tr>
<td>Microbial diversity</td>
<td>0.42</td>
<td>0.96</td>
<td>0.86</td>
<td>1.06</td>
<td>0.54</td>
<td>0.053</td>
<td>0.036</td>
<td>0.008</td>
</tr>
<tr>
<td>Presence of any S. aureus</td>
<td>0.02</td>
<td>0.34</td>
<td>0.14</td>
<td>0.81</td>
<td>0.54</td>
<td>0.056</td>
<td>0.021</td>
<td>0.012</td>
</tr>
<tr>
<td>MRS A</td>
<td>0.31</td>
<td>0.77</td>
<td>0.46</td>
<td>1.28</td>
<td>0.53</td>
<td>0.038</td>
<td>0.013</td>
<td>0.008</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>0.94</td>
<td>0.97</td>
<td>0.44</td>
<td>2.14</td>
<td>0.51</td>
<td>0.012</td>
<td>0.025</td>
<td>0.009</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.81</td>
<td>0.92</td>
<td>0.48</td>
<td>1.78</td>
<td>0.49</td>
<td>0.06</td>
<td>0.021</td>
<td>0.012</td>
</tr>
<tr>
<td>β-hemolytic Streptococcus</td>
<td>0.74</td>
<td>1.10</td>
<td>0.62</td>
<td>1.94</td>
<td>0.49</td>
<td>0.044</td>
<td>0.025</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Each line represents one model. Microbial load is the total number of CFUs per swab. Microbial diversity is the number of different species per swab. Presence of any is a dichotomous indicator for growth vs. no growth cultures. Presence of any Staphylococcus is an indicator for presence of the corresponding organism. A significant covariate P value indicates that the covariate is useful in the model. The c-statistic is the area under the ROC curve. A value for testing H0: RR = 1; UCL, upper 95% confidence limit for RR; P value, P value for testing H0: RR < 1; LCL, lower 95% confidence limit for RR.

strategies, a high microbial bioburden may have a greater impact on the rate of DFU healing.

To our knowledge, this is the first study to examine wound deterioration as an outcome in a prospective DFU study. Among the five (6.5%) DFUs that developed an infection-related complication, the dimensions of bioburden varied greatly. Four wounds deteriorated, while only one (1.3%) resulted in an amputation, a rate much better than the 4.9% amputation rate seen among Medicare beneficiaries with both neuropathic and ischemic foot ulcers (24). Our lower rate is likely due to an absence of significant macro- and microvascular compromise, as well as our routine practice of off-loading and debridement. Among five DFUs that developed an infection-related complication, microbial load ranged from $10^6$ to $10^7$ microbes/swab, a wider range than the often-cited threshold of $10^6$ organisms/g of tissue (25) believed to be indicative of infection. Microbial diversity also varied, ranging from one to nine microbial species. Of the nine species detected in one DFU, four were common pathogens, including β-hemolytic Streptococcus. Despite treatment with antibiotics, that DFU remained unhealed at the end of the 6-month follow-up period.

In our study, of the 28 ulcers that harbored S. aureus, including MRSA, only 4 developed an infection-related complication. (The DFU that harbored MRSA resulted in an amputation.) A previous study demonstrated that species isolated from ischemic DFUs were likely to contain antibiotic resistant S. epidermidis (90%) (26). Of 27 study ulcers that harbored proteobacteria (Gram-negative bacteria), the condition of only three deteriorated. Gram-negative bacteria, such as Pseudomonas aeruginosa, are
A potential limitation of this study is our culture-based method for measuring bioburden. We realize that standard culture methods are limited in delineating true bioburden (32), and our culture-based techniques might fail to detect microbial species and communities that contributed to ulcer outcomes. Nevertheless, our results are clinically relevant because although other methods for measuring bioburden (e.g., genomic techniques and molecular assays, such as PCR) are not yet widely available in the clinical setting. Therefore, the findings of this study are relevant in clinical settings that predominantly use culture-based techniques.

Table 4—Least-squares regression results for percent reduction in surface area per week (N = 77)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>P value</th>
<th>Coefficient</th>
<th>LCL</th>
<th>UCL</th>
<th>RSQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial load</td>
<td>0.24</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>Microbial diversity</td>
<td>0.32</td>
<td>−0.95</td>
<td>−2.83</td>
<td>0.93</td>
<td>0.01</td>
</tr>
<tr>
<td>Presence of any</td>
<td>0.08</td>
<td>−14.47</td>
<td>−30.73</td>
<td>1.79</td>
<td>0.04</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.32</td>
<td>−4.61</td>
<td>−13.79</td>
<td>4.58</td>
<td>0.01</td>
</tr>
<tr>
<td>MRSA</td>
<td>0.47</td>
<td>−5.33</td>
<td>−19.86</td>
<td>9.20</td>
<td>0.01</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>0.68</td>
<td>2.32</td>
<td>8.90</td>
<td>13.53</td>
<td>0.00</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.27</td>
<td>−5.20</td>
<td>−14.44</td>
<td>4.05</td>
<td>0.02</td>
</tr>
<tr>
<td>β-hemolytic Streptococcus</td>
<td>0.96</td>
<td>−0.26</td>
<td>−10.52</td>
<td>10.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

A second limitation of this study stems from controversy of how best to take a sample that assesses bioburden. One guideline suggests that tissue

Table 5—Bioburden of DFUs developing infection-related complications (N = 5)

<table>
<thead>
<tr>
<th>Identification number</th>
<th>Type of complication</th>
<th>Microbial load (total CFU/swab)</th>
<th>Microbial diversity (number of species/swab)</th>
<th>Potential pathogens</th>
<th>End of study reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>Wound deterioration</td>
<td>$1.1 \times 10^5$</td>
<td>7</td>
<td>MRSA</td>
<td>Lost to follow-up, unhealed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-hemolytic Streptococcus</td>
<td>β-hemolytic Streptococcus</td>
</tr>
<tr>
<td>141</td>
<td>Wound deterioration</td>
<td>$1.7 \times 10^7$</td>
<td>9</td>
<td>S. aureus</td>
<td>Unhealed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anaerobes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Proteobacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-hemolytic Streptococcus</td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>Wound deterioration</td>
<td>$6.0 \times 10^5$</td>
<td>1</td>
<td>S. aureus</td>
<td>Unhealed</td>
</tr>
<tr>
<td>304</td>
<td>Wound deterioration</td>
<td>$4.6 \times 10^4$</td>
<td>2</td>
<td>Proteobacteria</td>
<td>Lost to follow-up, unhealed</td>
</tr>
<tr>
<td>165</td>
<td>Amputation</td>
<td>$5.6 \times 10^5$</td>
<td>7</td>
<td>MRSA</td>
<td>Amputation</td>
</tr>
</tbody>
</table>
specimens must be obtained (1), while another suggests that validated swab specimens can be used (25). Cultures of tissue are impractical in many clinics because obtaining viable wound tissue suitable for culture is too invasive. Therefore, 54% of clinicians obtain swab specimens when assessing wound bioburden (33). We previously found swab cultures obtained using Levine’s technique—a technique that expresses wound fluid from deep tissue layers—accurately measures all three dimensions of bioburden (i.e., microbial load, microbial diversity, and presence of pathogens) when compared with cultures of wound tissue (34). Specifically, the accuracy of swab cultures for measuring microbial load was 0.80 compared with biopsy; microbial diversity based on swabs was 3.0 species/wound compared with 3.1 species/wound based on biopsy cultures; and the concordance in identifying potential pathogens was 96% for S. aureus, 99% for β-hemolytic Streptococcus, and 96% for P. aeruginosa.

A third limitation is that we did not consider how DFU outcomes might be influenced by biofilms. Biofilms are polymicrobial communities enclosed in a polysaccharide matrix that is secreted by the bacteria. This environment permits bacteria-to-bacteria signaling (i.e., quorum sensing) and a synergistic regulation of virulence factors that renders the bacteria in biofilms more resistant to host defenses and antimicrobial treatment (35). Increasingly, biofilms are believed to play an important role in wound chronicity. Measuring biofilms, however, is very complex and requires epifluorescence microscopy and assays of quorum-sensing signal molecules (35), techniques not widely available in clinical settings. Moreover, prospective studies have yet to define the relationship between biofilms and DFU outcomes (35).

Finally, we did not measure wound bioburden at times of ulcer deterioration. This might be problematic because the microbial milieu of a chronic wound typically evolves over time, in response to environmental pressure. Depending on the virulence of new inhabitants and their interactions within the microbial community, the wound may fail to heal altogether. Therefore, measuring bioburden over time may provide better insight than a single baseline assessment. Future studies of the association between bioburden and DFU outcomes should therefore use longitudinal designs.

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**Author Contributions.** S.E.G. designed the study, supervised study protocols, assisted with data analyses, and wrote the manuscript. A.H. wrote parts of the manuscript. Y.-L.J. collected research data, wrote parts of the manuscript, and conducted parts of data analysis. S.L.H. conducted data analyses and wrote parts of the manuscript. J.E.F. and P.P. enrolled subjects and managed subject care. K.P.H. completed all microbiological analyses. S.M.L. coordinated study protocols and edited the manuscript. C.L.F. managed study data and coordinated study protocols and edited the manuscript. Y.-L.J. wrote parts of the manuscript. S.M.L., C.L.F., and A.H. wrote parts of the manuscript. Y.-L.J. and S.M.L. edited the manuscript. S.E.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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