

Examining diabetic heel ulcers through an ecological lens: microbial community dynamics associated with healing and infection

Tim J. Sloan^{1,2,3*}, James C. Turton^{1,2}, Jess Tyson¹, Alison Musgrove⁴, Vicki M. Fleming², Michelle M. Lister², Matthew W. Loose¹, R. Elizabeth Sockett¹, Mathew Diggle², Frances L. Game^{4,5}, William Jeffcoate⁴

¹ School of Life Sciences, University of Nottingham, Nottingham, UK.

² Department of Clinical Microbiology, Queen's Medical Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK.

³ Path Links Pathology, Northern Lincolnshire and Goole NHS Foundation Trust, Lincolnshire, UK.

⁴ Foot Ulcer Trials Unit, Department of Diabetes and Endocrinology, Nottingham University Hospitals NHS Trust, Nottingham, UK.

⁵ Department of Diabetes and Endocrinology, Derby Teaching Hospitals NHS Foundation Trust, Derby, UK.

* Corresponding author

Email: tim.sloan@nottingham.ac.uk

Keywords: diabetic foot ulcers, chronic wounds, microbiome, wound healing, polymicrobial interactions

Repositories: Sequences are available at the European Nucleotide Archive www.ebi.ac.uk/ena under study accession reference PRJEB28661.

Subject category: Microbial Ecology and Health

Word count (main body): 5300

1 **Abstract**

2 **Purpose:** While some microorganisms, such as *Staphylococcus aureus*, are clearly
3 implicated in causing tissue damage in diabetic foot ulcers (DFUs), our knowledge of
4 the contribution of the entire microbiome to clinical outcomes is limited. We profiled
5 the microbiome of a longitudinal sample series of 28 people with diabetes and DFUs
6 of the heel in an attempt to better characterise the relationship between healing,
7 infection and the microbiome.

8

9 **Methodology:** 237 samples were analysed from 28 DFUs, collected at fortnightly
10 intervals for six months or until healing. Microbiome profiles were generated by 16S
11 rRNA analysis, supplemented by targeted nanopore sequencing.

12

13 **Results/Key findings:** DFUs which failed to heal during the study period (20/28,
14 71.4%) were more likely to be persistently colonised with a heterogeneous community
15 of microorganisms including anaerobes and *Enterobacteriaceae* (log-likelihood ratio
16 9.56, $p=0.008$). During clinically apparent infection, a reduction in the diversity of
17 microorganisms in a DFU was often observed due to expansion of one or two taxa,
18 with recovery in diversity at resolution. Modelling of the predicted species interactions
19 in a single DFU with high diversity indicated that networks of metabolic interactions
20 may exist that contribute to the formation of stable communities.

21

22 **Conclusion:** Longitudinal profiling is an essential tool for improving our understanding
23 of the microbiology of chronic wounds, as community dynamics associated with

24 clinical events can only be identified by examining changes over multiple time points.
25 The development of complex communities, particularly involving *Enterobacteriaceae*
26 and strict anaerobes, may be contributing to poor outcomes in DFUs and requires
27 further investigation.

28 Introduction

29 It is generally acknowledged that diabetic foot ulcers (DFUs) present a considerable
30 clinical and economic burden (1, 2). They have multiple and various overlapping
31 causes, including peripheral artery disease (PAD) and different modalities of
32 neuropathy. As intact skin provides a natural barrier, ulceration and exposure of
33 nutrient-rich tissues to the surface predisposes to colonisation with a wide array of
34 microbes. Clinically apparent infection with known pathogens, such as
35 *Staphylococcus aureus*, is recognised as an important cause of deterioration of pre-
36 existing DFUs and delayed healing (3, 4) and best practice is to sample DFUs with
37 clinical signs of infection to identify them (5). However, little is known of interactions
38 which may occur within a diverse community of micro-organisms (and/or with the host)
39 which could result in delayed healing, subclinical tissue damage or predisposition to
40 clinical infection.

41

42 Advances in high throughput next generation sequencing (NGS) has enabled the
43 molecular characterisation of entire microbial communities from any particular site,
44 termed the microbiome. Profiling of marker genes, such as the 16S ribosomal RNA
45 gene, or sequencing all the genetic material present in a sample provides an overview
46 of the microbiome - including microorganisms which may be difficult to culture or be
47 present in very low numbers (6). These large datasets can be analysed with data-
48 mining techniques to uncover relationships between the presence of various groups
49 of microorganisms and clinical variables. In a cross-sectional study of soft tissue
50 samples from 40 people with DFUs, Dowd (7) described a broader spread of taxa than
51 previously identified by culture based methods, while in a more recent study of 52

52 surface swabs from non-infected plantar neuropathic DFUs, Gardner and colleagues
53 (8) reported clustering of identified bacterial taxa into three broad groups,
54 demonstrating significant associations between bacterial groups and DFU depth, area
55 and overall quality of glycaemic control. Further insight was provided by a study of 100
56 subjects with DFUs from whom repeated samples were obtained in a subpopulation
57 (9). The authors described four different types of bacterial community based on the
58 dominant population of identified bacteria and also studied the extent to which these
59 populations changed. They suggested that more frequent change in community types
60 was a feature of those that healed within 12 weeks of observation.

61

62 A deeper understanding of polymicrobial interactions and impact on host tissues could
63 inform risk stratification tools for DFUs and facilitate the design of targeted
64 interventions aimed at altering the colonising microbiota such as phage therapy. In the
65 setting of infection, this may also enable more selective use of antibiotics where
66 clinically appropriate to reduce broad spectrum antibiotic exposure. Sequencing costs,
67 technical limitations and analytical barriers are constraining immediate application of
68 microbiome profiling to the management of DFUs. However, developments in
69 sequencing technologies, particularly those with potential point of care applications
70 such as Oxford nanopore sequencing, promise to minimise these limitations in the
71 foreseeable future (10).

72

73 The data presented here were collected as a sub-study of a clinical trial investigating
74 the effectiveness of a simple off-loading device in patients with DFUs affecting the
75 heel (11). Swab samples were taken at the time of recruitment and at fortnightly

76 intervals up to a maximum of 24 weeks until DFU healing or withdrawal from the parent
77 study. Associations were sought between clinical measures and the microbiota, both
78 at baseline and over the course of the study. Data available from the parent study
79 provided baseline details of the participants as well as clinical information relating to
80 DFU status at each study visit.

81

82 **Methods**

83 Participants in the parent trial were people with diabetes complicated by DFUs on the
84 heel and were randomised to management either with standard good care plus
85 lightweight fiberglass heel casts or with standard good care alone, and were reviewed
86 in a specialist clinical research service every two weeks. The results of this parent
87 study have been reported in full elsewhere (11). The present study was approved by
88 Derby Research Ethics Committee (IRAS ID 137934). Participating centres were
89 selected from those that were recruiting well to the parent study and which agreed to
90 undertake the additional sampling. All participating patients provided additional
91 informed consent. Since no difference in any outcome measure was observed
92 between the intervention and the usual care arms of the parent study, samples from
93 patients in both groups were combined for the present analyses.

94

95 **Details of sampling and sample handling**

96 Samples were obtained using flock swabs and the Z technique after cleansing and
97 sharp debridement of the wound surface. Samples were identified only by study centre
98 and study number. Each swab tip was retained in the collection tube and submerged
99 in a PBS-based buffer solution (MoBio Powersoil Collection Fluid, CARLSBAD, CA)
100 to maintain constant pH. For transport, the samples were packed in a protective plastic
101 casing with a cold pack, boxed and sealed in a prepaid envelope and posted first class
102 within 24 hours to a central microbiology laboratory at Nottingham University Hospitals
103 NHS Trust. Samples were subsequently stored at -80 °C until extraction.

104

105 **DNA extraction**

106 DNA was extracted from the whole sample using the MoBio Powersoil Kit (since
107 renamed to DNeasy PowerSoil Kit) as per manufacturer's guidelines with a pre
108 extraction homogenisation step (4500 rpm, 45 sec x 3). DNA was stored at -80 °C to
109 await subsequent analysis. Obtained DNA was quantified using the Quant-iT™
110 PicoGreen™ dsDNA Assay Kit (Invitrogen, Waltham, MA) and analysed, in duplicate,
111 on the ABI7500 (Applied Biosystems, Ca, USA).

112

113 **16S rRNA gene amplification, library preparation and sequencing**

114 Amplicons were generated for the V4 region of the 16S ribosomal RNA gene using
115 the above extracted DNA as a template. Previously validated primers were used to
116 amplify a 359bp amplicon of the V4 hypervariable region of the prokaryotic 16S
117 Ribosomal RNA gene
118 (515F:TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCG
119 GTAA,
120 806R:GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWT
121 CTAAT) (12). 237 out of 256 samples received were successfully amplified.

122 PCR clean-up was performed using the AMPure XP beads (Beckman Coulter, IN,
123 USA) before proceeding to index PCR, which attached barcodes to individual samples
124 and Illumina sequencing adapters using the Nextera XT Index Kit, as per Illumina (CA,
125 USA) guidelines. Negative controls were included to account for reagent
126 contamination (13). After index PCR, clean-up was repeated as above and amplicons
127 were individually quantified using the Agilent Bioanalyser (CA, USA), prior to
128 normalisation and pooling. The barcoded amplicon pool was run on the Illumina MiSeq

129 using 2x250bp chemistry. Raw .fastq files were output for subsequent bioinformatics
130 analysis. The sequencing data are publicly available in the European Nucleotide
131 Archive (ENA, acc.no. PRJEB28661).

132

133 **16S rRNA sequence pre-processing and taxonomic assignment**

134 DNA sequence data was pre-processed with the *mare* package (14) in R (Foundation
135 for Statistical Computing, Vienna, Austria) as follows: Paired-end reads were merged
136 with quality filtering and trimmed to a uniform length (290 bp). After chimera filtering
137 and clustering with USEARCH (15), reads were assigned to taxonomic labels by
138 alignment with the RDP database (16) using UTX and a confidence level of 0.6. Taxa
139 below this cut off were annotated as unclassified at that taxonomic level and assigned
140 to the next highest available level. Unique reads occurring at a frequency of less than
141 1:100,000 were excluded to avoid OTU inflation. Two halophilic genera *Halomonas*
142 and *Shewanella* were identified from negative controls as possible contaminants and
143 excluded from the analysis.

144

145 **Oxford nanopore library preparation and sequencing**

146 Swab DNA extracts were treated with a NEBNext Microbiome DNA Enrichment Kit
147 (New England Biolabs) to enrich microbial DNA prior to sequencing (17). Library
148 preparation was performed with a Rapid Low Input by PCR Barcoding Kit (SQK-
149 RLB001, Oxford Nanopore Technologies) and libraries sequenced on the MinION
150 platform (Oxford Nanopore Technologies). Base calling was performed with Albacore
151 Sequencing Pipeline Software (version 2.1.2, Oxford Nanopore Technologies) with
152 raw .fastq files output for subsequent analysis.

153

154 **Direct sample sequencing analysis**

155 MinION reads were screened against the human genome reference hg19 (GenBank
156 assembly GCA_000001405.1) using Minimap2 (18) to remove host DNA
157 contamination. The resulting non-human sequences were annotated with Centrifuge
158 1.0.3 (19) using the default database for bacteria and archaea. A minimum hit length
159 of 50 and hit length covering at least 5 % of the read were used to filter the resulting
160 annotations. For detection of antimicrobial resistance genes, all of the non-human
161 reads for the sample were assembled using Canu 1.7 (20). The resulting contiguous
162 sequences were assigned a taxonomic identification by Centrifuge and then input to
163 ResFinder 3.0 (21) searching for acquired antimicrobial genes with a 95 % identity
164 threshold.

165

166 **Statistical and network analysis**

167 Alpha diversity (Inverse Simpson Index, variation within any one sample) and beta
168 diversity (Bray-Curtis Dissimilarity (BCD), variation between samples) were calculated
169 using the vegan package (22) in R (Foundation for Statistical Computing, Vienna), with
170 Principal Coordinates Analysis of BCD and PERMANOVA used for analysis of
171 multivariate microbiota count data. Associations between diversity and clinical
172 categories were tested for using unpaired t-test or ANOVA after assessing for
173 normality with the Shapiro-Wilk method.

174 To enable analysis of changes occurring within individual DFUs over time, samples
175 were assigned to clusters based on taxonomic profile. Sample clusters were created
176 with the default k-means clustering function in R using relative abundances of taxa at

177 family level with 150 maximum iterations and 10 simulated starts. After examining the
178 relationship between variance explained and cluster number, six clusters were chosen
179 to best represent the data (Supplementary Fig. 1).

180 Once samples had been assigned to one of the six clusters, a Markov Model was
181 constructed by counting all cluster transitions over consecutive visits and the counts
182 converted to proportional probabilities by the dividing of total transitions from that
183 cluster. If swab data were unavailable for the subsequent visit, the next available visit
184 was used as the destination cluster. The final cluster for each subject was then used
185 to calculate the probability of transition from each cluster to a healed or unhealed state
186 at study end. Differences in transition probabilities between healed and unhealed
187 DFUs were calculated using a log likelihood ratio test.

188 A metabolic complementarity network was created from the MinION data by using the
189 NetSeed pairwise metabolic complementarity index (MCI) previously calculated by
190 Levy and Borenstein for 154 species found in the human microbiome (23, 24). Briefly,
191 NetSeed requires as input a metabolic network for each species, based on a list of
192 KEGG reactions per genome available from the Integrated Microbial Genomes project
193 (IMG, <http://img.jgi.doe.gov>) and generated as described previously (25). The seed
194 set of the network identified by NetSeed represents exogenously acquired compounds
195 which appear as reaction substrates but not products. The MCI is a pairwise
196 comparison of the proportion of the seed set from one species which are products but
197 not seeds in a partner species network, thereby representing complementary by-
198 products. The most closely related species for which an MCI score was available was
199 identified for each of the most abundant taxa in the DFU. These scores were used to
200 create a network with each species represented by a node, and each pairwise MCI

201 score with a value greater than 0.5 represented by a directed edge towards the
202 species from which benefit is predicted to be derived.

203

204 Results

205 DFU characteristics and microbiota composition

206 Two hundred and thirty-seven samples were analysed from the index DFU from 28
207 individuals, of which eight healed during the study period (Supplementary Table 1).
208 The median (IQR) age of the participants was 70.5 (60-77) years. Twenty (71%) were
209 male and 24 (86%) had Type 2 diabetes. The median (IQR) DFU area at
210 randomisation was 161.5 (69-593) mm².

211 Characterisation of the microbiota of each sample by 16S rRNA sequencing identified
212 63 distinct genera from 37 families, of which the 10 most abundant families accounted
213 for 89.6 % of the overall composition (Supplementary Table 2). The most abundant
214 taxonomic groups were *Corynebacterium* (22.7 %), *Staphylococcus* (15.2 %),
215 unclassified *Enterobacteriaceae* (10.6 %), *Anaerococcus* (6.4 %), *Pseudomonas* (6.1
216 %) and *Streptococcus* (4.6 %). The majority of the variation between the samples
217 could be explained by subject, indicating that the microbiome of any one DFU tended
218 to be distinct from any other (PERMANOVA, 63.7 % variance explained, $p < 0.001$).
219 There was no correlation between baseline DFU area or NPUAP depth score and
220 microbial diversity (alpha diversity, Inverse Simpson Index, ISI).

221 Clustering analysis indicated that samples could be separated into six distinct groups
222 based on broad compositional differences, with one or two highly abundant taxa
223 characterising each cluster (Supplementary Fig. 2). Clusters were assigned a letter
224 based on dominant taxa; cluster 1 ($n = 20$, *Pseudomonaceae* 58.1 % – P), cluster 3
225 ($n = 49$, *Corynebacteriaceae* 68.3 % – C), cluster 4 ($n = 17$, *Micrococcaceae* 87.2 %
226 – M) and cluster 6 ($n = 34$, *Staphylococcaceae* 74.6 % - S) were relatively
227 homogenous while cluster 2 ($n = 61$, *Enterobacteriaceae* 33.4 % – E) and cluster 5 (n

228 = 56, *Clostridiales Incertae Sedis XI* 38.2 % and other anaerobes ~ 20 % - A) exhibited
229 greater taxonomic diversity.

230

231 **Variations in microbial community dynamics between healing and non-healing** 232 **DFUs**

233 We sought to investigate whether the microbiota of non-healing DFUs were different
234 to healing DFUs in both overall composition and changes over time.

235 Of all the 51 samples taken from the 8 DFUs which healed, the majority of samples
236 fell into either the C (17/51, 33.3 %) or S (11/51, 21.6 %) clusters while of the 186
237 samples taken from 28 unhealed DFUs, the E (52/186, 28.0 %) and A (48/186, 25.8
238 %) clusters were most frequently represented. Mean baseline alpha diversity in
239 healing DFUs was 2.3 (+/- 0.9) compared to 2.9 (+/- 1.5) for those that did not heal,
240 but this difference was not statistically significant ($p = 0.19$).

241 For each DFU, the transition in the microbiota between clusters over subsequent visits
242 was used to develop a Markov model (Supplementary Fig. 3 and Supplementary Table
243 3). The microbiota of individual DFUs tended to remain within the same cluster over
244 subsequent visits with probabilities ranging from 53 % of samples in the S cluster to
245 71 % in the M cluster. No transitions were observed between the S and P cluster while
246 transitions frequently occurred from the A to E cluster (Probability = 20 %) although
247 less frequently from E to A (8 %).

248 Specific features of healing and non-healing DFUs were identified from the changes
249 in the microbiota profiles over time. Non-healing DFUs with profiles of either E or A
250 were less likely to transition away from these to other clusters than healing DFUs
251 (probably per visit; non-healing 21 % vs healing 59 %, log likelihood ratio 9.56, $p =$

252 0.008). In DFUs which healed, high rates of transition were observed from E towards
253 several clusters, particularly S (33 %) and C (22 %) so that in the final visit before
254 healing, 75 % healed DFUs had a microbiota profile of either S (3/8) or C (3/8) with
255 only 1 each in the E and P clusters and none in either M or A.

256 Individual profiles from DFUs which were consistent with these overall trends are
257 illustrated in Fig. 1. In Fig. 1(a), the DFU was persistently colonised by a high
258 proportion of *Corynebacterium* and *Staphylococcus* with two episodes of infection
259 treated with co-amoxiclav before finally healing. Fig. 1(b) is an example of one of two
260 DFUs where 3 consecutive samples fell into the E or A clusters with high proportions
261 of anaerobes and *Enterobacteriaceae* observed, accompanied by visible slough,
262 before emergence of *Corynebacterium* and *Staphylococcus* immediately prior to
263 healing. In the profile displayed in Fig. 1(c), *Enterobacteriaceae* and anaerobes are
264 highly abundant during several visits before gradual replacement with *Pseudomonas*
265 pre-healing. Fig. 1(d) is an example of a non-healing DFU where various anaerobes
266 and *Enterobacteriaceae* are present throughout.

267

268 **Changes in the microbiota associated with infection and antibiotic therapy**

269 Forty-six percent (13/28) of subjects had at least one episode of clinical infection, with
270 a total of 20 discrete episodes of infection identified covering 44 visits.

271 Infection and antibiotic use often co-occurred such that their relative associations with
272 sample diversity could not be readily distinguished. Overall, samples from infected
273 DFUs exhibited lower microbial diversity than those from uninfected DFU (infected 2.2
274 +/- 0.9 vs uninfected 3.1 +/- 1.5, $p = 1 \times 10^{-6}$), while diversity also appeared to be lower
275 for visits where subjects were receiving antibiotic therapy (antibiotics 2.4 +/- 1.2 vs no

276 antibiotics 3.1 +/- 1.5, p = 0.001). Analysing the contribution of both variables indicated
277 that infection was likely to be a greater contributor to the fall in diversity (ANOVA, F =
278 14.0, p < 0.001) than antibiotic exposure (F = 2.1, p = 0.15).

279 To further investigate the changes in microbial diversity associated with individual
280 episodes of infection, samples from visits occurring either immediately preceding,
281 during and following an episode of clinical infection were aligned and the microbial
282 diversity plotted. This demonstrated a reduction in microbial diversity at the onset of
283 infection with a corresponding recovery in diversity beginning from the final visit where
284 infection was noted clinically (Fig. 2).

285 Across all samples taken from infected DFUs, cluster E (24/44, 54.5 %) and C (11/44,
286 25.0 %) were the most prevalent overall, and this was the same for the first visit of
287 each episode of infection (cluster E: 10/20, 50.0 %; cluster C: 4/20, 20.0 %). Despite
288 the recognised importance of *Staphylococcus aureus* as a pathogen in this context,
289 only 1 DFU had a high proportion of *Staphylococcus* at infection onset, falling into the
290 S cluster. There were 3 and 2 episodes of infection beginning with profiles in the P
291 and A clusters, respectively.

292 Further individual profiles demonstrating temporal changes in the microbiota
293 associated with episodes of clinical infection are shown in Fig. 3. In the DFU profile
294 shown in Fig. 3(a), colonisation at baseline with a mixture of *Kocuria* and other
295 *Micrococcaceae* was observed with an initial alpha diversity index of 1.96. At visit 7,
296 this fell to 1.15 following the emergence and expansion of staphylococci within the
297 DFU, associated with an episode of clinical infection. After commencement of anti-
298 staphylococcal antibiotic therapy (flucloxacillin), the profile reverted to the taxa which
299 were originally dominant.

300 Several episodes of infection occurred in the DFU shown in Fig. 3(b) which had a high
301 baseline diversity index of 4.79, falling to 1.37 at visit 5 due to an expansion of
302 *Streptococci*, with associated clinical infection from visits 3 to 6. Recovery of microbial
303 diversity to 4.6 at visit 7 with emergence of *Enterobacteriaceae* and later
304 *Pseudomonas* were also observed.

305 In Fig. 3(c), a DFU colonised with a high proportion of anaerobes over successive
306 visits from 4 to 6 with high diversity (visit 6: 7.81) underwent a dramatic reduction in
307 diversity during an episode of clinical infection at visit 10 (2.58) accompanied by an
308 expansion of *Anaerococcus* and *Peptonophilus*. After a course of co-amoxiclav, the
309 anaerobes were no longer abundant at visit 11, having been replaced by
310 *Enterobacteriaceae*.

311 The profile shown in Fig. 3(d) shows a DFU colonised by *Enterobacteriaceae*,
312 *Pseudomonas* and *Corynebacterium*. Onset of infection at visit 8 and empirical
313 treatment with doxycycline was followed later by expansion of *Streptococci* from visit
314 8 to 9. After a second course of ciprofloxacin and metronidazole, diversity was
315 markedly reduced from 3.8 at visit 9 to 1.1 at visit 11 as *Corynebacterium* replaced the
316 other taxa to become the dominant organism in the DFU.

317 In addition to the cases described above, abrupt changes were also noted in certain
318 DFUs without any obvious cause such as antibiotic exposure or clinical infection. In
319 other cases the clinical diagnosis of infection was documented but there were no
320 obvious associated changes in the microbiota (Supplementary Fig. 4).

321

322 **Direct sample sequencing identifies individual species and resistance genes**

323 Short-read sequencing of 16S rRNA amplicons enables high throughput community
324 profiling but is limited by taxonomic resolution and does not provide any additional
325 information about the genetic capability of a community. To complement the 16S
326 profiles, we conducted longer read sequencing using the Oxford Nanopore MinION on
327 the swab DNA extracts for the profile shown in Fig. 3(b). This profile was chosen as it
328 was one of the most diverse profiles containing a mixture of taxa including
329 *Streptococci*, *Enterobacteriaceae*, *Pseudomonas* and several strict anaerobes.

330 Seven samples were sequenced from alternate visits (1, 3, 5, 7, 9, 11, and 13). Despite
331 a microbiome enrichment step, only 3 samples yielded > 1000 reads once host DNA
332 sequences had been discarded (V3 16900, V5 2353, V9 2127). After performing
333 sequence annotation with the taxonomic classifier, Centrifuge, (19) and applying strict
334 cut-offs to the output to optimise the accuracy of taxonomic identification, only a small
335 proportion of reads were eventually assigned to a bacterial species (V3 1548, V5 426,
336 V9 172). Taxa identified from V3 compared well to the 16S profile (Fig. 4a). While read
337 numbers were low for V5 and V9, the species identified were also consistent with the
338 16S results, including *Streptococcus anginosus* (V5, V9), *Escherichia coli* (V5, V9),
339 *Enterococcus faecalis* (V5), *Staphylococcus aureus* (V5), *Bacteroides fragilis* (V9) and
340 *Porphyromonas asaccharolytica* (V9).

341 Analysing the V3 sample for the presence of antimicrobial resistance genes using the
342 ResFinder tool (21) identified *tet*, *erm* and *cfx* genes. Assembling the sequences from
343 this sample and performing taxonomic annotation on the resulting assembly enabled
344 the matching of *tetM*, *ermA* and *ermB* to *Streptococcus anginosus* and *cxmA* and *tetQ*
345 to *Prevotella intermedia* based on the presence of these genes in continuous DNA
346 sequence with a high quality taxonomic assignment.

347 Finally, using the more detailed taxonomic profile created from this DFU, we compiled
348 a network of potential inter-species interactions based on a metabolic complementarity
349 index (MCI) previously calculated by Levy *et al.* (23) using the NetSeed algorithm (24)
350 (Fig. 4b). Higher scores on the MCI indicate a species is predicted to derive metabolic
351 benefit from the metabolic by-products of the partner species. The strict anaerobes,
352 particularly *Porphyromonas asaccharolytica*, are predicted to derive benefit from a
353 number of other species, particularly *E. coli* and *P. aeruginosa*, whereas *E. coli* is
354 predicted to confer benefit on all the strict anaerobes, *E. faecalis* and *S. anginosus*,
355 but may not itself derive much metabolic benefit from the presence of these species.

356

357 Discussion

358 We have characterised the variation in the microbiota of 28 DFUs using a high-
359 resolution longitudinal sampling approach over a 6 month period, or until DFU healing.
360 Successive longitudinal sampling is particularly valuable in this context as we were
361 able to observe changes in the DFU microbiota occurring over short time frames and
362 in conjunction with clinical events such as impending healing, infection or antibiotic
363 administration.

364

365 The most abundant taxa identified in this study were similar to those found in other
366 molecular studies of DFUs, although the proportions vary between studies (8, 9, 26).
367 We observed a dominance of Gram positive organisms including *Corynebacteria* (22.7
368 %), *Staphylococci* (15.2 %) and anaerobic members of the families *Clostridiales*
369 *Incertae Sedis X* (total 13.4 %, including *Anaerococcus*, *Finegoldia* and *Peptinophilus*)
370 and *Micrococcaceae* (7.9 %), with a lower abundance of Gram negative organisms;
371 predominantly the family *Enterobacteriaceae* (11.5 %) and *Pseudomonas* (6.1 %). By
372 comparison, Wolcott *et al.* (26) identified *Staphylococcus aureus* (15.0 %) and
373 *Staphylococcus epidermidis* (10.7 %) as the most abundant species in 910 DFUs of
374 all types using 16S amplicon pyrosequencing with high proportions of *Pseudomonas*
375 *aeruginosa*, *Corynebacterium spp.*, *Enterococcus spp.*, *Finegoldia magna* and
376 *Anaerococcus vaginalis* also present. The proportions observed here are likely to
377 reflect the patient group and DFU type selected for the parent study.

378

379 There are likely to be a large number of factors which are responsible for shaping the
380 microbiota of an individual DFU. These will include bacteria already present on intact
381 skin and elsewhere in the body prior to ulceration, as well as the microenvironment
382 influenced by oxygen tension, glycaemic control, chronicity of the wound and historical
383 antibiotic exposure (8, 27, 28). This would explain why samples from any one DFU
384 tended to be similar to each other, but often varied greatly in composition to other
385 DFUs. By contrast, the gut microbiota are heavily influenced by dietary intake and
386 tends to be composed of a core group of taxa in individuals who share a similar diet
387 (29).

388

389 To overcome the challenge of comparing a heterogeneous group of samples from a
390 limited DFU population, we used a clustering approach similar to that employed in
391 other molecular studies of DFUs (8, 9). Clustering at family level facilitated the
392 grouping of organisms with overlapping genomic content and growth requirements
393 such as the variety of closely related anaerobes within the *Clostridiales Incertae Sedis*
394 X. The resulting six clusters were relatively homogeneous, consistent with the most
395 abundant taxa identified and captured a significant proportion of the overall variation
396 in the data. Repeat sampling from individual DFUs yielded profiles from the same
397 cluster on at least 50 % of visits, consistent with the observation that individual DFUs
398 tended to have unique profiles and retained the same combinations of taxa over time.

399

400 Impaired healing of DFUs is influenced by a variety of factors notably ischaemia and
401 neuropathy, but also subtle cellular deficits, particularly impaired leucocyte function
402 and alterations in the normal inflammatory response (4, 30-32). There is a growing

403 body of evidence that the composition of the chronic wound microbiome may also
404 have an impact, but the possible mechanisms for this are not well understood. In a
405 murine wound model comparing wild-type to diabetic mice, Grice *et al.* (33) observed
406 delayed healing in diabetic mice with differences in gene expression associated with
407 the immune response, and increased abundance of *Staphylococcus*, *Aerococcus* and
408 taxa within the *Enterobacteriaceae* and *Porphyromonadaceae*. In a compelling study,
409 Wolcott *et al.* (34) sampled the microbiota from 43 chronic wounds and observed that
410 83% of these interfered with healing when transplanted into a murine chronic wound
411 model.

412

413 In this study, we observed a tendency towards persistent colonisation with a
414 heterogeneous population of bacteria including *Enterobacteriaceae* and several
415 groups of Gram positive and Gram negative anaerobes in DFUs which failed to heal.
416 DFUs which healed tended to be colonised with high proportions of Gram positive
417 aerobes, particularly staphylococci and corynebacteria, prior to healing. An obvious
418 explanation for these findings is that the microbiota are simply determined by the DFU
419 environment; chronic non-healing wounds promoting the growth of organisms
420 preferring moist sites while DFUs which have almost healed supporting organisms
421 commonly found on intact skin. The alternative possibility that must also be considered
422 however, particularly in light of other studies in this area, is that colonisation with
423 certain combinations of microbes may itself be a contributor to delayed healing. In a
424 murine chronic wound model, Dalton *et al.* (35) found that a polymicrobial mixture of
425 Gram positive and Gram negative aerobes and anaerobes was associated with
426 delayed healing compared to wounds colonised with a single organism. Loesche *et al.*
427 (9) also studied longitudinal profiles of the microbiota in DFUs and while they did not

428 identify specific groups of bacteria associated with poor healing, they also observed
429 that non-healing DFUs tended to have a stable microbiome with less variation over
430 time than healing DFUs.

431

432 In addition to environmental factors, competition for nutrients and metabolic synergy
433 between microbes is another key determinant of community composition (36).
434 Analysing the predicted metabolic overlap between species co-occurring in one DFU
435 in this study indicated that strict anaerobes are the likely principal beneficiaries of such
436 metabolic overlap. Anaerobes such as *Porphyromonas* benefit from poor tissue
437 oxygenation in diabetes and possibly also increased levels of metabolic substrates
438 such as alpha-ketoglutarate (37, 38). They may also benefit from micro-anaerobic
439 climates created by other bacteria lowering redox potential, forming surface layers
440 including biofilm and exchange of metabolic by-products. Studies of the microbiome
441 in periodontal disease have already demonstrated the importance of groups of
442 organisms, including anaerobes, working together to impair the host response,
443 degrade connective tissue and promote chronic inflammatory states (39).

444

445 Diversity of individual samples tended to be lower during episodes of clinical infection
446 and on antibiotic therapy. In some instances this appeared to indicate the proliferation
447 of potentially pathogenic organisms which may have resulted in the clinical symptoms
448 observed. Alternatively, it could be that inflamed wounds represent a changed local
449 environment in which certain bacteria are able to flourish while others are not. Certain
450 bacteria, such as *E. coli*, are able to use inflammatory mediators, for example nitric
451 oxide, as metabolic substrates potentially giving them a survival advantage in pro-

452 inflammatory environments (40). Interactions between microbes may result in the
453 promotion or suppression of certain species with virulence potential. Another possible
454 mechanism by which community dynamics may influence the onset of infection is
455 through modulation of virulence potential by inter-species interaction. Ramsey *et al.*
456 observed that *Corynebacterium striatum* was capable of interfering with quorum
457 sensing in *Staphylococcus aureus* with the effect of suppressing virulence factor
458 production (41). While high proportions of staphylococci were only observed in 1 in 20
459 cases of developing infection, it is possible that proliferation of other species may have
460 altered virulence factor expression by less abundant pathogens such as
461 *Staphylococcus aureus*, resulting in tissue damage, inflammation and clinically
462 apparent infection.

463

464 16S rRNA amplicon sequencing enables the identification of organisms that are
465 difficult to isolate with conventional culture, while giving an estimate of the proportion
466 of bacterial taxa present in the sample. Despite potential high throughput and low cost,
467 the principal limitation of short-read sequencing is that taxa cannot be reliably
468 identified to the lowest taxonomic levels, particularly species but also genus in some
469 cases. As higher level taxonomic groupings, such as the family *Enterobacteriaceae*,
470 can contain a wide range of organisms with greatly differing virulence potential, this is
471 a significant drawback. Nonetheless, this form of molecular survey is capable of
472 yielding important insights into the bacterial community dynamics of a DFU and how
473 this relates to clinical events, more so than phenotypic testing. Microbiota profiles were
474 often similar over multiple visits, both in the most abundant taxa identified and their
475 relative proportions. This suggests that the approaches used for sampling, extraction,
476 amplification and sequencing were likely to be relatively consistent between samples.

477

478 To complement the 16S analysis, we also analysed swab DNA extracts directly with
479 Oxford nanopore sequencing technology, which is the first time to our knowledge that
480 this has been applied to diabetic wounds. Sequencing the total pool of the DNA in a
481 sample enabled greater taxonomic resolution and identification of antibiotic resistance
482 genes. The amount of information which could be determined from a sample appeared
483 to be directly proportional to the ratio of host to bacterial DNA recovered, as any host
484 DNA sequenced reduced bacterial read depth. The high proportion of host DNA in
485 many of the samples limited a comprehensive characterisation of the microbiota.
486 Technical challenges to be overcome include the optimisation of sample extraction
487 methods and reduction in the proportion of host DNA, however the capacity for rapid
488 sequencing is an appealing prospect for diagnostic utility.

489

490 Given the longitudinal study design and need for fortnightly sampling, ulcer base
491 swabs were chosen over tissue sampling. Aside from drawbacks of the sequencing
492 technologies used, this is another potential study limitation as surface swabs may not
493 detect the invasion and proliferation of bacteria in tissue layers during infection. In a
494 cross sectional study of wound swab versus tissue sampling, Nelson *et al.* detected a
495 higher proportion of tissue samples containing at least one recognised pathogen (86.1
496 %) vs. swabs (70.1 %) with fewer less pathogenic organisms in tissue (42). Host DNA
497 proportions are likely to be even higher in tissue samples than from swabs, however
498 longitudinal microbiota characterisation from tissue samples could potentially give
499 clearer insight into the dynamics of infection if these considerable practical and
500 technical difficulties can be overcome.

501

502 This study has yielded important information of the microbiology of difficult to heal
503 DFUs of the heel, including differences in microbial diversity and changes over time in
504 infected, healing and non-healing DFUs. While heel ulcers are a subtype of DFUs,
505 they represent a wide range of DFU types from the small and superficial to the deep
506 and anaerobic. We anticipate that building on this and similar studies by analysing a
507 greater number and employing a wide range of sequencing strategies, including full
508 metagenomics and transcriptional analysis, will add to our understanding of the
509 mechanisms underlying non-healing DFUs with implications for future basic and
510 translational research studies in this field.

511

512 **Author statements**

513 **Author contributions**

514 Conceptualisation, TJS, JCT, MD, VMF, FLG, WJ; Methodology, TJS, JCT, VMF;
515 Investigation, TJS, JCT, VMF, JT, AM, MWL; Visualization, TJS; Writing – Original
516 Draft, TJS, WJ; Writing – Review & Editing – TJS, JCT, VMF, JT, MML, MD, FLG, WJ;
517 Funding Acquisition, RES, MD, FLG, WJ; Resources, JCT, AM, VMF, MML, MWL,
518 RES, MD, FLG, WJ; Supervision, RES, MD, FLG, WJ.

519 **Funding information**

520 The parent trial ISRCTN62524796 ‘Evaluation of lightweight fibreglass heel casts in
521 the management of ulcers of the heel in diabetes’ was funded by the National Institute
522 for Health Research Health Technology Assessment 09/01/53.

523 This study was funded by research funds held by the Foot Ulcer Trials Unit and
524 Department of Clinical Microbiology at Nottingham University Hospitals NHS Trust.

525 **Acknowledgements**

526 The support of the parent study team, including the Nottingham Clinical Trials Unit,
527 was essential in facilitating this study. In particular, Mara Sprengel and Vivienne Turtle-
528 Savage facilitated the matching of data from the clinical trial with sample data. Illumina
529 and Oxford Nanopore Sequencing was carried out by Deep Seq, School of Life
530 Sciences, University of Nottingham. Discussions with Dr. Bonnie Hurwitz, University
531 of Arizona, were also invaluable in refining the Markov model and other data

532 visualisations. We also thank the clinical sites which collected and promptly delivered
533 the swab samples.

534 **Conflicts of interest**

535 The authors report no conflicts of interest.

536 **Ethical statements**

537 All participants gave written, informed consent for inclusion in the parent trial, and for
538 molecular analysis to be performed on DFU swabs. Researchers working on sample
539 analysis were given participant data in pseudo-anonymised form by study identifier.
540 Any sequences identified as human from direct sample sequencing were counted and
541 discarded prior to downstream analysis.

542

References

- 544 1. Boulton AJ, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. The global burden of
545 diabetic foot disease. *Lancet*. 2005;366(9498):1719-24.
- 546 2. Kerr M. Diabetic footcare in England, An economic case study. *Diabetes UK*. 2017
547 [Available from:
548 [https://www.diabetes.org.uk/Upload/Shared%20practice/Diabetic%20footcare%20in%20En](https://www.diabetes.org.uk/Upload/Shared%20practice/Diabetic%20footcare%20in%20England,%20An%20economic%20case%20study%20(January%202017).pdf)
549 [gland,%20An%20economic%20case%20study%20\(January%202017\).pdf](https://www.diabetes.org.uk/Upload/Shared%20practice/Diabetic%20footcare%20in%20England,%20An%20economic%20case%20study%20(January%202017).pdf)
- 550 3. Prompers L, Schaper N, Apelqvist J, Edmonds M, Jude E, Mauricio D, et al. Prediction
551 of outcome in individuals with diabetic foot ulcers: focus on the differences between
552 individuals with and without peripheral arterial disease. The EURODIALE Study.
553 *Diabetologia*. 2008;51(5):747-55.
- 554 4. Ince P, Abbas ZG, Lutale JK, Basit A, Ali SM, Chohan F, et al. Use of the SINBAD
555 classification system and score in comparing outcome of foot ulcer management on three
556 continents. *Diabetes Care*. 2008;31(5):964-7.
- 557 5. Lipsky BA, Aragon-Sanchez J, Diggle M, Embil J, Kono S, Lavery L, et al. IWGDF
558 guidance on the diagnosis and management of foot infections in persons with diabetes.
559 *Diabetes Metab Res Rev*. 2016;32 Suppl 1:45-74.
- 560 6. Mizrahi-Man O, Davenport ER, Gilad Y. Taxonomic classification of bacterial 16S
561 rRNA genes using short sequencing reads: evaluation of effective study designs. *PLoS One*.
562 2013;8(1):e53608.
- 563 7. Dowd SE, Wolcott RD, Sun Y, McKeenan T, Smith E, Rhoads D. Polymicrobial nature
564 of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX
565 amplicon pyrosequencing (bTEFAP). *PLoS One*. 2008;3(10):e3326.
- 566 8. Gardner SE, Hillis SL, Heilmann K, Segre JA, Grice EA. The neuropathic diabetic foot
567 ulcer microbiome is associated with clinical factors. *Diabetes*. 2013;62(3):923-30.
- 568 9. Loesche M, Gardner SE, Kalan L, Horwinski J, Zheng Q, Hodkinson BP, et al. Temporal
569 Stability in Chronic Wound Microbiota Is Associated With Poor Healing. *J Invest Dermatol*.
570 2017;137(1):237-44.
- 571 10. Jain M, Olsen HE, Paten B, Akeson M. The Oxford Nanopore MinION: delivery of
572 nanopore sequencing to the genomics community. *Genome Biol*. 2016;17(1):239.
- 573 11. Jeffcoate W, Game F, Turtle-Savage V, Musgrove A, Price P, Tan W, et al. Evaluation
574 of the effectiveness and cost-effectiveness of lightweight fibreglass heel casts in the
575 management of ulcers of the heel in diabetes: a randomised controlled trial. *Health Technol*
576 *Assess*. 2017;21(34):1-92.
- 577 12. Gilbert JA, Field D, Swift P, Thomas S, Cummings D, Temperton B, et al. The
578 taxonomic and functional diversity of microbes at a temperate coastal site: a 'multi-omic'
579 study of seasonal and diel temporal variation. *PLoS One*. 2010;5(11):e15545.
- 580 13. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and
581 laboratory contamination can critically impact sequence-based microbiome analyses. *BMC*
582 *Biol*. 2014;12:87.
- 583 14. Korpela K. mare: Microbiota Analysis in R Easily. R package version 1.0. Zenodo.
584 <http://doi.org/10.5281/zenodo.50310> 2016.
- 585 15. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads.
586 *Nat Methods*. 2013;10(10):996-8.

- 587 16. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database
588 Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.*
589 2014;42(Database issue):D633-42.
- 590 17. Feehery GR, Yigit E, Oyola SO, Langhorst BW, Schmidt VT, Stewart FJ, et al. A method
591 for selectively enriching microbial DNA from contaminating vertebrate host DNA. *PLoS One.*
592 2013;8(10):e76096.
- 593 18. Li H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics.* 2018.
- 594 19. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: rapid and sensitive
595 classification of metagenomic sequences. *Genome Res.* 2016;26(12):1721-9.
- 596 20. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable
597 and accurate long-read assembly via adaptive k-mer weighting and repeat separation.
598 *Genome Res.* 2017;27(5):722-36.
- 599 21. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al.
600 Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother.*
601 2012;67(11):2640-4.
- 602 22. Dixon P. VEGAN, a package of R functions for community ecology. *Journal of*
603 *Vegetation Science.* 2003;14(6):927-30.
- 604 23. Levy R, Borenstein E. Metabolic modeling of species interaction in the human
605 microbiome elucidates community-level assembly rules. *Proc Natl Acad Sci U S A.*
606 2013;110(31):12804-9.
- 607 24. Carr R, Borenstein E. NetSeed: a network-based reverse-ecology tool for calculating
608 the metabolic interface of an organism with its environment. *Bioinformatics.*
609 2012;28(5):734-5.
- 610 25. Borenstein E, Kupiec M, Feldman MW, Ruppin E. Large-scale reconstruction and
611 phylogenetic analysis of metabolic environments. *Proc Natl Acad Sci U S A.*
612 2008;105(38):14482-7.
- 613 26. Wolcott RD, Hanson JD, Rees EJ, Koenig LD, Phillips CD, Wolcott RA, et al. Analysis of
614 the chronic wound microbiota of 2,963 patients by 16S rDNA pyrosequencing. *Wound*
615 *Repair Regen.* 2016;24(1):163-74.
- 616 27. Smith K, Collier A, Townsend EM, O'Donnell LE, Bal AM, Butcher J, et al. One step
617 closer to understanding the role of bacteria in diabetic foot ulcers: characterising the
618 microbiome of ulcers. *BMC Microbiol.* 2016;16:54.
- 619 28. Gontcharova V, Youn E, Sun Y, Wolcott RD, Dowd SE. A comparison of bacterial
620 composition in diabetic ulcers and contralateral intact skin. *Open Microbiol J.* 2010;4:8-19.
- 621 29. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet
622 rapidly and reproducibly alters the human gut microbiome. *Nature.* 2014;505(7484):559-63.
- 623 30. Blakytyn R, Jude E. The molecular biology of chronic wounds and delayed healing in
624 diabetes. *Diabet Med.* 2006;23(6):594-608.
- 625 31. Zykova SN, Jenssen TG, Berdal M, Olsen R, Myklebust R, Seljelid R. Altered cytokine
626 and nitric oxide secretion in vitro by macrophages from diabetic type II-like db/db mice.
627 *Diabetes.* 2000;49(9):1451-8.
- 628 32. Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, et al. An
629 unrestrained proinflammatory M1 macrophage population induced by iron impairs wound
630 healing in humans and mice. *J Clin Invest.* 2011;121(3):985-97.
- 631 33. Grice EA, Snitkin ES, Yockey LJ, Bermudez DM, Liechty KW, Segre JA, et al.
632 Longitudinal shift in diabetic wound microbiota correlates with prolonged skin defense
633 response. *Proc Natl Acad Sci U S A.* 2010;107(33):14799-804.

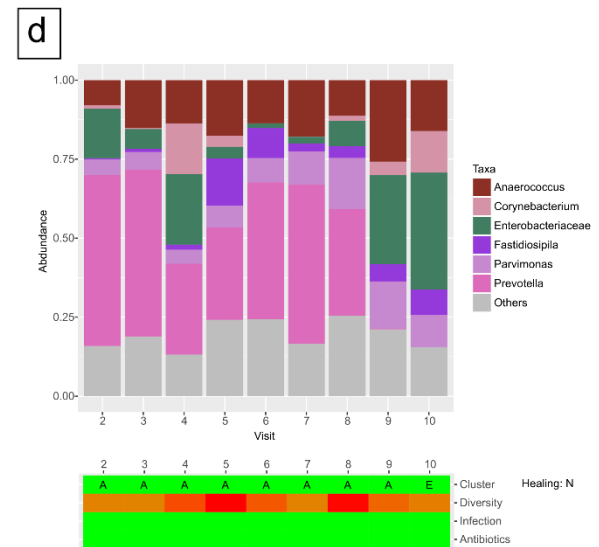
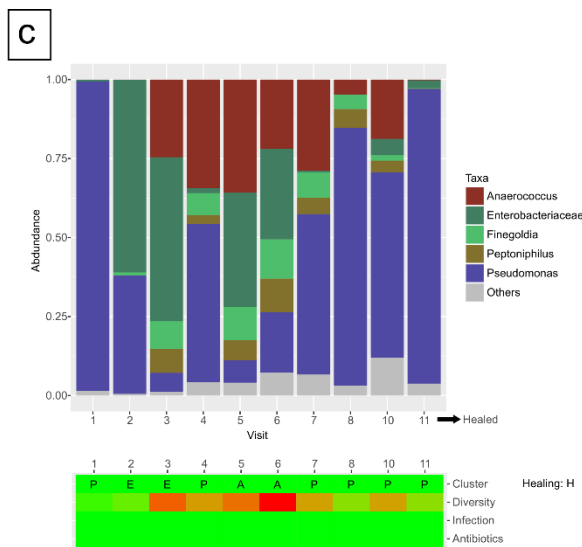
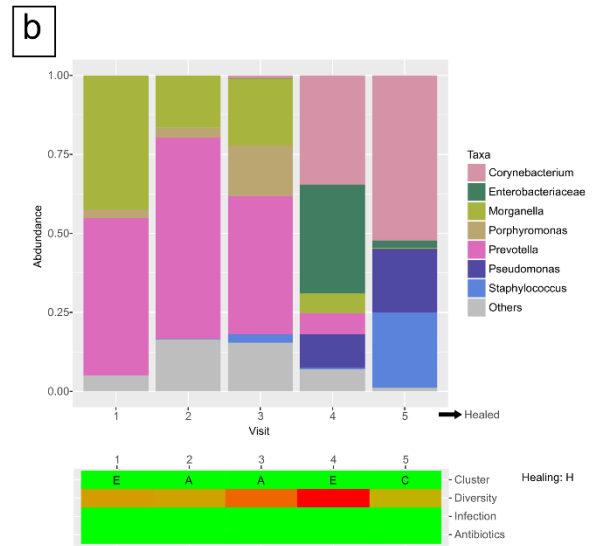
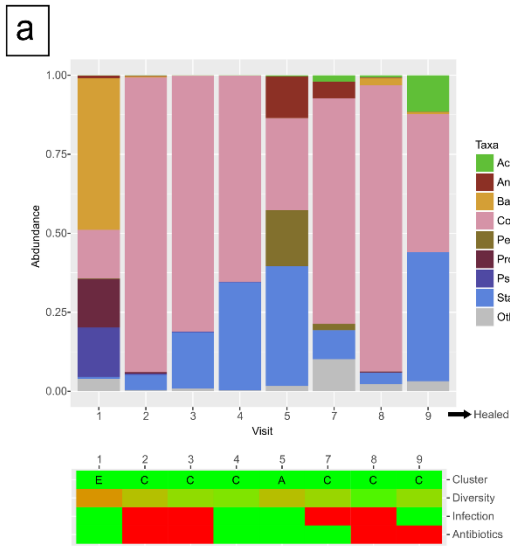
- 634 34. Wolcott R, Sanford N, Gabriliska R, Oates JL, Wilkinson JE, Rumbaugh KP. Microbiota
635 is a primary cause of pathogenesis of chronic wounds. *J Wound Care*. 2016;25(Sup10):S33-
636 S43.
- 637 35. Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, et al. An in vivo
638 polymicrobial biofilm wound infection model to study interspecies interactions. *PLoS One*.
639 2011;6(11):e27317.
- 640 36. Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, Patil KR. Metabolic
641 dependencies drive species co-occurrence in diverse microbial communities. *Proc Natl Acad
642 Sci U S A*. 2015;112(20):6449-54.
- 643 37. Milner P, Batten JE, Curtis MA. Development of a simple chemically defined medium
644 for *Porphyromonas gingivalis*: requirement for alpha-ketoglutarate. *FEMS Microbiol Lett*.
645 1996;140(2-3):125-30.
- 646 38. Tan Q, Wang W, Yang C, Zhang J, Sun K, Luo HC, et al. alpha-ketoglutarate is
647 associated with delayed wound healing in diabetes. *Clin Endocrinol (Oxf)*. 2016;85(1):54-61.
- 648 39. Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of
649 periodontal disease and caries. *Immunol Lett*. 2014;162(2 Pt A):22-38.
- 650 40. Winter SE, Lopez CA, Baumler AJ. The dynamics of gut-associated microbial
651 communities during inflammation. *EMBO reports*. 2013;14(4):319-27.
- 652 41. Ramsey MM, Freire MO, Gabriliska RA, Rumbaugh KP, Lemon KP. *Staphylococcus*
653 *aureus* Shifts toward Commensalism in Response to *Corynebacterium* Species. *Front
654 Microbiol*. 2016;7:1230.
- 655 42. Nelson A, Wright-Hughes A, Backhouse MR, Lipsky BA, Nixon J, Bhogal MS, et al.
656 CODIFI (Concordance in Diabetic Foot Ulcer Infection): a cross-sectional study of wound
657 swab versus tissue sampling in infected diabetic foot ulcers in England. *BMJ Open*.
658 2018;8(1):e019437.
659

660 **Abbreviations**

| | | |
|-----|-----------|--|
| 661 | ANOVA | Analysis of Variance |
| 662 | bp | Base pairs |
| 663 | BCD | Bray-Curtis Dissimilarity |
| 664 | DFU | Diabetic foot ulcer |
| 665 | IMG | Integrated Microbial Genomes |
| 666 | ISI | Inverse Simpson Index |
| 667 | MCI | Metabolic Complementarity Index |
| 668 | NPUAP | National Pressure Ulcer Advisory Panel |
| 669 | OTU | Operational Taxonomic Unit |
| 670 | PERMANOVA | Permutational Analysis of Variance |
| 671 | PBS | Phosphate buffered saline |

672

673 **Tables and figures**



675 **Figure 1. Examples of DFU profiles where healing occurred during the study.**

676 (a) Healing DFU profile largely dominated by *Corynebacterium* and *Staphylococcus*.

677 (b) Healing DFU with transition from anaerobes and *Enterobacteriaceae* to

678 *Corynebacterium* and *Staphylococcus*. (c) Healing DFU with disappearance of

679 *Enterobacteriaceae* and anaerobes in the visits preceding healing. (d) Example of a

680 non-healing DFU persistently colonised by mixed anaerobes and *Enterobacteriaceae*.

681 Infection, antibiotic exposure or high microbial diversity is indicated by red on the

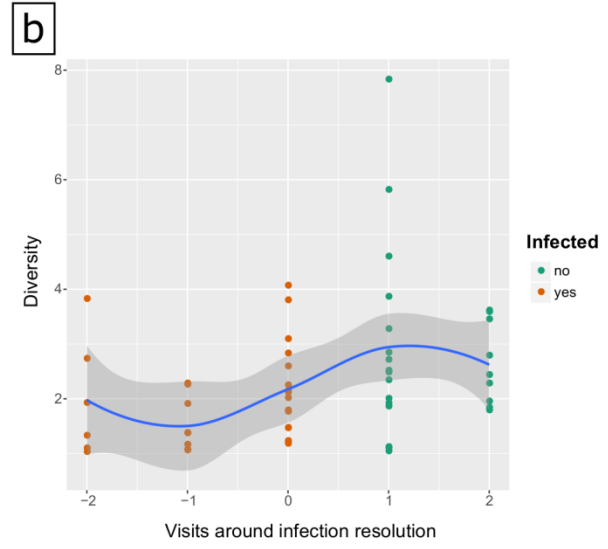
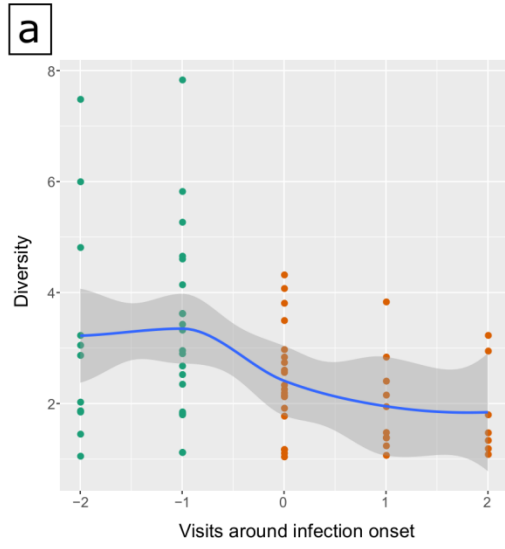
682 colour map, while absence of infection, antibiotic exposure or low microbial diversity

683 is indicated by green. DFU cluster designations; P = *Pseudomonaceae*, E =

684 *Enterobacteriaceae*, C = *Corynebacteriaceae*, S = *Staphylococcaceae*, A =

685 Anaerobes, M = *Micrococcaceae*, N = Non-healing at study end, H = Healed.

686



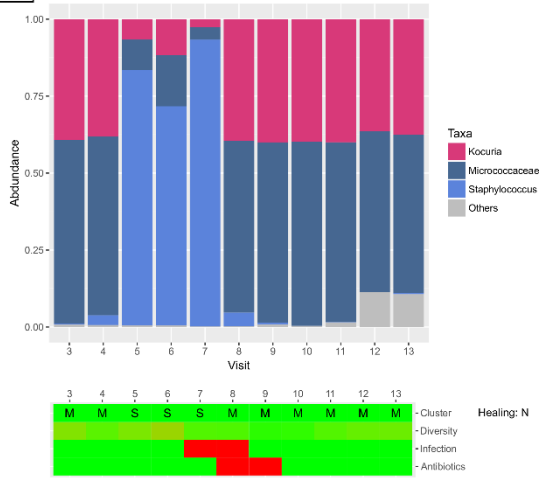
687
688

689 **Figure 2. Changes in diversity associated with onset and resolution of infection.**

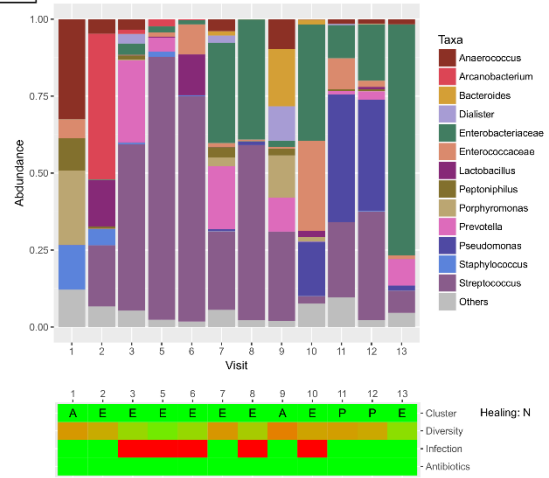
690 Alpha diversity (Inverse Simpson Index) plotted for DFU samples taken before, during
691 and after all documented episodes of infection. (a) Fall in alpha diversity associated
692 with onset of infection where visit 0 is the first visit where a new infection is observed
693 clinically, visit -2 and -1 the two preceding visits with no infection and visit 1 and 2 any
694 subsequent visits with persistent infection. (b) Recovery in alpha diversity associated
695 with resolution of infection where visit 0 is the final visit where infection is observed
696 clinically, visit -2 and -1 any preceding visits with infection and visit 1 and 2 any
697 subsequent visits where infection had resolved. The trend line indicates changing
698 diversity calculated by local polynomial regression fitting with the shaded area
699 indicating the 95% confidence intervals.

700

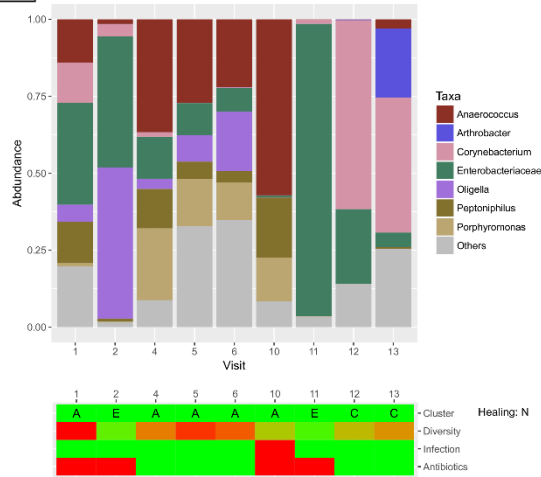
a



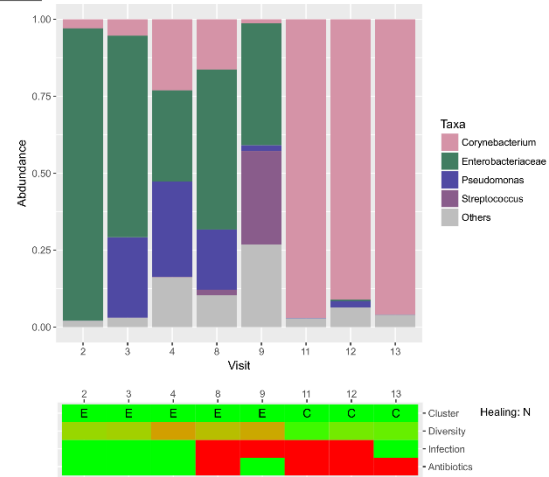
b



c



d

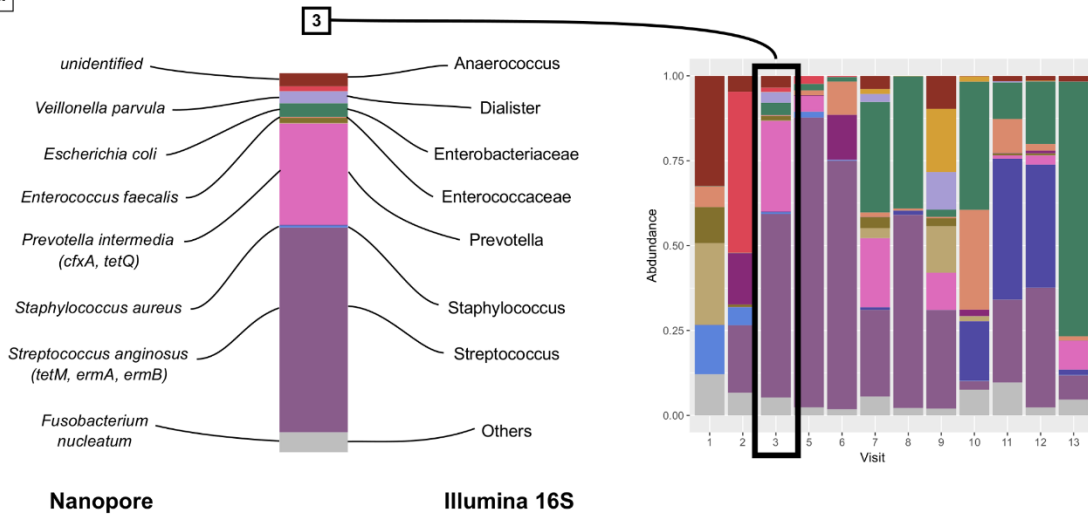


702 **Figure 3. Examples of DFU profiles where clinical infection occurred.**

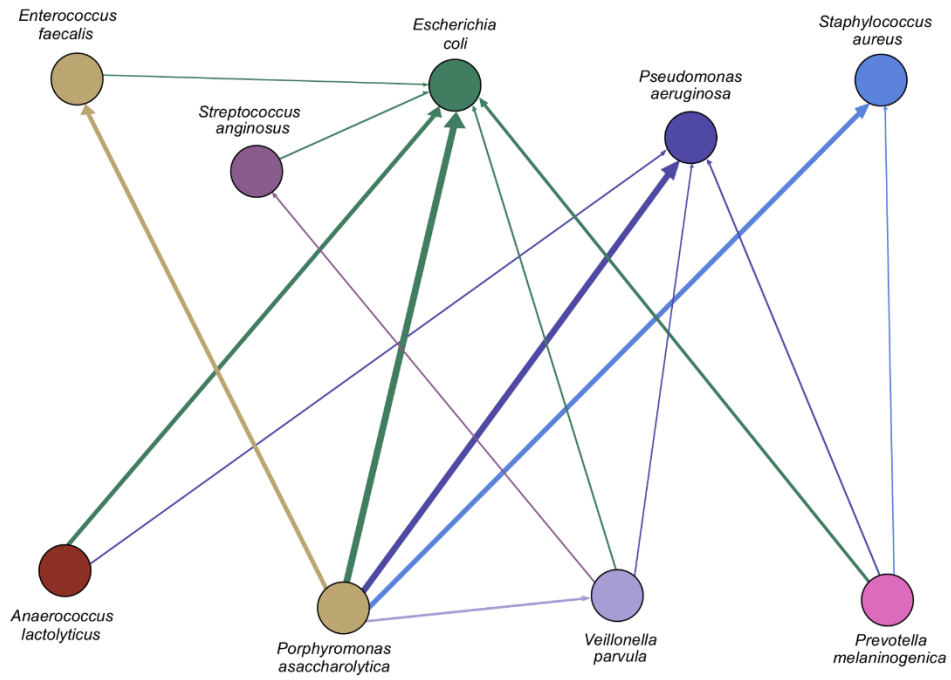
703 (a) Expansion of *Staphylococcus* associated with clinical infection at visit 7 and
704 subsequent decline following treatment with flucloxacillin at visit 8. (b) Episodes of
705 clinical infection observed in a profile dominated by *Streptococcus* with later
706 emergence of *Pseudomonas* and *Enterobacteriaceae*. (c) High diversity seen at visit
707 6 markedly reduced at visit 10, with other anaerobes replaced by *Anaerococci* and
708 *Peptonophilus*. An episode of clinical infection was treated with co-amoxiclav with
709 subsequent expansion of *Enterobacteriaceae*. (d) Infection associated with
710 emergence and expansion of *Streptococcus* followed by treatment with ciprofloxacin
711 after visit 9 and accompanying elimination of *Streptococcus* and *Enterobacteriaceae*.
712 Infection, antibiotic exposure or high microbial diversity is indicated by red on the
713 colour map, while absence of infection, antibiotic exposure or low microbial diversity
714 is indicated by green. DFU cluster designations; P = *Pseudomonaceae*, E =
715 *Enterobacteriaceae*, C = *Corynebacteriaceae*, S = *Staphylococcaceae*, A =
716 Anaerobes, M = *Micrococcaceae*, N = Non-healing at study end, H = Healed.

717

a



b



718

719

720 **Figure 4. Direct sample sequencing enables identification of species and**
721 **resistance genes**

722 (a) Detailed analysis of the DFU profile shown in Fig. 3(b), comparing taxonomic
723 identifications based on Illumina 16S rRNA and Oxford Nanopore MinION direct
724 sample sequencing taken during an episode of clinical infection at visit 3.

725 (b) Potential network of inter-species interaction for representative species based on
726 this DFU profile, created using the Metabolic Complementary Index (23). Edges are
727 shown for all scores greater than 0.5. Higher scores are reflected by greater edge
728 thickness with arrows directed towards the partner species from which metabolic
729 benefit is predicted to derive.

730

731 **Supplementary Table 1. Patient demographics and DFU details.**

| Age | Sex | DFU depth (NPUAP) | DFU size (mm ²) | Healed | Infection | Alpha diversity (ISI) | Profile figure reference |
|-----|-----|-------------------|-----------------------------|--------|-----------|-----------------------|--------------------------|
| 37 | M | II | 70 | Yes | No | 3.25 | |
| 63 | M | II | 261 | No | No | 2.05 | |
| 62 | M | III | 618 | No | Yes | 3.53 | |
| 84 | M | III | 30 | No | Yes | 5.25 | |
| 60 | M | III | 1203 | No | Yes | 5.73 | Fig. 3(c) |
| 58 | M | III | 50 | No | No | 1.60 | |
| 77 | F | III | 251 | No | No | 5.22 | |
| 74 | M | III | 103 | No | No | 1.44 | |
| 45 | M | III | 1713 | No | Yes | 3.21 | Supp. Fig. 4(c) |
| 68 | M | III | 700 | Yes | No | 2.33 | Fig. 1(b) |
| 73 | M | II | 891 | No | No | 3.03 | Fig. 1(d) |
| 71 | M | II | 19 | No | No | 3.15 | Supp. Fig. 4(a) |
| 42 | F | II | 109 | Yes | No | 1.04 | Fig. 1(c) |
| 81 | F | II | 930 | No | Yes | 1.11 | Fig. 3(d) |
| 71 | M | III | 344 | No | Yes | 2.93 | |
| 61 | M | III | 146 | Yes | Yes | 3.30 | Fig. 1(a) |
| 79 | F | III | 287 | No | Yes | 1.10 | Supp. Fig. 4(d) |
| 81 | F | III | 114 | No | Yes | 1.96 | Fig. 3(a) |
| 56 | M | II | 107 | No | No | 3.28 | |
| 88 | M | II | 61 | No | No | 3.22 | |
| 62 | M | III | 25 | Yes | Yes | 2.85 | |
| 70 | M | III | 37 | No | Yes | 3.03 | |
| 56 | M | III | 33 | No | No | 1.05 | |
| 76 | F | III | 177 | No | No | 1.09 | Supp. Fig. 4(b) |
| 73 | M | IV | 990 | Yes | No | 1.74 | |
| 79 | M | III | 69 | Yes | Yes | 1.10 | |
| 50 | F | III | 593 | No | Yes | 4.79 | Fig. 3(b), Fig. 4 |
| 85 | F | II | 184 | Yes | No | 2.49 | |

732

733 Baseline patient demographics and DFU details for all 28 study participants. DFUs
 734 which healed or developed clinical infection during the study are indicated by 'Yes' or
 735 'No' in the Healed and Infection columns respectively. NPUAP = National Pressure
 736 Ulcer Advisory Panel depth grade. ISI = Inverse Simpson Index.

737 **Supplementary Table 2. Highly abundant taxa.**

| Family | Abundance (%) | Dominant genera |
|---------------------------------------|----------------------|---|
| <i>Corynebacteriaceae</i> | 22.7 | <i>Corynebacterium</i> (22.7 %) |
| <i>Staphylococcaceae</i> | 15.3 | <i>Staphylococcus</i> (15.2 %) |
| <i>Clostridiales Incertae Sedis X</i> | 13.4 | <i>Anaerococcus</i> (6.4 %), <i>Finnegoldia</i> (2.4 %), <i>Helcococcus</i> (1.8 %) |
| <i>Enterobacteriaceae</i> | 11.5 | Unclassified (10.6 %), <i>Morganella</i> (0.5 %) |
| <i>Micrococcaceae</i> | 7.9 | <i>Kocuria</i> (3.9 %), Unclassified (3.1 %), <i>Arthrobacter</i> (0.8 %) |
| <i>Pseudomonadaceae</i> | 6.1 | <i>Pseudomonas</i> (6.1 %) |
| <i>Streptococcaceae</i> | 4.6 | <i>Streptococcus</i> (4.6 %) |
| <i>Moraxellaceae</i> | 3.2 | <i>Acinetobacter</i> (3.0 %) |
| <i>Prevotellaceae</i> | 2.8 | <i>Prevotella</i> (2.8 %) |
| <i>Actinomycetaceae</i> | 2.1 | <i>Arcanobacterium</i> (0.9 %), <i>Actinobaculum</i> (0.8 %) |

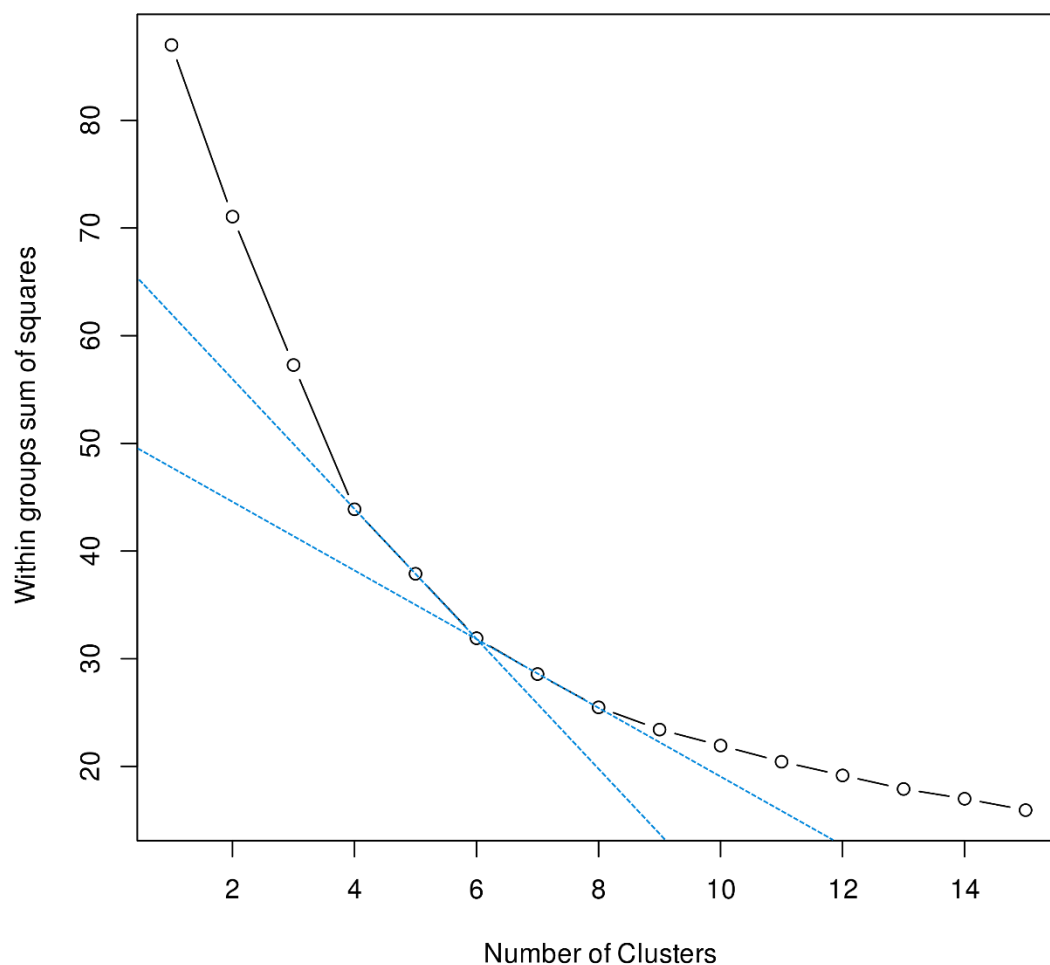
738

739 The 10 most abundant taxa at family level are shown according to mean proportional
740 abundance across all 237 DFU samples. Dominant genera are indicated for each
741 family.

742 **Supplementary Table 3. DFU cluster transition probabilities**

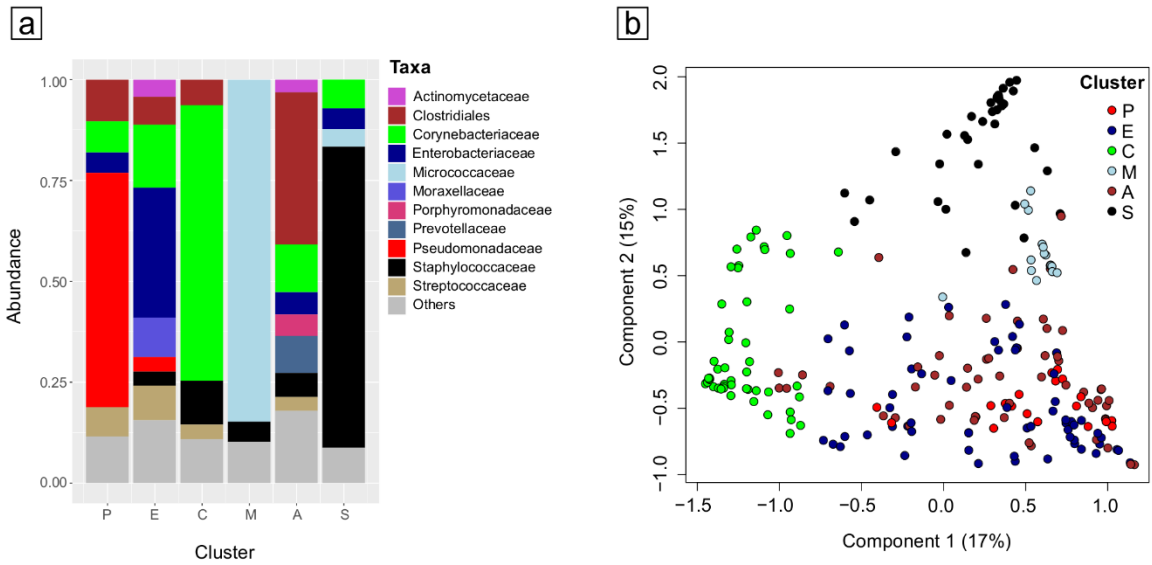
| All DFUs | | | | | | | | | |
|---------------|-------------|-------------|-------------|-------------|-------------|-------------|------|------|-------|
| From | To | | | | | | | | Count |
| | P | E | C | M | A | S | N | H | |
| P | <i>0.65</i> | 0.15 | 0.05 | 0.00 | 0.05 | 0.00 | 0.05 | 0.05 | 20 |
| E | 0.05 | <i>0.56</i> | 0.11 | 0.00 | 0.08 | 0.08 | 0.10 | 0.02 | 61 |
| C | 0.02 | 0.06 | <i>0.67</i> | 0.00 | 0.06 | 0.02 | 0.10 | 0.06 | 49 |
| M | 0.00 | 0.00 | 0.00 | <i>0.71</i> | 0.00 | 0.18 | 0.12 | 0.00 | 17 |
| A | 0.02 | 0.20 | 0.04 | 0.00 | <i>0.64</i> | 0.04 | 0.07 | 0.00 | 56 |
| S | 0.00 | 0.09 | 0.06 | 0.09 | 0.09 | <i>0.53</i> | 0.06 | 0.09 | 34 |
| Healed DFUs | | | | | | | | | |
| From | To | | | | | | | | Count |
| | P | E | C | M | A | S | N | H | |
| P | <i>0.50</i> | 0.17 | 0.00 | 0.00 | 0.17 | 0.00 | 0.00 | 0.17 | 6 |
| E | 0.11 | <i>0.11</i> | 0.22 | 0.00 | 0.11 | 0.33 | 0.00 | 0.11 | 9 |
| C | 0.00 | 0.00 | <i>0.65</i> | 0.00 | 0.12 | 0.06 | 0.00 | 0.18 | 17 |
| M | 0.00 | 0.00 | 0.00 | <i>0.00</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0 |
| A | 0.13 | 0.25 | 0.25 | 0.00 | <i>0.38</i> | 0.00 | 0.00 | 0.00 | 8 |
| S | 0.00 | 0.09 | 0.09 | 0.00 | 0.00 | <i>0.55</i> | 0.00 | 0.27 | 11 |
| Unhealed DFUs | | | | | | | | | |
| From | To | | | | | | | | Count |
| | P | E | C | M | A | S | N | H | |
| P | <i>0.71</i> | 0.14 | 0.07 | 0.00 | 0.00 | 0.00 | 0.07 | 0.00 | 14 |
| E | 0.04 | <i>0.63</i> | 0.10 | 0.00 | 0.08 | 0.04 | 0.12 | 0.00 | 52 |
| C | 0.03 | 0.09 | <i>0.69</i> | 0.00 | 0.03 | 0.00 | 0.16 | 0.00 | 32 |
| M | 0.00 | 0.00 | 0.00 | <i>0.71</i> | 0.00 | 0.18 | 0.12 | 0.00 | 17 |
| A | 0.00 | 0.19 | 0.00 | 0.00 | <i>0.69</i> | 0.04 | 0.08 | 0.00 | 48 |
| S | 0.00 | 0.09 | 0.04 | 0.13 | 0.13 | <i>0.52</i> | 0.09 | 0.00 | 23 |

743
744 Transition probabilities for movement between DFU clusters over subsequent visits
745 for all DFUs (n = 28) and also separated by DFUs which went on to heal (n = 8) and
746 those that did not (n = 20). Probability of movement between clusters is shown from 0
747 (no probability) to 1 (guaranteed probability) with the number of samples falling into
748 each of the 6 clusters shown in the count column. Self-transitions are italicised. P =
749 *Pseudomonaceae*, E = *Enterobacteriaceae*, C = *Corynebacteriaceae*, M =
750 *Micrococcaceae*, A = Anaerobes, S = *Staphylococcaceae*, N = non-healing state
751 (study end), H = healed DFU.



752
753

754 **Supplementary Figure 1. Cluster variance explained by increasing number of**
 755 **clusters.** Plot of variance explained by increasing the numbers of clusters used,
 756 showing the trade-off between clustering complexity and fit to the data. The change in
 757 trajectory in moving from 5 to 6 clusters compared to 6 to 7 clusters is shown with
 758 dashed lines indicating the diminishing returns associated with using a higher number
 759 of clusters.
 760

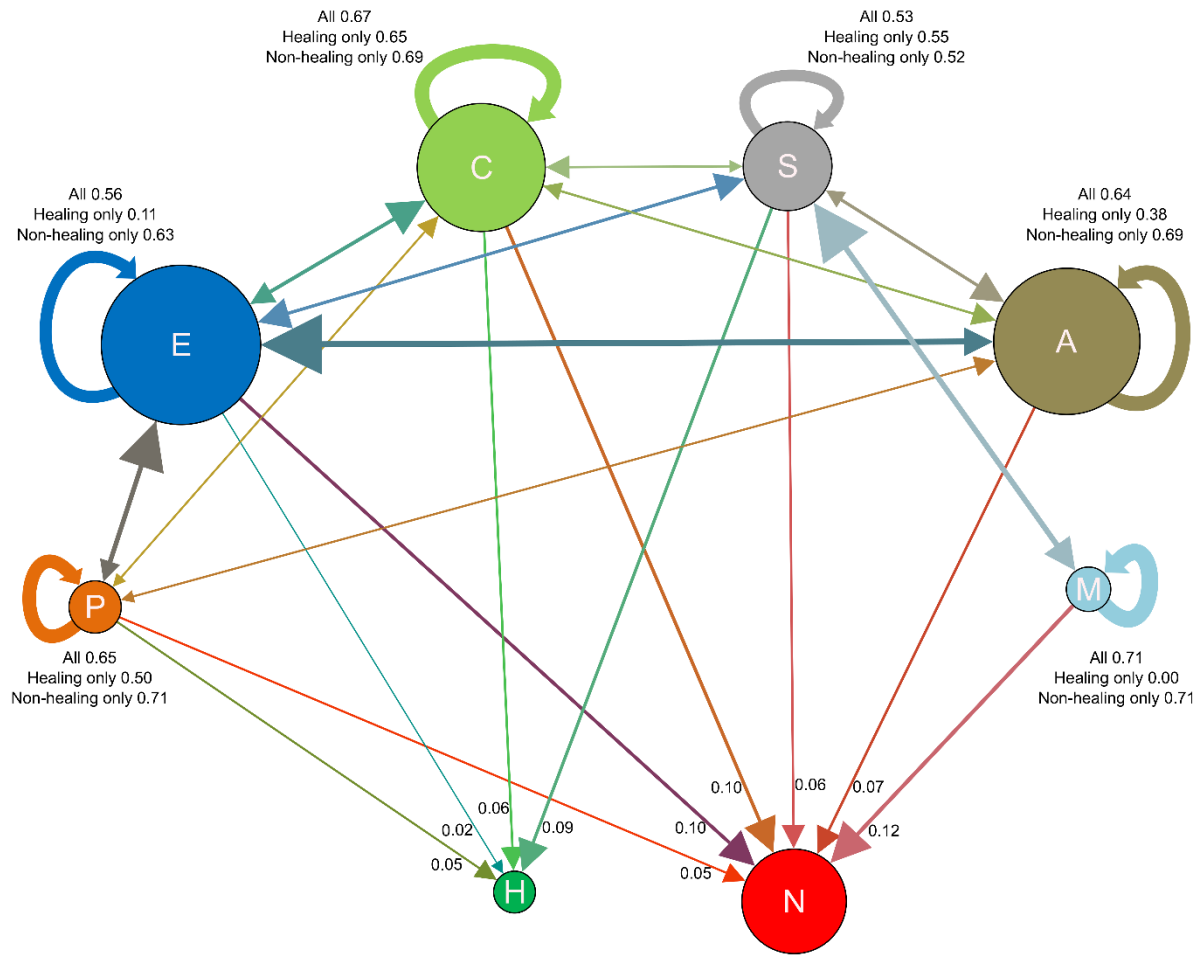


761
762

763 **Supplementary Figure 2. Clustering of DFU samples by major taxonomic**
764 **groups.**

765 DFU samples were clustered at family level by k-means clustering and assigned to
766 one of 6 clusters, labelled according to most abundant taxa. DFU cluster designations;
767 P = *Pseudomonaceae*, E = *Enterobacteriaceae*, C = *Corynebacteriaceae*, S =
768 *Staphylococcaceae*, A = Anaerobes, M = *Micrococcaceae*. (a) Mean proportional
769 abundance of the taxa in each cluster. The E and A clusters contained were the most
770 heterogeneous, representing a more diverse combination of different taxa. (b)
771 Principal coordinates analysis showing the distribution of samples at genus level, with
772 samples coloured by cluster. Samples in the low diversity C, M and S clusters were
773 well discriminated by the first two principal components while samples from clusters
774 E, A and P tended to overlap and were more widely spread. DFU clusters accounted
775 for a significant proportion of the overall variance when analysed with PERMANOVA
776 (7 % variance explained, $p = 0.001$).

777

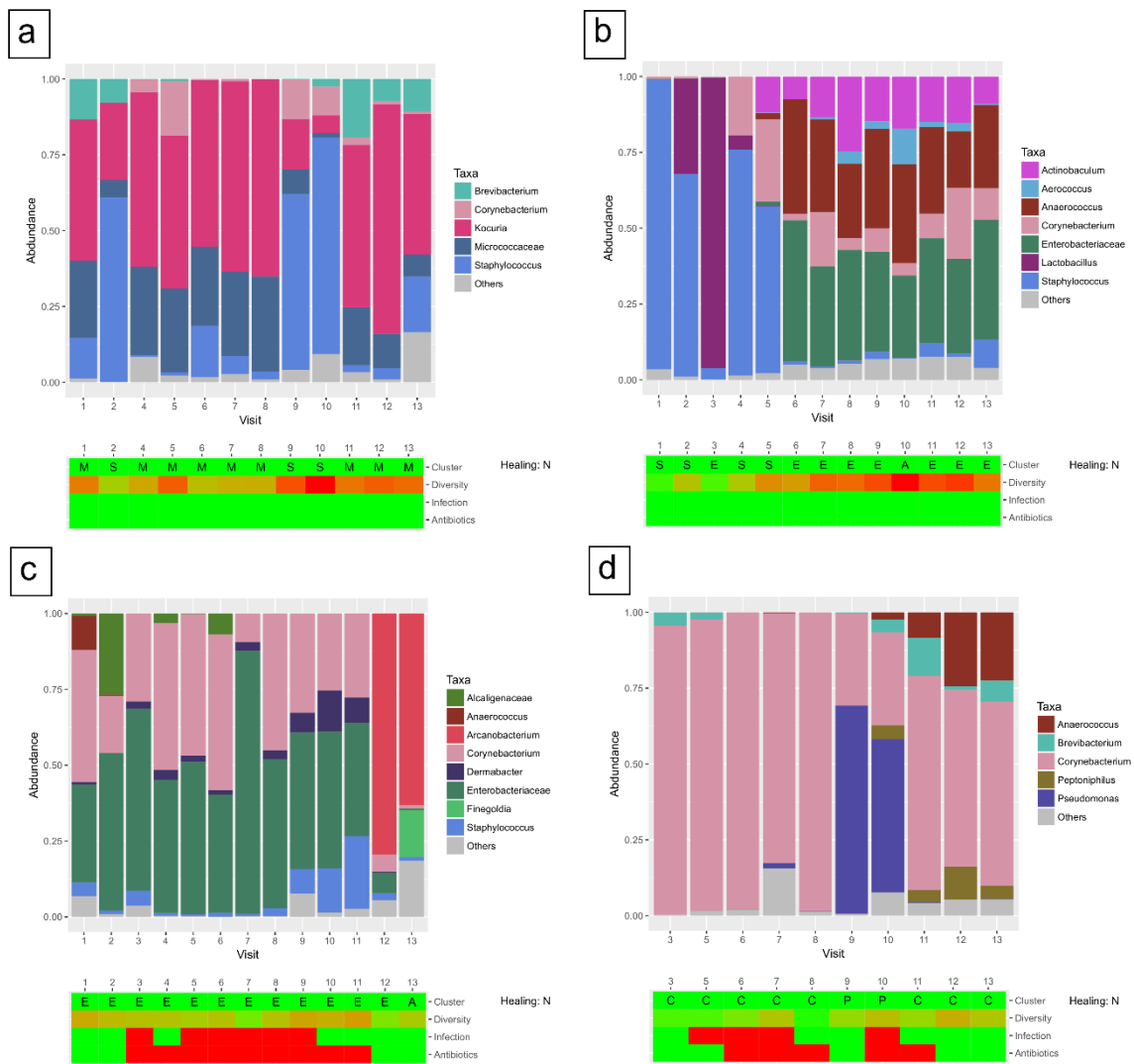


778
779

780 **Supplementary Figure 3. Transition between DFU clusters during the study.**

781 A Markov model demonstrating the transition between DFU clusters between visits
 782 until either healing (H) or non-healing at study end (N) for 237 samples from 28
 783 subjects combining both healing (n=8) and non-healing (n=20) DFUs. The size of the
 784 nodes indicates the number of samples in each state while the edge and arrow weights
 785 represent the probability of transition between states. Transition probabilities within
 786 nodes or to the endpoints (healing or non-healing) are labelled, with self-transition
 787 probabilities separated by group also shown. The full transition data are also shown
 788 in Supplementary Table 3. DFU cluster designations; P = *Pseudomonaceae*, E =
 789 *Enterobacteriaceae*, C = *Corynebacteriaceae*, S = *Staphylococcaceae*, A =
 790 *Anaerobes*, M = *Micrococcaceae*.

791



793

794 **Supplementary Figure 4. Additional examples of DFU profiles.**

795 Examples of DFU profiles with unexplained abrupt changes in microbial profile, or
 796 static profiles despite clinical infection. (a) Proliferation of *Staphylococcus* at visits 2
 797 and 9 without clinically apparent infection, (b) Transition from colonisation with
 798 *Staphylococcus* to *Enterobacteriaceae* and anaerobes at visit 6, (c) Stable
 799 colonisation with *Enterobacteriaceae* and *Corynebacterium* despite clinical infection
 800 and antibiotic exposure, (d) Colonisation with *Corynebacterium* unchanged by
 801 infection or antibiotic exposure, with a second episode of infection associated with
 802 proliferation of *Pseudomonas*. Infection, antibiotic exposure or high microbial diversity
 803 is indicated by red on the colour map, while absence of infection, antibiotic exposure
 804 or low microbial diversity is indicated by green. DFU cluster designations; P =
 805 *Pseudomonaceae*, E = *Enterobacteriaceae*, C = *Corynebacteriaceae*, S =
 806 *Staphylococcaceae*, A = Anaerobes, M = *Micrococaceae*, N = Non-healing at study
 807 end, H = Healed.