

What is Slough?

A pilot study to define the proteomic and microbial composition of wound slough and its implications for wound healing.

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35 **Abstract**
36 Slough is a well-known feature of non-healing wounds. This study aims to determine the proteomic and
37 microbiologic components of slough as well as interrogate the associations between wound slough
38 components and wound healing. Twenty-three subjects with slow-to-heal wounds and visible slough
39 were enrolled. Etiologies included venous stasis ulcers, post-surgical site infections, and pressure
40 ulcers. Patient co-morbidities and wound healing outcome at 3-months post-sample collection was
41 recorded. Debrided slough was analyzed microscopically, through untargeted proteomics, and high-
42 throughput bacterial 16S-ribosomal gene sequencing. Microscopic imaging revealed wound slough to
43 be amorphous in structure and highly variable. 16S-profiling found slough microbial communities to
44 associate with wound etiology and location on the body. Across all subjects, slough largely consisted
45 of proteins involved in skin structure and formation, blood-clot formation, and immune processes. To
46 predict variables associated with wound healing, protein, microbial, and clinical datasets were
47 integrated into a supervised discriminant analysis. This analysis revealed that healing wounds were
48 enriched for proteins involved in skin barrier development and negative regulation of immune
49 responses. While wounds that deteriorated over time started off with a higher baseline Bates-Jensen
50 Wound Assessment Score and were enriched for anaerobic bacterial taxa and chronic inflammatory
51 proteins. To our knowledge, this is the first study to integrate clinical, microbiome, and proteomic data
52 to systematically characterize wound slough and integrate it into a single assessment to predict wound
53 healing outcome. Collectively, our findings underscore how slough components can help identify
54 wounds at risk of continued impaired healing and serves as an underutilized biomarker.

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56
57 **Introduction**
58 Chronic, non-healing wounds impose a significant, underappreciated burden to affected individuals and
59 the healthcare system. An estimated 2 - 10% of the general population in Australia, the United Kingdom
60 and the United States suffer from chronic wounds.¹⁻⁴ Individuals with conditions known to impair wound
61 healing, such as peripheral arterial disease, venous insufficiency, immune-compromised, obesity,
62 diabetes, impaired sensation, and spinal cord injuries are at the highest risk for developing chronic
63 wounds.⁵ With the prevalence of these comorbidities on the rise, chronic wounds are anticipated to
64 pose a growing burden for patients and the healthcare system.^{2,3} Thus, identifying biomarkers to
65 distinguish chronic wounds that are likely to heal from those that may benefit from intensive therapies
66 to promote healing is a critical imperative.

67 A hallmark feature of chronic wounds is the presence of slough, which mainly consists of devitalized
68 tissue that overlays the wound bed. Slough is hypothesized to arise as a byproduct of prolonged wound
69 inflammation.⁶⁻⁸ On a macro level, slough has highly variable physical characteristics ranging in
70 consistency, color, odor, and attachment to the wound bed even across a single wound's surface.^{6,8}
71 Subsequently the appearance varies widely wound-to-wound and patient-to-patient. Although, to date,
72 there are no studies interrogating slough directly, assessments of exudative fluid from surface of chronic
73 wounds and wound biopsies suggest that the wound surface and associated slough is enriched for
74 various types of collagen, extracellular matrix proteins, matrix metalloproteases, and proteins related to
75 inflammatory immune responses.⁹⁻¹⁴ Slough can also be infiltrated by an array of bacterial either as
76 single cells or by forming aggregates and biofilm.¹⁵⁻²⁰ However, due to the highly variable appearance
77 and inconsistencies in even defining slough between providers, it is difficult to distinguish slough with
78 or without microbial biofilm from infected wound exudate.^{15,21} Ultimately, slough's variable nature has
79 led to inconsistent clinical approaches to wound management.

80
81 One dominant theory proposes that slough inhibits wound healing by prolonging the inflammatory phase
82 of healing, preventing the formation of granulation tissue and subsequent wound contraction. Slough is
83 commonly associated with biofilm, although limited evidence exists to support the idea that slough is
84 primarily microbial in nature. Slough may serve as a reservoir attractive to bacteria on the wound bed
85 that subsequently promotes biofilm formation, however this is also challenging to quantify.⁶ In the
86 absence of conclusive data, standard chronic wound care focuses on proper debridement to remove
87

88 devitalized tissue, reduce potential surface microbial burden, and ideally return the wound to an acute
89 state to stimulate tissue repair.²² However, less than 50% of wounds respond or go on to heal following
90 debridement.²³ Conversely, some wounds with slough present will heal without debridement,
91 suggesting that the presence of slough does not always indicate that healing is disrupted.⁸

92
93 Despite it being a common feature of chronic wounds, a detailed molecular characterization of the host
94 and microbial components within slough from different wound etiologies is missing. A systematic
95 analysis of slough composition and factors associated with wound healing outcomes could shape
96 wound treatment strategies and aide in triaging high risk patients into specialty care.

97
98 With this pilot study, we aim to characterize the human and microbial components of slough collected
99 from wounds of various etiologies. Collectively we show that wound slough is primarily composed of
100 proteins associated with the structure and formation of the skin, blood clot formation, and various
101 immune responses. Wound slough is highly polymicrobial and exhibits signatures associated with both
102 wound etiology and location on the body. Finally, slough protein profiles from wounds with a healing
103 trajectory are significantly different than slough protein profiles from non-healing wounds, suggesting
104 they may serve as a prognostic marker. Rather than being discarded, slough may be a critical indicator
105 to predict if a wound is more likely on the trajectory toward healing or at risk of deteriorating.

106
107
108 **Methods**

109 Subject Identification and Enrollment

110 Adults 18 years or older with chronic wounds were recruited from UW-Health Wound Care Clinics under
111 an IRB approved protocol (Study ID: 2020-1002). Examples of wounds identified for possible inclusion
112 included and were not limited to, chronic or non-healing diabetic ulcers, pressure ulcers, venous ulcers,
113 surgical or procedural wounds, trauma wounds, burn wounds, and wounds of unknown or other etiology.
114 On the day of sample collection, subject wounds were measured, evaluated and scored according to
115 the Bates-Jensen Wound Assessment Tool.²⁴ Information related to the wound's etiology and care,
116 wound measurements from the most recent previous visit, and patient co-morbidities were extracted
117 from the medical record. Digital photos of the wound were taken before and after the debridement
118 procedure. Swabs for microbiome analysis of the wound edge and center were collected using Levine's
119 technique and placed into 300 μ l of DNA/RNA Shield (Zymo Research, Irvine, CA) and stored at -80°C
120 until further processing. Swabs were spun down using DNA IQ Spin Baskets (Promega, Madison, WI)
121 and DNA was extracted. Swabs designated for microbial culture were taken from the wound center
122 using Levine's technique into 1 ml of liquid Amies (Copan Diagnostics Inc., Murrieta, CA). Swabs were
123 stored at 4°C for less than 2 hours before being processed for microbial culture.

124
125 All subjects received sharp debridement of their wounds. Prior to debridement wounds were washed
126 with soap and rinsed with water. Debridement was performed by a skilled practitioner with surgical
127 instruments such as scalpel, curette, scissors, rongeur, and/or forceps. Removed slough material was
128 collected into 1ml of DNA/RNA Shield (Zymo Research, Irvine, CA) and stored at 4°C before sectioning
129 for scanning electron microscopy (SEM), fluorescence in situ hybridization (FISH), and proteomics.
130 Remaining slough material was stored at -80°C.

131
132 Samples from South Western Sydney Hospital were collected and processed as described by Malone
133 et al.²⁵ Adults 18 years or older presenting with a diabetes-related foot ulcer with visible signs of slough
134 were recruited for the study. The collection of samples and their corresponding patients was undertaken
135 as a sub-analysis of a larger clinical study, with samples being obtained following written consent. Ethics
136 approval for the larger clinical study and the slough sub-analysis was approved by South Western
137 Sydney LHD Research and Ethics Committee. All DFUs were debrided and rinsed with 0.9% NaCl prior
138 to specimen collection. For DNA sequencing, patient wound slough was removed from the ulcer base
139 with a dermal curette and immediately stored in RNA Shield (Zymo Research, Irvine, CA) at 4°C for 24
140 hours before being frozen at -80°C until further processing. For PNA-FISH, tissue specimens were

141 obtained through a dermal ring curette from the wound bed of each DFU. Following removal, tissue
142 specimens were rinsed vigorously in a phosphate buffer solution (PBS) bath to remove any coagulated
143 blood and to reduce the number of planktonic microorganisms. Tissue specimens were immediately
144 fixed in 4% paraformaldehyde overnight at 4°C, then transferred into 70% ethanol and stored at -20°C
145

146 **Microbial Culture and Bacterial Isolate Identification**

147 Swabs designated for microbial culture were spun down using DNA IQ Spin Baskets (Promega,
148 Madison, WI). A portion of each sample was serially diluted with 1X phosphate buffered saline and
149 plated onto Tryptic Soy Agar (TSA) with 5% sheep blood (BBL, Sparks, MD) for quantitative bacterial
150 culture. Plates were incubated at 35°C overnight. To isolate culturable bacteria, colonies with distinct
151 morphology were isolated and incubated at 35°C overnight on TSA with 5% sheep blood then single
152 colonies were inoculated into liquid Tryptic Soy Broth (TSB) for overnight incubation. To identify each
153 bacterial isolate, a portion of the overnight TSB culture underwent DNA extraction and sanger
154 sequencing (Functional Biosciences, Madison, WI) of the bacterial 16S ribosomal RNA gene. The
155 remaining portion of the isolate culture was stored in glycerol at -80°C.
156

157 **DNA/RNA extraction, library construction, sequencing**

158 DNA extraction on samples collected in the USA was performed as previously described with minor
159 modifications.²⁶ Briefly, 300 µl of yeast cell lysis solution (from Epicentre MasterPure Yeast DNA
160 Purification kit), 0.3 µl of 31,500 U/µl ReadyLyse Lysozyme solution (Epicentre, Lucigen, Middleton,
161 WI), 5 µl of 1 mg/ml mutanolysin (M9901, Sigma-Aldrich, St. Louis, MO), and 1.5 µl of 5 mg/ml
162 lysostaphin (L7386, Sigma-Aldrich, St. Louis, MO) was added to 150 µl of swab liquid before incubation
163 for one hour at 37°C with shaking. Samples were transferred to a tube with 0.5 mm glass beads (Qiagen,
164 Germantown, Maryland) and bead beat for 10 min at maximum speed followed by a 30 min incubation
165 at 65°C with shaking, 5 min incubation on ice. The sample was spun down at 10,000 rcf for 1 min and
166 the supernatant was added to 150 µl of protein precipitation reagent (Epicentre, Lucigen, Middleton,
167 WI). Remaining steps followed the recommended PureLink Genomic DNA Mini Kit (Invitrogen,
168 Waltham, MA) protocol for DNA extraction and purification. 16S rRNA gene amplicon libraries targeting
169 the V1-V3 or V4 region were constructed using a dual-indexing method and sequenced on a MiSeq
170 with a 2x300 bp run format (Illumina, San Diego, CA). Reagent-only negative controls were carried
171 through the DNA extraction and sequencing process.
172

173 Swabs obtained from DFUs in Australia were defrosted on ice prior to DNA extraction. Genomic DNA
174 was extracted using Qiagen DNeasy PowerBiofilm kit (Cat No./ID: 24000-50) following the
175 manufacturer's instructions. Preparation of the 16S library and DNA sequencing was carried out by a
176 commercial laboratory (Ramaciotti Centre for Genomics, University of New South Wales, Australia) on
177 the Illumina MiSeq platform (2x300bp) targeting the V1-V3 (27f/519r) 16S region.
178

179 **Sequence analysis**

180 The QIIME2²⁷ environment was used to process DNA-based 16S rRNA gene amplicon data. Paired
181 end reads were trimmed, quality filtered, and merged into amplicon sequence variants (ASVs) using
182 DADA2. Taxonomy was assigned to ASVs using a naive Bayes classifier pre-trained on full length 16S
183 rRNA gene 99% operational taxonomic unit (OTU) reference sequences from the Greengenes database
184 (version 13_8). Using the qiime2R package, data was imported into RStudio (version 1.4.1106) running
185 R (version 4.1.0) for further analysis using the phyloseq package.²⁸ Negative DNA extraction and
186 sequencing controls were evaluated based on absolute read count and ASV distribution in true patient
187 samples. Abundances were normalized proportionally to total reads per sample. Data was imported into
188 RStudio running R (version 4.2.1) for analysis. Relative abundance plots were produced using the
189 package ggplot2, where taxa below 1% relative abundance were pooled into an "Other" category.
190

191 **Proteomics**

192 Debrided slough tissue samples were weighed and placed in PowerBead tubes containing 1.4mm
193 ceramic beads (Qiagen, Germantown, Maryland) for tissue homogenization, proteomic processing, and

194 analysis at the University of Wisconsin Mass Spectrometry and Proteomics Core Facility. In brief,
195 samples were labeled and pooled for multiplex relative mass spectrometry (MS) quantification with the
196 TMTpro 16plex labeling kit (ThermoFisher Scientific, Waltham, MA) and underwent Liquid
197 Chromatography with tandem mass spectrometry on an Orbitrap Elite mass spectrometer
198 (ThermoFisher Scientific). Protein sequences were matched to known human and bacterial proteins.
199 Functions associated with each protein were gathered from the Gene Ontology (GO) database, KEGG
200 Pathways, Reactome Pathways, and WikiPathways databases. Data was imported into RStudio for
201 analysis. To determine the most enriched proteins and their associated biologic processes within
202 slough, abundances were normalized proportionally to total abundance per sample and the ranked
203 dataset was analyzed via the Gene Ontology enrichment analysis (GORILA) and visualization tool.²⁹
204 Differential protein expression between subject groups was assessed via DEqMS.³⁰ To determine the
205 key biologic processes for smaller sets of proteins, such as those enriched within subject groups or the
206 proteins within each of the k-means protein clusters (see *Integration of Biologic Data Sets* below), small
207 sets of proteins were submitted as unranked lists to the GO Enrichment Analysis tool.^{31,32}

208

209 *Fluorescence in situ hybridization (FISH)*

210 Formalin-fixed paraffin-embedded (FFPE) histological sections were deparaffinized in xylene and
211 rehydrated in a series of ethanol washes (100%, 99%, 95%, and 0%). Subsequently, the samples were
212 allowed to hybridize at 46°C for 4 hours in hybridization solution (900 mM NaCl, 20 mM Tris pH 7.5,
213 0.01% SDS, 20% formamide, 2 µM FISH probe). The FISH probe used was a DNA oligonucleotide
214 (EUB388 sequence) with a 3'-conjugated TEX615 fluorophore (Integrated DNA Technologies,
215 Coralville, IA, USA). Samples were washed in excess wash buffer (215 mM NaCl, 20 mM Tris pH 7.5,
216 5 mM EDTA) at 48°C for 15 mins, dipped into ice cold water, 100% ethanol, drained, and air-dried.
217 Slides were mounted with Prolong Glass antifade mounting medium with NucBlue counterstain (Thermo
218 Fisher Scientific, Waltham, MA, USA) and a glass coverslip of #1.5 thickness and stored flat to cure
219 overnight in the dark. Micrographs were acquired using a Zeiss 780 confocal laser scanning microscope
220 on the red TEX615, blue Hoescht, and green GFP (tissue autofluorescence) channels using 5x and 63x
221 objectives. Zeiss Zen software was used to analyze tiled images, z-stacks, and generate maximum
222 intensity projections.

223

224 *PNA-FISH*

225 As described by Nadler et al.³³, formalin-fixed, paraffin-embedded samples were cut, deparaffinized and
226 rehydrated following standard procedures. Subsequently, the samples were stained with a PNA-FISH-
227 TexasRed-5-conjugated universal bacterial (BacUni) 16s rRNA probe (AdvanDx, Woburn, MA, US),
228 incubated and then counterstained with 3 µM 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies,
229 Eugene, OR, USA). The samples were afterward mounted (ProLong™ Gold Antifade Mountant, Life
230 Technologies) and a coverslip was added (Marienfeld, Lauda-Königshoffen, Germany). Slides were
231 evaluated using a CLSM (Axio Imager.Z2, LSM880 CLSM; Zeiss, Jena, Germany). Images were taken
232 using 405 nm (DAPI) and 561 nm (TexasRed-5) lasers, as well as a 488 nm laser for visualizing the
233 green autofluorescence of the surrounding tissue. Images were subsequently processed with IMARIS
234 9.2 (Bitplane, Zurich, Switzerland) and presented using “Easy 3D”.

235

236 *Scanning Electron Microscopy*

237 Wound slough specimens were rinsed with PBS and fixed overnight in 5 mL of 1.5% glutaraldehyde in
238 0.1 M sodium phosphate buffer (pH 7.2) at 4°C. Samples were rinsed, treated with 1% osmium tetroxide
239 for 1 h, and then washed again in buffer. Samples were dehydrated through a series of ethanol washes
240 (30–100%) followed by critical point drying (14 exchanges on low speed) and were subsequently
241 mounted on aluminum stubs with a carbon adhesive tab and carbon paint. Samples were left to dry in
242 a desiccator overnight. Following sputter coating with platinum to a thickness of 20 nm, samples were
243 imaged in a scanning electron microscope (Zeiss LEO 1530-VP) at 3 kV.

244

245 *Integration of Biologic Data Sets*

246 To reduce the complexity of the proteomics data for integrative analysis protein abundances were
247 normalized, mean centered and grouped via k-means clustering. The optimal number of protein clusters
248 was determined by the Gap-Statistics method. Since there was no significant difference in the microbial
249 community composition at the wound edge or center, taxa relative abundances from the wound edge
250 and center were averaged to create a summative wound slough microbiome for each subject. The 15
251 microbial ASVs with greater than 1% relative abundance in at least two summative subject slough
252 microbiome samples were included for this analysis. To predict the variables associated with wound
253 healing, the protein cluster, summative slough microbiome, and the numerical Bates-Jensen Wound
254 Assessment score datasets were integrated into a supervised Partial Least Squares - Discriminant
255 Analysis (PLS-DA, aka. Data Integration Analysis for Biomarker discovery using Latent variable
256 approaches for Omics studies [DIABLO]) via the via MixOmics³⁴ R-studio package.
257

258 Statistical analyses

259 Statistical analyses were conducted in R studio running R (version 4.2.1). Bates Jensen wound
260 assessment scores were analyzed via Prism (version 9.2.0).

261 Data availability

262 Sequence reads for this project can be found under NCBI BioProject PRJNA1021648. Code for
263 analysis and generation of figures can be found on GitHub at https://github.com/Kalan-Lab/Townsend_et al_WhatIsSlough .
264

265 **Results**

266 Subject and Wound Characteristics

267 Twenty-three subjects with wounds of various etiologies were included in this study. To address
268 potential inconsistencies between sites and batch effects, the main analysis focuses on ten patients
269 recruited in the United States (Table 1, Table S1). Data from the remaining thirteen patients is available
270 in supplementary materials. For the ten patients, prior to sample collection wounds were measured,
271 evaluated and scored according to the Bates-Jensen Wound Assessment Tool (Table 2, Table S1).³⁵
272 Overall Bates-Jensen Wound Assessment scores ranged from 26 to 46 (mean 37.4) out of 60 points
273 with higher scores indicating greater wound degeneration. Photos of the wounds before sharp
274 debridement of the superficial wound slough are in [supplemental figure 1](#).
275

276 Wound status at 3 months post-sample collection was recorded (Table 1). At this time, 3 of the subjects'
277 wounds healed, 4 were ongoing yet stable in size and clinical assessment, and 3 wounds had
278 deteriorated (e.g. significantly increased in size, depth, and/or continued antibiotic resistant infection).
279 The total Bates-Jensen Wound Assessment Score, and several of the sub-scores trended higher in
280 wounds that deteriorated compared to those that went on to heal (p-values < 0.1, yet > 0.05, Mann-
281 Whitney t-test. Table 2). However, none of these comparisons reached statistical significance, likely
282 due to the relatively small number of subjects within each group.
283

284 Slough protein composition is associated with wound age and healing trajectory

285 Slough tissue was first characterized by untargeted proteomics to determine the overall protein
286 composition (Table S2). 11,058 peptide fragments (7,302 unique peptide groups) corresponding to
287 1,447 unique protein signatures were detected. To identify the biologic processes, molecular functions,
288 and cellular components that associated with protein features, abundant proteins identified across all
289 samples were analyzed using the Gene Ontology enrichment analysis (GORILLA) and visualization
290 tool.²⁹ This demonstrated that wound slough is enriched for proteins derived from both intracellular and
291 extracellular components, and notably enriched for proteins specific to skin tissue, such as the cornified
292 envelope and keratin filaments (Fig. 1, Fig. S2, Table S3). Molecular pathway analysis determined
293 slough samples are enriched for proteins involved in ion and metabolite binding. This analysis further
294 confirmed that wound slough is significantly enriched for proteins involved in skin barrier formation,
295

298 wound healing, blood clotting, as well as various immune functions including responding to bacteria,
299 acute inflammatory responses, immune effector cell responses, and humoral immunity (Fig. 1).
300

301 To determine if the protein composition of slough is associated with clinical features and wound healing
302 outcomes, hierarchical clustering using Euclidean distances was performed to identify patterns across
303 the dataset. Clustering appeared to be driven by the wound age at sample collection and clinical
304 outcome at 12 weeks-post collection defined as healed, ongoing but stable, or deteriorating (Fig. 2A).
305 Proteins differentially abundant in healing wounds compared to those that were stable or deteriorated,
306 were then determined using DEqMS.³⁰ Forty-eight proteins were differently abundant between healing
307 wounds and deteriorating wounds, while thirty-two proteins were differently abundant between healing
308 wounds and those that were ongoing yet stable (Fig. 2B-D, Table S4). GO Enrichment Analysis³² shows
309 that healing wounds are enriched for proteins involved in skin barrier development (e.g. cornifin-B, and
310 14-3-3 protein sigma), wound healing (e.g. beta-2-glycoprotein 1), blood clot formation (e.g. coagulation
311 factor XIII) and responses to bacteria and external stress (e.g. immunoglobins, cystatin-F, and
312 peroxiredoxin-6). Conversely, deteriorating wounds are enriched for proteins involved in immune
313 responses categorized as chronic inflammatory responses (e.g. AP-1 complex subunit gamma-1 and
314 NLR family proteins) and the compliment cascade (e.g. Complement factor H). Finally, differential
315 protein analysis between newer or older wounds found newer wounds (defined as being present for
316 less than 1 year) are enriched in proteins involved in epithelial barrier formation and integrity (e.g.
317 epithelial cell division and epithelial cell-to-cell adhesion), neutrophil degranulation, and response to
318 bacteria (Fig. S3).
319

320 Wound Slough is Polymicrobial and Associated with Wound Etiology and Body Site

321 To assess the microbial bioburden of slough samples, swabs were collected from the wound surface,
322 prior to washing and removal of slough via debridement. Bacterial bioburden was assessed by both
323 quantitative bacterial culture and quantitative-PCR of the bacterial 16S ribosomal RNA gene (Table S5).
324 Slough bioburden was generally high across all samples ranging from 1.0×10^2 to 8.0×10^7 colony forming
325 units (CFU) and 4.2×10^3 to 4.6×10^8 bacteria per inch² by qPCR. The quantity of bacteria determined by
326 qPCR and the quantity of bacteria detected through quantitative bacterial culture are highly concordant
327 (Fig. 3A, spearman r = 0.84, p-value < 0.01).
328

329 To determine the composition and spatial variation of bacterial communities within slough, samples
330 collected from slough at the edge and center of the wound were assessed through high-throughput
331 sequencing of the bacterial 16S ribosomal RNA marker gene (Fig. 3B, Fig. S4). Due to pain, subject-
332 001 did not have a sample collected from the wound center for this analysis. The major bacterial genera
333 detected were consistent with previous wound microbiome studies. Collectively, the most abundant taxa
334 from wound samples include *Corynebacterium* spp., *Pseudomonas* spp., and *Staphylococcus* spp. (Fig.
335 3B, Fig. S4). Overall microbiome community structure was generally consistent between the wound
336 edge and wound center. However, in some cases microbiome composition drastically differed, such as
337 in subject-008, where a single species appears to dominate the wound center while the wound edge
338 harbors a much more diverse microbiome. Microbial communities dominated by few taxa within the
339 center of the wound more often occurred in the subjects with large (surface area > 25 cm²), deep (> 10
340 cm³) wounds.
341

342 Principle component analysis was conducted to reduce the dimensionality of the microbiome data set
343 and explore the variability of samples (Fig. 3C-E). For this analysis any bacterial amplicon sequence
344 variants (ASVs) present in only one sample or averaged less than 1% of across all samples were
345 removed. Factors significantly associated with microbial community composition included the wound's
346 etiology and its location on the body (Type II permutation MANOVA $r^2 = 0.47$ and = 0.46 respectively,
347 both p-values < 0.01; Fig. 3C-D, Table S6). Notably, community composition was not associated with
348 spatial sampling at the wound edge or center, or the outcome of the wound 3 months following sample
349 collection (p-values > 0.05). The primary bacterial taxa that influenced sample position in the PCA plot
350 belong to *Corynebacterium*, *Pseudomonas*, *Staphylococcus*, and *Anaerococcus* species (Fig. 3E).

351

352 Detection of microbial aggregates in slough is highly variable

353 To evaluate potential commonalities in the microscopic structure of slough and associated microbial
354 aggregates, slough samples were visualized using both confocal scanning laser microscopy (CLSM)
355 and scanning electron microscopy (SEM). Overall, both techniques revealed slough to be highly
356 variable in structure and composition. CLSM of slough histological sections revealed heterogenous,
357 auto-fluorescent fibrinous tissue and DNA (Fig 4 and S5). SEM showed complex milli-, micron-, and
358 nano-meter scale features on the slough surface, consistent with fibrin and collagen fibrils, fibers, and
359 bundles (Fig S6). One semifluid sample (Subject-008) contained undefined crystalline structures.
360

361 Notably, microbes were only visible in three samples from the Wisconsin cohort, those from patients
362 with the highest slough bioburden, and four of the Australian subjects (Fig 4, S5). This indicates a lower
363 sensitivity of microscopy-based methods. Additionally, all three samples demonstrated different spatial
364 distributions of microbes. Subject-007 had small (~10 μ m) aggregates embedded in tissue localized to
365 a DNA-rich, layered region of solid slough (CLSM; Fig 4, S5). Subject-008 had large (>50 μ m) bacterial
366 aggregates surrounded by extracellular DNA and putative collage fibers within the core of the semifluid
367 slough, suggesting a biofilm community structure (CLSM; Fig 4). Subject-009 had putative collagen
368 bundles colonized with individual rods, cocci, and lancet-shaped bacteria (SEM; Fig S6). CLSM cross-
369 sections showed sparse bacteria in between tissue bundles (Fig 4).
370

371 Integrated analysis reveals key features of non-healing wounds

372 To predict the variables associated with wound healing outcome, an integrative analysis was pursued
373 encompassing protein clusters, microbial taxa relative abundance, and the numerical Bates-Jensen
374 Wound Assessment score. Datasets were integrated into a supervised Partial Least Squares -
375 Discriminant Analysis (PLS-DA).³⁴ To reduce the complexity of the proteomics dataset, K-means
376 clustering was first performed. Further, the top 15 microbial ASVs with greater than 1% relative
377 abundance in at least two subject samples were included (Table S7). PLS-DA revealed that the
378 proteomic and microbial composition of slough and Bates-Jensen scores can distinguish chronic
379 wounds that go on to heal versus those that deteriorate (Fig. 5A,B). Figure 4C illustrates the key
380 variables that help distinguish each outcome group along variate 1 of the PLS-DA plots. Wounds that
381 deteriorated were associated with a higher total Bates-Jensen Assessment score and sub-scores (e.g.
382 higher granulation tissue score, indicating smaller area of the wound bed covered by granulation tissue
383 and poor vascular supply; higher wound edge score, indicating more well-defined to thickened wound
384 edge; as well as greater wound depth); increased abundance of anaerobic taxa (e.g. *Finegoldia* ASV1,
385 *Peptoniphilus* ASV 2), and higher expression of protein clusters 6, 21, and 11. GO Enrichment Analysis
386 revealed that these clusters were enriched for proteins involved in immune responses, particularly
387 immune activation and responses to stimuli, cell motility, and intracellular processes (Fig. 6A).
388 Conversely, wounds that went on to heal were associated with higher abundance of *Acinetobacter* ASV
389 1 and protein clusters 22, 19, and 5 (Fig. 5C). These clusters were enriched for proteins involved in
390 metabolic and biosynthetic processing, gene expression, and regulation (including negative regulation)
391 of wound healing and responses to stress (Fig. 6C, Fig. S7, Table S8). Overall, the findings of this
392 integrative analysis highlight potentially fundamental differences in the microbial and proteomic
393 composition of slough from wounds that go on to heal compared to those at higher risk for progression.
394
395

396 Discussion

397 Slough is a highly common and burdensome feature of wounds. However, its definition and composition
398 remain poorly characterized. This pilot study aimed to characterize the host and microbial elements of
399 slough across a variety of wound etiologies. We also sought to identify key factors within slough
400 associated with wound healing trajectories. Our findings demonstrate that, i) the microscopic structure
401 of slough is heterogenous and unique to each wound; ii) across subjects wound slough is composed of
402 proteins involved in the structure and formation of the skin, blood clot formation, and various immune
403 responses; iii) the microbial community composition is diverse and corresponds to the wound's etiology

404 and location on the body; and iv) the composition of slough is associated with wound healing outcomes.
405 Collectively, these findings underscore how the composition of slough itself may be useful for
406 developing microbial and proteomic biomarkers prognostic of wound healing trajectories.
407
408 The clinical presentation of slough is highly variable. Slough can range in color from pale yellow to
409 yellow-green, tan, brown, or black to resemble eschar. It can also range in texture from mucous-wet to
410 thick and fibrinous, and range from loosely to firmly attached^{6,8} As expected with this variable clinical
411 presentation, confocal and scanning electron microscopic imaging reveals slough to be microscopically
412 heterogenous and different across wounds (Fig. S5-6). However, as noted by others, slough's intrinsic
413 gelatinousness consistency makes it easy to perturb and difficult to fix for microscopic assessment.³⁶
414 This likely limits our ability to ascertain additional three-dimensional features within slough that may be
415 pertinent to the wound surface environment.
416
417 Microbial biofilm is thought to be highly integrated within wound slough.²¹ In the clinical setting wound
418 slough is often mistaken for microbial biofilm.^{15,21} To address this, several studies have proposed wider
419 adoption of culture based, molecular (i.e. quantitative-PCR), and microscopic techniques into diagnostic
420 practice.³⁷⁻⁴⁰ At the time of sample collection, only one of the ten subjects was diagnosed with a current
421 wound infection and five had a history of infection in the sampled wound (Table 1). However, SEM and
422 CLSM imaging detected microbes in only three of nine samples tested (Fig. S5-6). Interestingly, subject-
423 009, who had no record of current or previous wound infection, was the only subject to have microbes
424 visualized via both SEM and CLSM. This speaks to the difficulty in identifying biofilm or even the
425 presence of single cells of bacteria using microscopy techniques as more sensitive molecular methods
426 indicated every sample contained a considerable bioburden of bacteria. Further, the detection rates for
427 microbial biofilm in this study are notably lower than previously reported for chronic wound samples.³⁹⁻⁴¹
428 This could be due to a number of factors, including spatial heterogeneity of bacterial aggregates
429 across the wound surface. Indeed, to saturate sampling efforts hundreds of slides and images would
430 need to be obtained. To improve detection rates the incorporation of methods that increase specificity
431 of bacterial detection such as immunogold labeling or gold in situ hybridization could be applied, but
432 remain impractical for routine clinical evaluation.^{42,43}
433
434 The quantity of bacteria determined via qPCR correlates with the bacterial burden as determined by
435 quantitative culture (Fig. 3A). The reference standard for clinical definition of a wound infection is 10^5 or
436 more cultured colony forming units (CFU)/ml.³⁷ By that metric, eight of the ten subjects meet definition
437 for clinical infection, despite an absence of clinical sign of infection (Fig. 3A). Indeed, only one subject
438 had a diagnosed infection. While the use of qPCR for detecting bacterial bioburden is more sensitive,
439 particularly for patients like subject-009 whose wounds may contain more anaerobic or difficult to culture
440 bacteria (Fig. 3A,B), this data suggests the use of such cutoffs are complicated and should be used
441 with caution. Indeed, wounds with high bacterial bioburden can go on to heal without intervention with
442 antibiotics.
443
444 Isolation and identification of bacteria from all subjects through both microbial culture and 16S
445 sequencing underscores that even in the absence of a clinical wound infection, slough contains complex
446 microbial communities (Fig. 3A-B, Table S5). Previous work evaluating the influence of sharp
447 debridement on the wound microbiome further has shown that these microbes are likely highly
448 integrated within and throughout wound slough.¹⁶ Here, the most frequently isolated via microbial
449 culture were *Corynebacterium*, *Staphylococcus*, and *Pseudomonas* species (Table S6).
450 *Corynebacterium*, *Staphylococcus*, and *Pseudomonas* were also the most abundant taxa identified via
451 16S profiling, comprising at least 30% of the microbial community in slough three of the ten subjects
452 respectively (Fig. 3B). Across wound etiologies *Corynebacterium*, *Staphylococcus*, and *Pseudomonas*
453 species appear to be the most abundant taxa within the chronic wound microbiome (Fig. 3B).^{16-18,44}
454 Contradicting some previous reports,^{16,18,44} we find slough microbial community composition to be
455 associated wound etiology and location on the body (Fig. 3C-E, Table S6). The microbiome of healthy
456 intact skin naturally varies across body sites due to differences in the physiologic characteristics of the

457 local skin environment.⁴⁵⁻⁴⁹ It is plausible that these variations in the microbiome of the surrounding skin
458 influence the community structures within wound slough.

459

460 In terms of the human components of the wound, there are only a handful of reports on the proteomic
461 composition of tissue biopsies, granulation tissue, and exudative fluid from chronic pressure ulcers and
462 diabetic wounds.⁹⁻¹⁴ Broadly speaking, these studies find fluid and tissue from these wounds to contain
463 various types of collagen, extracellular matrix proteins, matrix metalloproteases, clotting factors and
464 proteins generally related to innate and acute immune responses.⁹⁻¹⁴ To our knowledge, this is the first
465 study to specifically evaluate the proteomic composition of wound slough. In line with these previous
466 studies, slough from chronic wounds is primarily composed of keratin and various types of collagen,
467 extracellular matrix proteins, matrix metalloproteases, clotting factors, and immune response proteins
468 (Table S2, Fig. 1). More specifically, slough is enriched for proteins involved in skin barrier integrity and
469 formation, wound healing, and immune functions ranging from innate compliment activation to acute
470 responses to stimuli (e.g. to bacteria) and humoral immune responses (Fig. 1, Fig. S2). The high
471 prevalence of intracellular and skin associated proteins combined with the relative absence of
472 enrichment for vascular and angiogenic pathways supports that hypothesis that slough is largely
473 devitalized tissue. However, many of these proteins may be functional in this environment. Further, the
474 collective abundance of proteins associated with inflammatory cells such as neutrophils, underscores
475 the leading theory that slough is a biproduct of prolonged inflammatory process.^{6,7}

476

477 Of the three main data sets assessed (proteomics, microbiome, and the Bates Jensen Wound
478 Assessment), the proteomics dataset had the strongest associations with wound healing outcome.
479 When assessed independently, Bates-Jensen Wound Assessment Tool (BWAT) scores were not
480 significant for wounds that deteriorated, nor were there associations between microbiome composition
481 and wound outcome (Table 2, Fig. 3). Although these analyses were likely limited due to low subject
482 numbers, differential Protein Expression Analysis (DeqMS)³⁰ found wounds that went on to heal were
483 enriched for proteins involved in skin barrier development, wound healing, blood clot formation, and
484 responses to bacteria and external stress (Fig. 2B-D, Table S4). Conversely, deteriorating wounds were
485 enriched for proteins involved in immune responses categorized as chronic inflammatory responses
486 and the compliment cascade. Of the proteins enriched in wounds that deteriorated; AP-1 is a notable
487 biphasic regulator of wound healing⁵⁰; NLR family proteins and Caveolase-associated protein 1 have
488 been associated with impaired wound healing in murine models⁵¹⁻⁵³; and CD177, compliment factor H,
489 and vasodilator-stimulated phosphoprotein have also been noted to be elevated in chronic wound fluid
490 and/or tissue.^{11,12} To identify variables associated with wound healing we incorporated proteomics,
491 microbiome, and the BWAT score datasets into a supervised Partial Least Squares - Discriminant
492 Analysis (PLS-DA).³⁴ In this model, slough from wounds that healed were enriched for proteins involved
493 in regulation, particularly negative regulation, of immune responses and wound healing as well as the
494 aerobic microbial taxa *Acinetobacter* (Fig. 5-6). Conversely, wounds that deteriorated contained slough
495 enriched with inflammatory proteins, particularly those involved in immune activation, responding to
496 stimuli and chronic inflammation. Wounds that deteriorated were also associated with greater
497 abundance of anaerobic microbial taxa, *Finegoldia* and *Peptoniphilus*, as well as higher Bates-Jensen
498 wound assessment scores, indicating a more severe wound state.

499

500 Overall, our model's findings are consistent with related literature. For instance, they underscore
501 BWAT's clinical utility in a well-rounded wound evaluation, and suggest that high sub-scores for
502 granulation, wound edge, wound depth, and exudate amount may hold the strongest predictive potential
503 for identifying a wound likely to deteriorate (Fig. 5).^{24,35,54} From a microbiological perspective, high
504 abundance of anaerobic taxa and select *Staphylococci* species are frequently associated with impaired
505 wound healing and poor outcomes.^{16,17,55,56} Similar proteomic investigations with wound fluid and tissue
506 biopsies also find elevated inflammatory proteins and enrichment of proteases and matrix
507 metalloproteinases in wounds that do poorly as well as enrichment of extracellular matrix proteins and
508 keratin in healing wounds.^{10,12} This work demonstrates that slough, which is often regularly debrided as
509 a part of standard care, provides a readily available, underutilized, high protein concentration biomarker

510 reservoir. Most of the proteins identified through independent DeqMS assessment also fall within the
511 protein clusters that distinguish between healed and non-healing wounds in the comprehensive model
512 (Fig. 2 and 5, Tables S2, S4 and S8), suggesting these identified proteins have the greatest potential
513 as biomarker targets to predict wound healing.
514

515 The primary limitation of this pilot study is the small number of samples enrolled. Future investigations
516 intend to expand upon these methods, potentially with even more targeted proteomic and microbiologic
517 approaches, to validate the predicted features associated with wound healing outcome in a larger
518 cohort. This study is also limited in the collection of tissue samples from the wound bed itself, after
519 removal of slough. Future studies should consider collecting both slough and wound tissue samples to
520 understand the proportion of slough proteins that overlap with proteins also found in the wound bed.
521 There were also several factors that inhibited the microscopic detection of microorganisms via SEM
522 and CLSM. For instance, initial cleansing of the wound with soap and water prior to debridement per
523 standard of care, may have removed superficial microbial aggerates. With SEM, there are no efficient
524 algorithms for distinguishing individual microbes or microbial biofilm from background collagen fibers
525 and tissue. The heterogeneous and often gelatinous texture of slough along with the ability to only view
526 a histological cross-section, may have also limited microbial detection via CLSM. Our microbial
527 assessment with 16S amplicon sequencing only provides genus level resolution, and not all species
528 within a genus have propensity to cause infection (e.g. *Staphylococcus aureus* vs. *Staph. hominis*).
529 Evaluating chronic wound metagenomes would provide species level resolution and detect the
530 presence of virulence and antibiotic resistance genes. However, to date there are very few
531 investigations into wound microbial metagenomes as this method remains limited due to cost.¹⁷
532

533 In conclusion, slough is an underutilized reservoir for potential microbial and proteomic biomarkers. To
534 our knowledge this is the first study to integrate clinical wound assessment, microbiome, and proteomic
535 data into a single assessment for the prediction of wound healing outcome. Future studies intend to
536 utilize these and similar methods to further explore the biomarkers within slough in a larger cohort with
537 appropriate statistical power. Utilization of a comprehensive patient-centered assessment will lead to
538 more effective identification of high-risk patients wounds for triage into specialty care, ultimately,
539 reducing the healthcare, financial, and personal burden of living with hard to heal wound.
540
541

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Tables

Table 1: Subject and Wound Characteristics

	All Subjects (n = 10)
Age (yr: Mean \pm SD)	66 \pm 11
Biologic Sex (M:F)	4 : 6
Race	
White	9
Black / African American	1
Wound Characteristics	
Wound Age (yr: Mean \pm SD)	2.4 \pm 4.5
Wound Etiology	
Pressure Ulcer	2
Surgical Infection	2
Trauma	2
Venous Stasis Ulcer	4
Wound Location	
Coccyx	2
Shin	4
Posterior Lower Leg	1
Ankle	3
History of the Wound	
Previously Debrided	6
Previously Infected	5
Current Infection	1
Wound Outcome at 12 weeks	
Healed	3
Ongoing, Stable	4
Deteriorated	3
Patient Comorbidities	
BMI (Mean \pm SD)	43.0 \pm 18.6
Anemia	2
Heart Disease	3
Pre-Diabetes	1
Diabetes	4
Hypertension	5
Lymphedema	2
Neuropathy	1
Paraplegia	2
Peripheral Vascular disease	4
Former or Current Smoker	4
History of Alcohol Use Disorder	1

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Table 2: Bates-Jensen Wound Assessment Scores By Wound Healing Outcome at 3 months.
Data represented as mean +/- standard deviation

	Healed (n=3)	Ongoing (n=4)	Deteriorated (n = 3)	All Subjects (n = 10)
Size	2.00 ± 1.00	3.25 ± 1.71	3.00 ± 2.00	2.80 ± 1.55
Depth	2.67 ± 0.58	2.75 ± 0.5	4.00 ± 1.00	3.10 ± 0.88
Edges	2.67 ± 1.15	3.25 ± 0.5	3.67 ± 0.58	3.20 ± 0.79
Undermining	2.33 ± 2.31	1.50 ± 1.00	2.33 ± 2.31	2.00 ± 1.70
Necrotic Tissue Type	2.67 ± 0.58	3.00 ± 0.82	3.00 ± 1.00	2.90 ± 0.73
Necrotic Tissue Amount	2.67 ± 0.58	2.75 ± 0.5	2.67 ± 2.08	2.70 ± 1.06
Exudate Type	2.67 ± 0.58	3.25 ± 0.5	3.67 ± 1.15	3.20 ± 0.79
Exudate Amount	3.33 ± 0.58	3.50 ± 0.58	4.33 ± 0.58	3.70 ± 1.32
Skin Color Surrounding Wound	1.00 ± 0	1.75 ± 1.50	2.67 ± 1.53	1.80 ± 1.32
Edema	1.67 ± 1.15	2.50 ± 1.73	2.33 ± 1.53	2.20 ± 1.40
Induration	1.00 ± 0	1.75 ± 0.96	2.00 ± 1.73	1.60 ± 1.08
Granulation	2.00 ± 1.00	3.75 ± 0.50	4.00 ± 0	3.30 ± 1.06
Epithelialization	5.00 ± 0	4.75 ± 0.50	5.00 ± 0	4.90 ± 0.32
Total Score	31.67 ± 4.93	37.75 ± 3.86	42.67 ± 3.06	37.4 ± 5.7

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752 **Figure Legends**

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754 **Figure 1: Wound slough is enriched for proteins involved in skin barrier formation, wound**

755 **healing, blood clotting, and various immune functions including responding to bacteria.**

756 Debrided slough tissue was sent for proteomic characterization via mass spectrometry. The most

757 abundant proteins across all samples were input as a ranked list to the Gene Ontology enrichment

758 analysis (GORILA) and visualization tool.²⁹ Significantly enriched GO terms are listed by their

759 description and ordered by their FDR-qValue. GO terms associated with extracellular and cellular

760 components are in the top section (reds), those associated with molecular functions are in the middle

761 (oranges to yellows), and those associated with biologic processes are in the bottom section (yellow-

762 greens to blues). Associated color-coded trimmed directed acyclic graphs (DAG) of all significantly

763 enriched GO terms as grouped by component, function, and biologic process are in [supplemental figure](#)

764 [2](#). More detail, including GO term annotations, descriptions, enrichment, number of proteins (Uniprot

765 Genes) involved from our dataset involved in each GO Term, and FDR-qValues are in [supplemental](#)

766 [table 3](#).

767

768 **Figure 2: Wounds that go on to heal, are ongoing, or deteriorate are enriched for different**

769 **proteins.** A) heat map demonstrating each proteins' relative expression across all subjects

770 demonstrates that samples largely grouped by the wounds relative age and outcome 3 months after

771 the sample collection (e.g whether the wound went on to heal, is ongoing but stable, or continued to

772 deteriorate). B-D) Subjects were grouped by the wound's outcome and groups were assessed for

773 differential protein expression via DEqMS. Volcano plots indicating the proteins with significantly greater

774 expression in; B) wounds that continued to deteriorate vs. wounds that went on to heal; C) wounds that

775 were stable but on going vs. those that healed; D) and wounds that continued to deteriorate vs. wounds

776 that were ongoing but stable. Biologic functions of these highly expressed proteins were determined by

777 the Gene Ontology Database. In brief, wounds that healed are enriched for proteins involved in skin

778 barrier development, wound healing, blood clot formation, responses to bacteria and external stress.

779 Wounds that deteriorated have higher expression of proteins involved in chronic inflammatory

780 responses, the compliment cascade and a *pseudomonas* histone kinase. [Supplemental figure 3](#)

781 displays the volcano plot for differential protein expression in younger vs. older wounds.

782

783 **Figure 3: Microbial communities at a wound surface are largely dictated by the body site where**

784 **the wound is located and the wound's etiology.** Swabs of the surface microbiome were collected

785 from the wound edge and wound center. Subject-001 did not have a sample collected from the wound

786 center due to pain. A) The number of bacteria per inch² determined by quantitative-PCR (qPCR)

787 strongly correlates with the number of bacteria detected through quantitative bacterial culture (measured

788 in bacterial colony forming units [CFU]). Points are colored by subject. B) Relative abundance of

789 bacterial genera on the surface of each subject's wound center (top) and wound edge (bottom) based

790 on high-throughput sequencing of the bacterial 16S ribosomal gene. For each sample, bacterial taxa

791 that were < 1% abundant were grouped into the "Other" category along with any un-classified bacterial

792 sequences. Genera are grouped by the phyla in which they belong; Actinomycetota (blues), Bacillota

793 (greens to orange-yellows), Bacteroidota (oranges), Campylobacter (deep orange-red),

794 Pseudomonadota (reds), and Thermodesulfobacteriota (deep red). Relative abundance of genera

795 within bolded indicates that the genera comprises > 10% of at least one sample. An * indicates that the

796 genera comprises > 30% of at least one sample. C-D) Principal component analysis indicated that

797 wound surface microbial communities cluster by both the wound's etiology (C) and the wounds location

798 on the body (D). Plots C and D are the same but colored differently to highlight the sample groupings

799 by etiology and body site respectively. Microbiome samples did not cluster by whether the swab was

800 taken from the wound edge or center, the wounds age, or the wounds outcome 3 months after the

801 sample collection (e.g whether the wound went on to heal or did not). E) A vector plot indicating the

802 primary bacterial ASVs that dictated a points position in the PCA plot C-D. These ASV's belong to

803 *Corynebacterium*, *Pseudomonas*, *Staphylococcus*, and *Anaerococcus* species.

804

805 **Figure 4: Confocal scanning laser microscopy of bacteria aggregates in slough.** Formalin-fixed,
806 paraffin-embedded (FFPE) slough samples were stained with a universal bacterial 16S rRNA probe
807 (red) and for double stranded DNA (DAPI, blue) then visualized with confocal scanning laser microscopy
808 (CSLM). Autofluorescence of the surrounding tissue was visualized in green. Only specimens with
809 detected bacterial aggregates are shown here. Images from specimens with no bacterial aggregates
810 are in [supplemental figure 5](#).
811

812 **Figure 5: Chronic wounds that go on to heal can be distinguished from those that deteriorate**
813 **via the proteomic, microbial, and clinical features of slough.** To predict the variables associated
814 with wound healing, the protein cluster, microbial, and the Bates - Jensen Wound Assessment datasets
815 were integrated into a supervised Partial Least Squares - Discriminant Analysis (PLS-DA) via the
816 MixOmics package. To simplify the proteomics dataset, proteins were grouped into 23 k-means clusters
817 via the gap-stat method ([Table S2](#)). Since there was no significant difference in the microbial community
818 composition at the wound edge or center, samples were combined to create a summative wound slough
819 microbiome for each subject. The “key” 14 microbial ASVs with greater than 1% relative abundance in
820 at least two subjects’ slough samples were included in this integrative analysis ([Table S7](#)). A) PLS-DA
821 plots for the protein cluster, microbial ASV, and wound assessment data set respectively. Each dataset
822 contains variables that can distinguish chronic wounds that go on to heal from those that deteriorate.
823 Outcome groups most clearly separate along variate 1 for each of the datasets. B) PLS-DA plot for all
824 the data sets combined. The asterisk indicates the centroid position where the subject’s slough sample
825 falls considering variables from all three datasets. Arrows from the centroid indicate the direction that
826 variables from each individual dataset pull the subject’s datapoint. C) Variable plots of the protein
827 clusters (blue), microbial taxa (orange) and wound assessment criteria (red) that distinguish each
828 outcome group along variate 1 of the PLS-DA plots. A longer vector to the right indicates a variable with
829 greater influence pulling samples to the right along the variate 1 axis. The enriched GO biologic
830 processes for representative slough protein clusters that distinguish slough from wounds with each
831 outcome are in [figure 5](#).
832

833 **Figure 6: Slough from wounds that go on to deteriorate are enriched for immune activation and**
834 **inflammatory immune responses.** To determine the key biologic processes associated with each
835 protein k-means cluster, proteins within each cluster were submitted as unranked lists to the GO
836 Enrichment analysis tool for evaluation with the PANTHER Overrepresentation test. Details for this
837 analysis are included in [supplemental table 8](#). This figure displays the top 25 most enriched GO biologic
838 process for three representative protein clusters that distinguish slough from wounds that deteriorated
839 three months following sample collection from those that ([Fig. 4](#)). For each cluster the biologic
840 processes are ordered from most significantly enriched at the top to least enriched at the bottom. Color
841 of the point indicates the broader biologic classification. A) Wounds that deteriorated are enriched for
842 immune cell activation and inflammatory immune responses, responses to stimuli and stress, cell
843 motility, intracellular transport and intracellular processes. B) Wounds that were stable but ongoing were
844 enriched for responses to stress, metabolic processes, and gene expression. C) Wounds that went on
845 to heal were enriched for metabolic and biosynthetic processing, gene expression, and regulation
846 (particularly negative regulation) of wound healing and responses to stress. [Supplemental figure 5](#)
847 displays the significantly enriched GO biologic processes for all 23 k-means clusters.
848
849

850 Supplemental Figure Legends

851 **Supplemental Figure 1: Photos of subject wounds before debridement procedure.**

852 **Supplemental Figure 2: Directed Acyclic Graph (DAG) of the significantly enriched gene**
853 **ontology (GO) terms grouped by biologic processes (A) molecular functions (B) and cellular**
854 **components (C) within wound slough.** The most abundant proteins across all slough debridement
855 tissue samples were input as a ranked list to the Gene Ontology enrichment analysis (GORILA) and
856

858 visualization tool.²⁹ Figure 1 displays the enriched GO terms associated with each DAG. The
859 significantly enriched GO terms for each DAG are displayed. Box colors indicate p-values; white > 10⁻³
860 ; yellow 10⁻³ – 10⁻⁵, yellow-orange 10⁻⁵ – 10⁻⁷, orange 10⁻⁷ – 10⁻⁹, Red < 10⁻⁹. More detail, including GO
861 term annotations, descriptions, enrichment, number of proteins (Uniprot Genes) involved from our
862 dataset involved in each GO Term, and FDR-qValues are in [Supplemental Table 3](#).
863

864 **Supplemental Figure 3: Chronic wounds present less than 1 year are enriched for proteins**
865 **involved in epithelial barrier formation, neutrophil degranulation, and response to bacteria.**
866 Conversely, wounds present for more than 1 year are enriched for proteins involved in iron
867 sequestration and tRNA metabolism. Subjects were grouped the age of the wound at the time of sample
868 collection. Wounds present for less than 1 year were considered “young”, and those present for more
869 than 1 year were considered “old.” Groups were assessed for differential protein expression via
870 DEqMS. This volcano plot displays the proteins with significantly greater expression in younger or older
871 wounds.
872

873 **Supplemental Figure 4: The Abundance of key bacterial taxa is similar across wound slough**
874 **from two distinct subject cohorts from Wisconsin and Australia.** Datasets were generated using
875 amplicon sequencing of the V4 (panel A, Wisconsin) or V1V3 (panel B, Australia) regions of the 16S
876 rRNA gene, and were thus analyzed separately. ASVs were summed at the genus level. Note that the
877 taxonomic resolution for classification may differ by amplicon region. Genera are shown if present at
878 above 5% relative abundance in at least one specimen and are ordered by mean relative abundance
879 across all specimens within a dataset. Subjects are ordered by average linkage hierarchical clustering
880 of Bray-Curtis dissimilarities. In the Wisconsin cohort (panel A), subject taxa profiles are averaged from
881 multiple specimens.
882

883 **Supplemental Figure 5: Confocal scanning laser microscopy of slough samples without**
884 **bacterial aggregates.** Formalin-fixed, paraffin-embedded (FFPE) slough samples were stained with a
885 universal bacterial 16S rRNA probe (red) and for double stranded DNA (DAPI, blue) then visualized
886 with confocal scanning laser microscopy (CSLM). Autofluorescence of the surrounding tissue was
887 visualized in green. The specimens with detected bacterial aggregates are shown in figure 4. Here are
888 the remaining specimens from both patient cohorts that did not have identifiable bacterial aggregates.
889

890 **Supplemental Figure 6: Scanning electron microscopy finds slough to be variable in structure**
891 **and unique to the subject.** Debrided slough samples were evaluated via scanning electron
892 microscopy (SEM). Subjects-004, -005, and -006 did not have enough debridement tissue for SEM.
893 One subject, subject-009 had visible microorganisms on SEM. A majority of specimens were fibrous in
894 appearance, while one specimen had crystalline structures.
895

896 **Supplemental Figure 7: Enriched GO biologic processes for each of the 23 k-means protein**
897 **clusters.** To determine the key biologic processes associated with each protein k-means cluster,
898 proteins within each cluster were submitted as unranked lists to the GO Enrichment analysis tool for
899 evaluation with the PANTHER Overrepresentation test. Details for this analysis are included in
900 [supplemental table 8](#). This figure displays the top 25 most enriched GO biologic process each of the 23
901 k-means protein clusters. For each biologic processes are ordered from most significantly enriched at
902 the top to least enriched at the bottom. Color of the point indicates the broader biologic classification.
903
904

905 Supplemental Table Legends

906

907 **Supplemental table 1: Detailed subject and wound characteristics.** Ten subjects with chronic or
908 slow to heal wounds of various etiologies were enrolled from the UW-Health Wound Care Clinic.
909 Wounds were evaluated with the Bates-Jensen Wound Assessment Tool. Information on the wound
910 and patient comorbidities were extracted from the medical record at the time of sample collection.

911 Information on whether the wound went on to heal, was ongoing yet clinically stable, or deteriorated 3
912 months following sample collection was also recorded. This table serves as a compliment to [table 1 and](#)
913 [2](#), providing subject level detail on the wound and patient comorbidities.
914

915 **Supplemental Table 2: Proteomic composition of wound slough.** Normalized and means centered
916 protein peptide abundance within each subject's wound slough. Description, species of origin (eg.
917 Homosapiens or bacteria), broad GO biological process, GO cellular component, GO molecular
918 function, WikiPathways, Reactome Pathways, and KEGG pathways for each protein peptide accession
919 are also included. To simplify the proteomics dataset for integrative PLS-DA analysis proteins were
920 grouped into 23 clusters via the gap-stat method. The Kmeans cluster in which the protein falls is also
921 indicated.
922

923 **Supplemental Table 3: Data-frame of the GO terms that are significantly enriched in chronic**
924 **wound slough.** Debrided slough tissue was sent for proteomic characterization via mass spectrometry.
925 A) The most abundant proteins across all samples were input as a ranked list to the Gene Ontology
926 enrichment analysis (GORILA) and visualization tool.²⁹ Significantly enriched GO terms are listed with
927 their GO term annotations, descriptions, enrichment, number of proteins (Uniprot Genes) involved from
928 our dataset involved in each GO Term, and FDR-qValues. Visual representations of this data can be
929 found in [figure 1 and supplemental figure 2](#).
930

931 **Supplemental Table 4: Proteins with significantly greater expression subjects grouped by**
932 **outcome or wound age.** Subjects were grouped by the wound's outcome (healed, ongoing,
933 deteriorated), or wound age (young [wounds present < 1 year], or old [wounds present > 1 year]) and
934 groups were assessed for differential protein expression via DEqMS. Only proteins with significantly
935 greater expression (\log_2 Fold change > 1 and \log_{10} P-value < 10^{-2}) are displayed. Details on the protein
936 description and associated GO terms, WikiPathways, Reactome Pathways, and KEGG pathways are
937 included. Volcano plots indicating the proteins with significantly greater expression in each of the
938 associated group comparisons are in [figure 2 B-D and supplemental figure 5](#).
939

940 **Supplemental Table 5: Bacterial bioburden and Identification of Cultured bacteria from the**
941 **wound surface.** Swabs of the wound slough microbiome were collected into either DNA/RNA Shield
942 or liquid ames broth. Bacterial DNA was extracted from the samples collected into DNA/RNA Shield,
943 and bacterial bioburden was assessed through quantitative PCR of the 16S ribosomal gene. Samples
944 collected into liquid ames broth were plated on to blood agar and grown overnight at 37C for quantitative
945 bacterial culture. Individual bacterial colonies with distinct morphologies were isolated and grown
946 overnight. To identify the genus of these isolates, bacterial DNA was extracted and sent for sanger
947 sequencing of the 16S bacterial ribosomal gene. Genera of successfully identified isolates are listed.
948

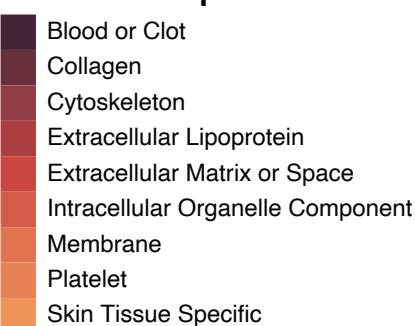
949 **Supplemental Table 6: Univariate type 2 permutation MANOVA results indicate that microbial**
950 **community composition were the wound's etiology and its location on the body.** Table of
951 univariate type 2 permutation MANOVA results. Each permutation MANOVA was run via the Euclidian
952 method with 9999 permutations using the Adonis 2 r package. * Indicates p-value less than 0.05. **
953 Indicates p-value less than 0.01.
954

955 **Supplemental Table 7: relative abundance for key microbial ASV's in wound slough.** Since there
956 was no significant difference in the microbial community composition at the wound edge or center (Type
957 II permutation MANOVA p-value > 0.5, [Table S6](#)), samples were combined to create a summative
958 wound slough microbiome for each subject. Relative abundance of each ASV in the wound center and
959 wound edge were averaged. This table displays the 14 microbial ASVs with greater than 1% relative
960 abundance in at least two summative subject slough microbiome samples, which were included in the
961 integrative PLS-SA analysis. Anaerococcus ASV 3 (6a418787996565e7641dbbf39b7d3e18) and
962 Staphylococcus ASV 1 (18af7b7f2b61429936fcfd63a453fcefd) from [figure 3](#) are not included here since
963 they were only present in samples from one subject (subject-006) and subsequently not included in the

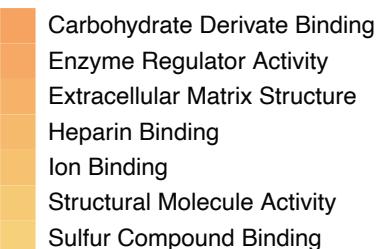
964 PLS-DA. Although not included in the PLS-DA analysis, the relative abundance of all other taxa, which
965 were either only present in samples from that subject, or present at < 1% is also indicated to highlight
966 the proportion of taxa within a subject that were of low abundance or unique to that subject.
967

968 **Supplemental Table 8: Most enriched GO biologic processes for each of the 23 k-means protein**
969 **clusters.** To determine the key biologic processes associated with each protein k-means cluster,
970 proteins within each cluster were submitted as unranked lists to the GO Enrichment analysis tool for
971 evaluation with the PANTHER Overrepresentation test. This table depicts the 25 most significantly
972 enriched GO biologic processes for each protein cluster and includes the associated go terms, the
973 broader classification, number of protein IDs in *Homo sapiens* reference database, number of IDs in
974 uploaded K-means cluster, the expected number of IDs, fold enrichment, p-value, and false discovery
975 rate (FDR) if it was able to be calculated. To determine the most enriched biologic processes, terms by
976 the smallest to largest FDR, followed by smallest to largest p-value if FDR was unable to be calculated.
977 A rank of 1 indicates that it was the most enriched biologic process in the protein cluster. [Supplemental](#)
978 [table 2](#) includes details on the proteins within each cluster.
979

Cellular Component



Molecular Function



Biologic Process



Figure 1: Wound slough is enriched for proteins involved in skin barrier formation, wound healing, blood clotting, and various immune functions including responding to bacteria. Debrided slough tissue was sent for proteomic characterization via mass spectrometry. The most abundant proteins across all samples were input as a ranked list to the Gene Ontology enrichment analysis (GORILA) and visualization tool.²⁷ Significantly enriched GO terms are listed by their description and ordered by their FDR-qValue. GO terms associated with extracellular and cellular components are in the top section (reds), those associated with molecular functions are in the middle (oranges to yellows), and those associated with biologic processes are in the bottom section (yellow-greens to blues). Associated color-coded trimmed directed acyclic graphs (DAG) of all significantly enriched GO terms as grouped by component, function, and biologic process are in supplemental figure 2. More detail, including GO term annotations, descriptions, enrichment, number of proteins (Uniprot Genes) involved from our dataset involved in each GO Term, and FDR-qValues are in supplemental table 3.

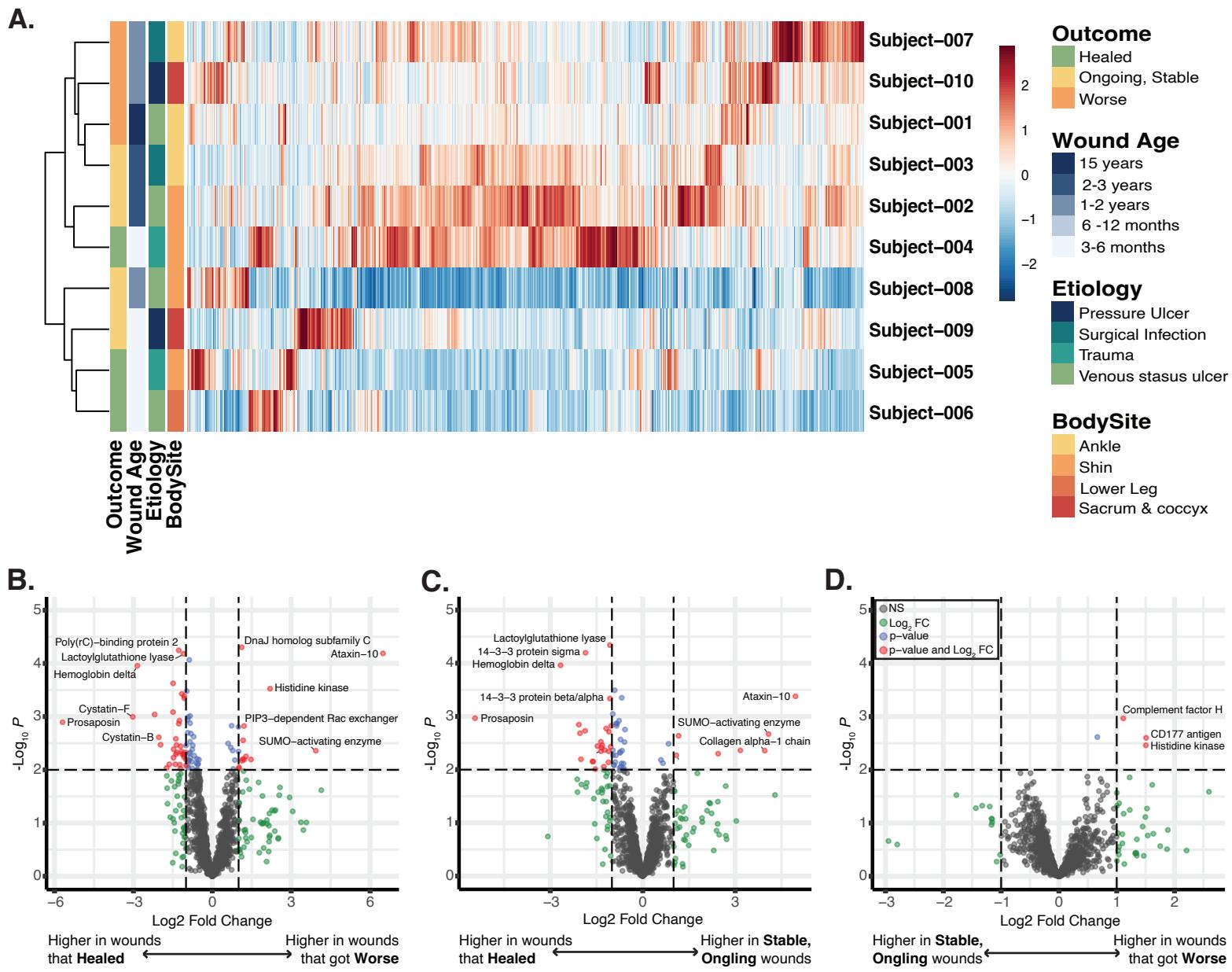
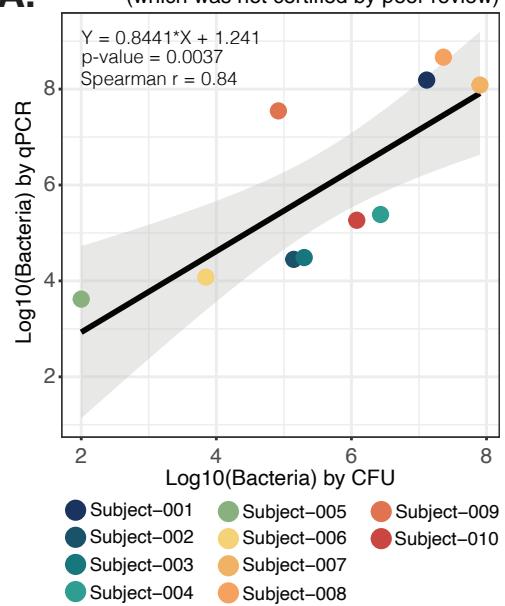
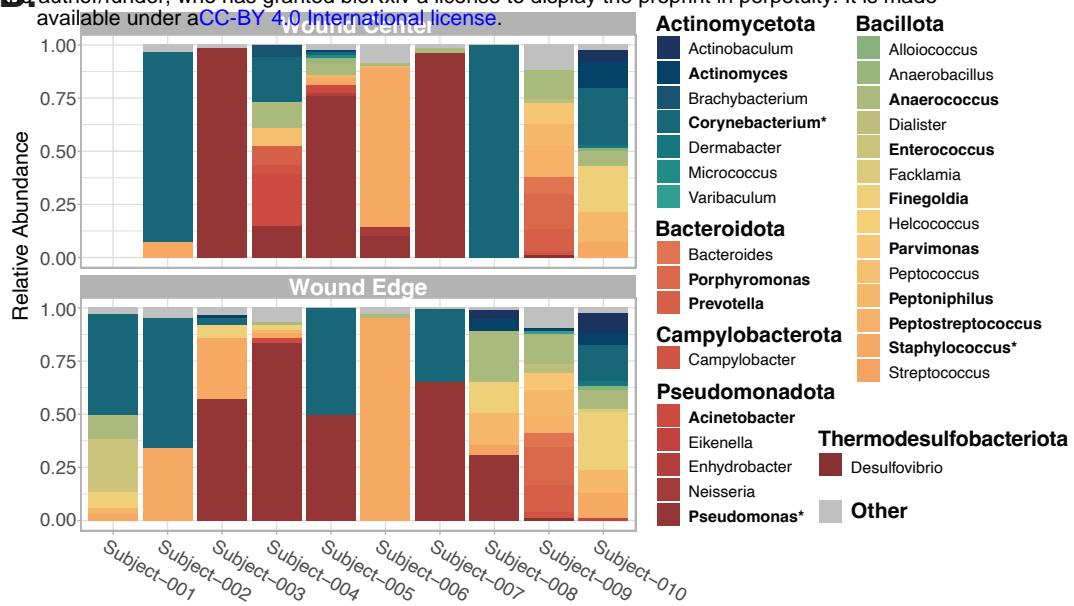


Figure 2: Wounds that go on to heal, are ongoing, or deteriorate are enriched for different proteins. A) heat map demonstrating each proteins' relative expression across all subjects demonstrates that samples largely grouped by the wounds relative age and outcome 3 months after the sample collection (e.g whether the wound went on to heal, is ongoing but stable, or continued to deteriorate). B-D) Subjects were grouped by the wound's outcome and groups were assessed for differential protein expression via DEqMS. Volcano plots indicating the proteins with significantly greater expression in; B) wounds that continued to deteriorate vs. wounds that went on to heal; C) wounds that were stable but ongoing vs. those that healed; D) and wounds that continued to deteriorate vs. wounds that were ongoing but stable. Biologic functions of these highly expressed proteins were determined by the Gene Ontology Database. In brief, wounds that healed are enriched for proteins involved in skin barrier development, wound healing, blood clot formation, responses to bacteria and external stress. Wounds that deteriorated have higher expression of proteins involved in chronic inflammatory responses, the compliment cascade and a pseudomonas histone kinase. Supplemental figure 3 displays the volcano plot for differential protein expression in younger vs. older wounds.

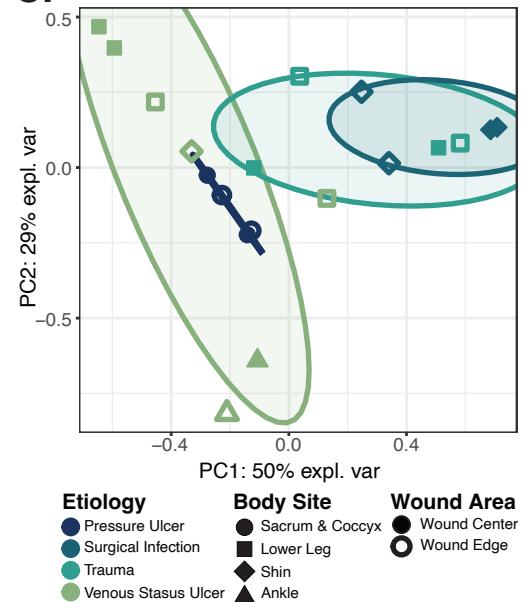
A.



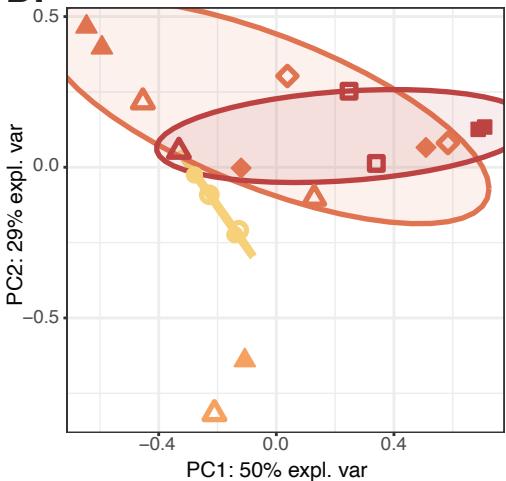
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C.



D.



E.

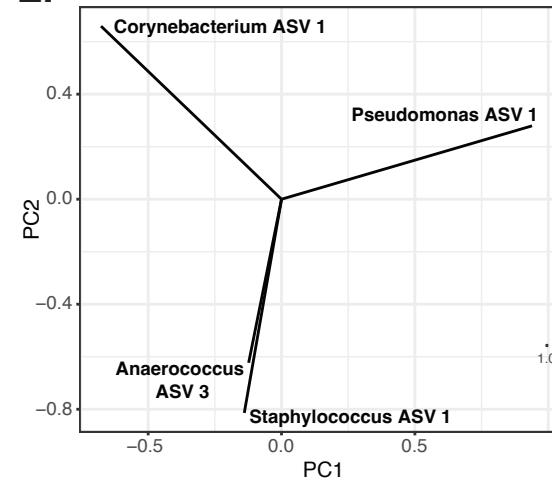
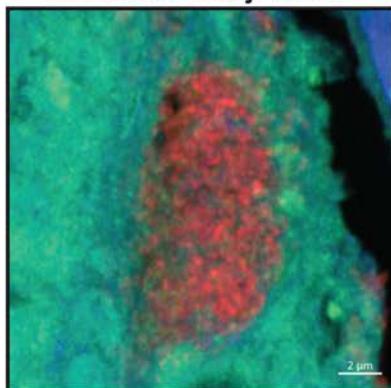
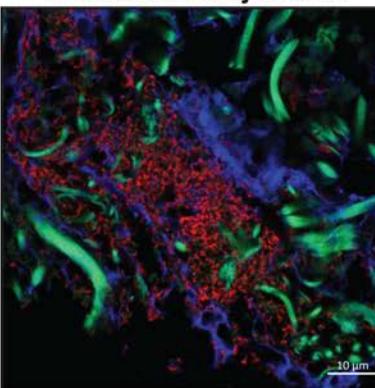


Figure 3: Microbial communities at a wound surface are largely dictated by the body site where the wound is located and the wound's etiology. Swabs of the surface microbiome were collected from the wound edge and wound center. Subject-001 did not have a sample collected from the wound center due to pain. A) The number of bacteria per inch² determined by quantitative-PCR (qPCR) strongly correlates with the number of bacteria detected through quantitative bacterial culture (measured in bacterial colony forming units [CFU]). Points are colored by subject. B) Relative abundance of bacterial genera on the surface of each subject's wound center (top) and wound edge (bottom) based on high-throughput sequencing of the bacterial 16S ribosomal gene. For each sample, bacterial taxa that were < 1% abundant were grouped into the "Other" category along with any un-classified bacterial sequences. Genera are grouped by the phyla in which they belong; Actinomycetota (blues), Bacillota (greens to orange-yellows), Bacteroidota (oranges), Campylobacter (deep orange-red), Pseudomonadota (reds), and Thermodesulfobacteriota (dark red). Relative abundance of genera within bolded indicates that the genera comprises > 10% of at least one sample. An * indicates that the genera comprises > 30% of at least one sample. C-D) Principal component analysis indicated that wound surface microbial communities cluster by both the wound's etiology (C) and the wound's location on the body (D). Plots C and D are the same but colored differently to highlight the sample groupings by etiology and body site respectively. Microbiome samples did not cluster by whether the swab was taken from the wound edge or center, the wound's age, or the wound's outcome 3 months after the sample collection (e.g. whether the wound went on to heal or did not). E) A vector plot indicating the primary bacterial ASVs that dictated a point's position in the PCA plot C-D. These ASVs belong to *Corynebacterium*, *Pseudomonas*, *Staphylococcus*, and *Anaerococcus* species.

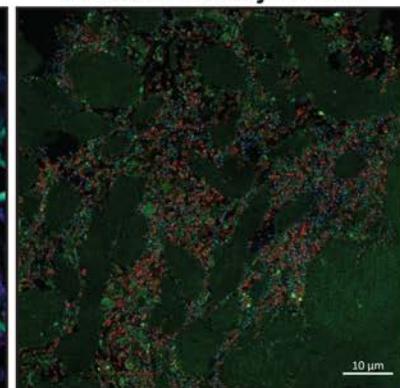
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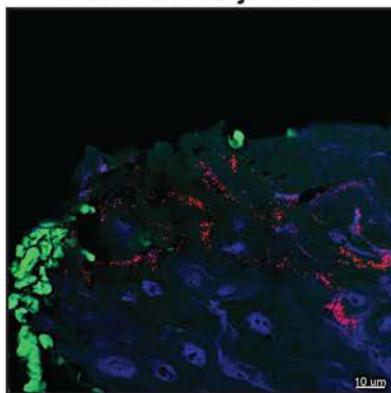
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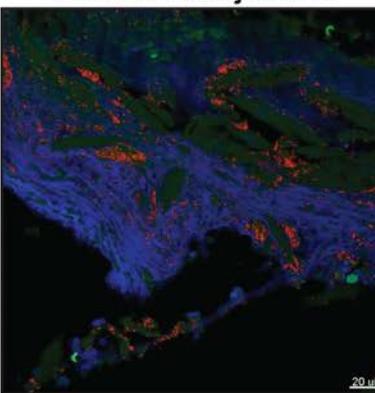
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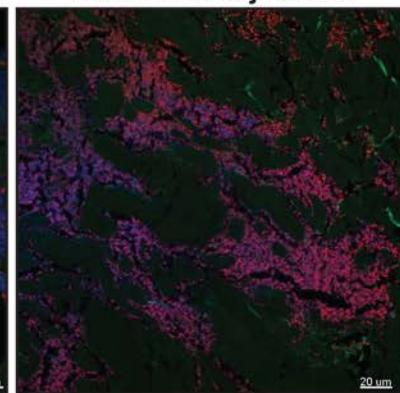
Australia Subject-006



Australia Subject-011



Australia Subject-013



Australia Subject-017

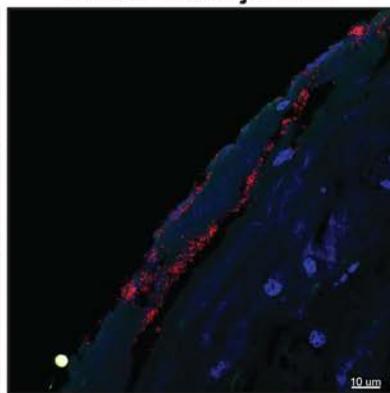


Figure 4: Confocal scanning laser microscopy of bacteria aggregates in slough. Formalin-fixed, paraffin-embedded (FFPE) slough samples were stained with a universal bacterial 16S rRNA probe (red) and for double stranded DNA (DAPI, blue) then visualized with confocal scanning laser microscopy (CSLM). Autofluorescence of the surrounding tissue was visualized in green. Only specimens with detected bacterial aggregates are shown here. Images from specimens with no bacterial aggregates are in supplemental figure 5.

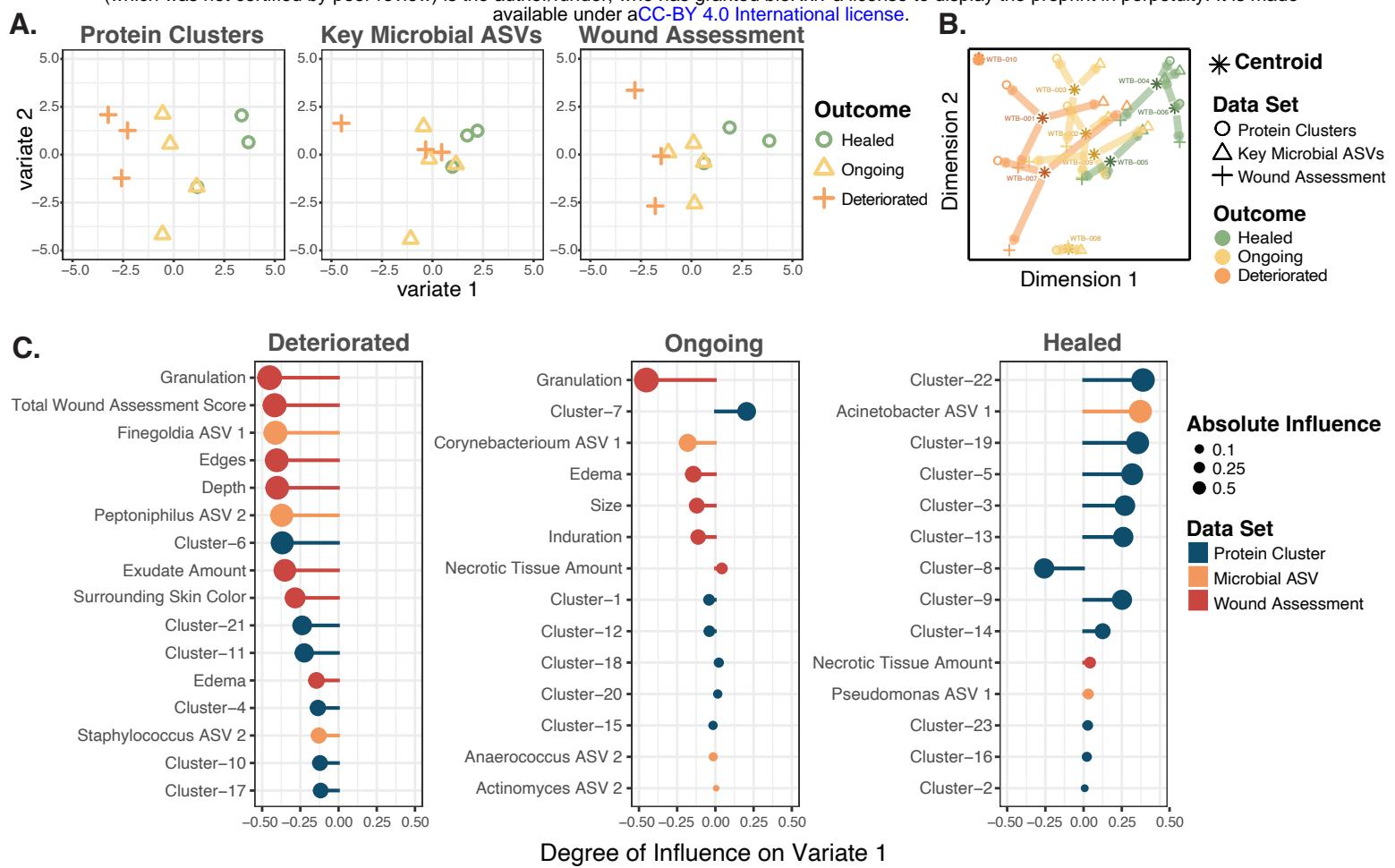
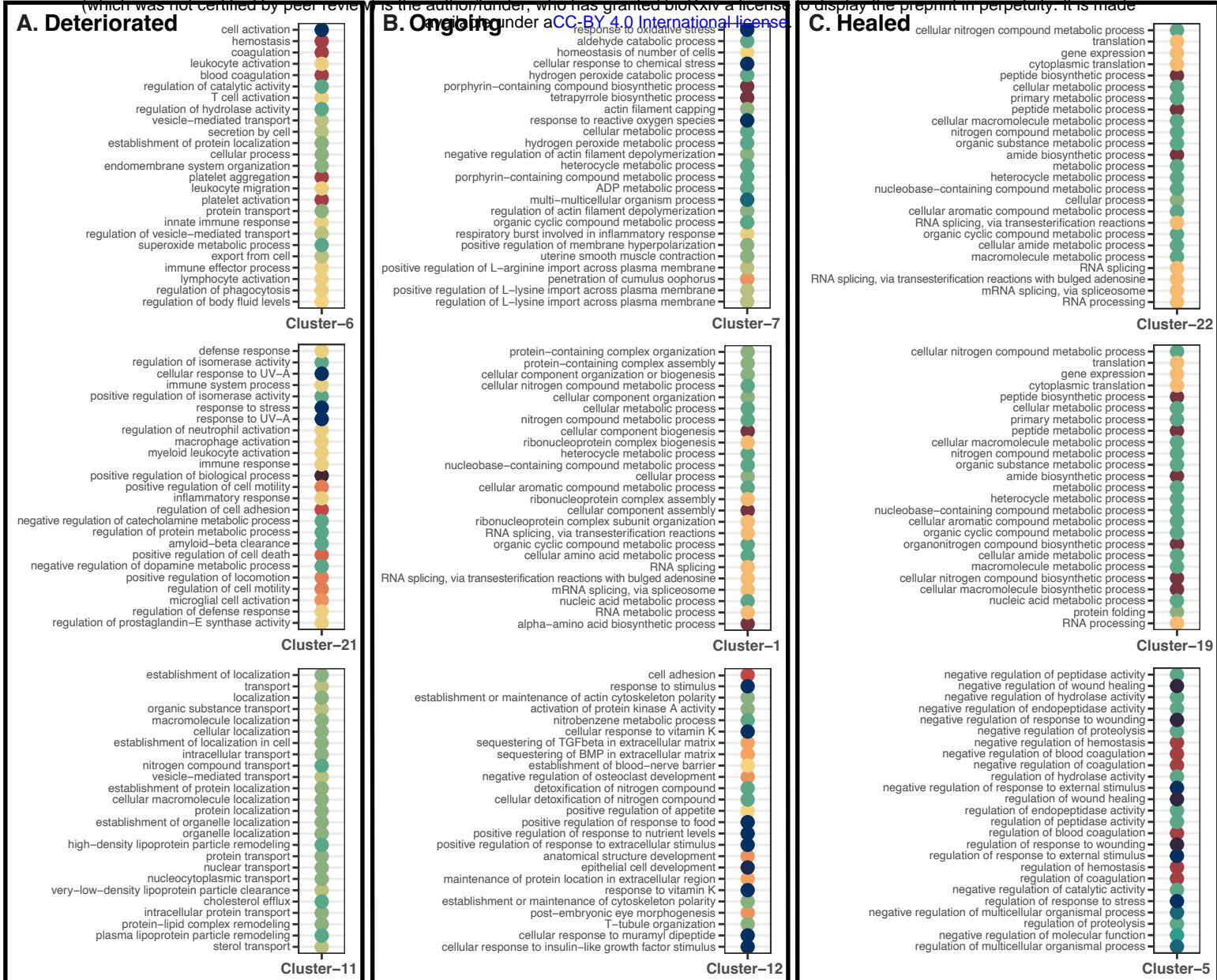


Figure 5: Chronic wounds that go on to heal can be distinguished from those that deteriorate via the proteomic, microbial, and clinical features of slough. To predict the variables associated with wound healing, the protein cluster, microbial, and the Bates - Jensen Wound Assessment datasets were integrated into a supervised Partial Least Squares - Discriminant Analysis (PLS-DA) via the MixOmics package. To simplify the proteomics dataset, proteins were grouped into 23 k-means clusters via the gap-stat method (Table S2). Since there was no significant difference in the microbial community composition at the wound edge or center, samples were combined to create a summative wound slough microbiome for each subject. The “key” 14 microbial ASVs with greater than 1% relative abundance in at least two subjects’ slough samples were included in this integrative analysis (Table S6). A) PLS-DA plots for the protein cluster, microbial ASV, and wound assessment data set respectively. Each dataset contains variables that can distinguish chronic wounds that go on to heal from those that deteriorate. Outcome groups most clearly separate along variate 1 for each of the datasets. B) PLS-DA plot for all the data sets combined. The asterisk indicates the centroid position where the subject’s slough sample falls considering variables from all three datasets. Arrows from the centroid indicate the direction that variables from each individual dataset pull the subject’s datapoint. C) Variable plots of the protein clusters (blue), microbial taxa (orange) and wound assessment criteria (red) that distinguish each outcome group along variate 1 of the PLS-DA plots. A longer vector to the right indicates a variable with greater influence pulling samples to the right along the variate 1 axis. The enriched GO biologic processes for representative slough protein clusters that distinguish slough from wounds with each outcome are in figure 6.



- Biologic Process
- Biosynthetic Process
- Blood Clotting
- Cell Adhesion
- Cell Death
- Cell Motility
- Developmental Process
- Extracellular Matrix
- Gene Expression
- Homeostasis
- Intercellular Transport
- Intracellular Process
- Metabolic Process
- Molecular Function
- Multicellular Organismal Process
- Response to Stimulus
- Skin Development
- Wound Healing

Figure 6: Slough from wounds that go on to deteriorate are enriched for immune activation and inflammatory immune responses. To determine the key biologic processes associated with each protein k-means cluster, proteins within each cluster were submitted as unranked lists to the GO Enrichment analysis tool for evaluation with the PANTHER Overrepresentation test. Details for this analysis are included in supplemental table 7. This figure displays the top 25 most enriched GO biological process for three representative protein clusters that distinguish slough from wounds that deteriorated three months following sample collection from those that (Fig. 4). For each cluster the biologic processes are ordered from most significantly enriched at the top to least enriched at the bottom. Color of the point indicates the broader biologic classification. A) wounds that deteriorated are enriched for immune cell activation and inflammatory immune responses, responses to stimuli and stress, cell motility, intracellular transport and intracellular processes. B) Wounds that were stable but ongoing were enriched for responses to stress, metabolic processes, and gene expression. C) Wounds that went on to heal were enriched for metabolic and biosynthetic processing, gene expression, and regulation (particularly negative regulation) of wound healing and responses to stress. Supplemental figure 5 displays the significantly enriched GO biological processes for all 23 k-means clusters.