

1 A Trifactor of New Insights into Ovine Footrot for Infection
2 Drivers, Immune Response and Host Pathogen Interactions.

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22

23 **Abstract**

24 Footrot is a polymicrobial infectious disease in sheep causing severe lameness,
25 leading to one of the industry's biggest welfare problems. The complex aetiology of
26 footrot makes in-situ or in-vitro investigations difficult. Computational methods offer a
27 solution to understanding the bacteria involved, how they may interact with the host
28 and ultimately providing a way to identify targets for future hypotheses driven
29 investigative work. Here we present the first combined global analysis of the bacterial
30 community transcripts together with the host immune response in healthy and
31 diseased ovine feet during a natural polymicrobial infection state using
32 metatranscriptomics. The intra tissue and surface bacterial populations and the most
33 abundant bacterial transcriptome were analysed, demonstrating footrot affected skin
34 has a reduced diversity and increased abundances of, not only the causative bacteria
35 *Dichelobacter nodosus*, but other species such as *Mycoplasma fermentans* and
36 *Porphyromonas asaccharolytica*. Host transcriptomics reveals a suppression of
37 biological processes relating to skin barrier function, vascular functions, and
38 immunosurveillance in unhealthy interdigital skin, supported by histological findings
39 that type I collagen (associated with scar tissue formation) is significantly increased
40 in footrot affected interdigital skin compared to outwardly healthy skin. Finally, we
41 provide some interesting indications of host and pathogen interactions associated
42 with virulence genes and the host spliceosome which could lead to the identification
43 of future therapeutic targets.

44

45 **Impact Statement**

46 Lameness in sheep is a global welfare and economic concern and footrot is the
47 leading cause of lameness, affecting up to 70% of flocks in the U.K. Current methods

48 for control of this disease are labour intensive and account for approximately 65% of
49 antibiotic use in sheep farming, whilst preventative vaccines suffer from poor efficacy
50 due to antigen competition. Our limited understanding of cofounders, such as strain
51 variation and polymicrobial nature of infection mean new efficacious, affordable and
52 scalable control measures are not receiving much attention. Here we examine the
53 surface and intracellular bacterial populations and propose potential interactions with
54 the host. Identification of these key bacterial species involved in the initiation and
55 progression of disease and the host immune mechanisms could help form the basis
56 of new therapies.

57

58 **Introduction**

59 Ovine footrot is a persistent animal welfare issue and has a significant financial
60 burden for farmers due to the cost of preventative footbaths, antibiotic treatments,
61 and reduced carcass weights at slaughter (1). The causative bacterium
62 *Dichelobacter nodosus* (*D. nodosus*) has received extensive attention since its
63 description in the initiation of footrot (2). However, it has been accepted since the
64 beginning of the 20th century that footrot is a polymicrobial disease, with
65 *Fusobacterium necrophorum* (*F. necrophorum*), *Spirochaeta penortha* (*S. penortha*)
66 (3), *Treponema podovis* (*T. podovis*) (4) and *Corynebacterium pyogenes* (*C.*
67 *pyogenes*) (5) proposed as species that can exacerbate the lesions.

68

69 Currently our understanding of bacterial populations associated with footrot is only
70 based on 16S rRNA analysis from the skin surface (6, 7). The highly abundant
71 genera identified in the footrot samples were congruent with those identified
72 previously by standard microbiological techniques (*Corynebacterium*, *Fusobacterium*,

73 *Dichelobacter* and *Treponema*). However, additional genera were also identified
74 (*Mycoplasma*, *Psychrobacter* and *Porphyromonas*) (7) and their absence using
75 traditional culture techniques, could be due to the fastidious nature of the bacteria (8)
76 or that they were not yet identified (9). Investigating the total bacterial load within
77 tissues, we have shown recently, that in healthy tissues, bacterial load is similar
78 throughout tissue depth and did not extend beyond the follicular depth in the reticular
79 dermis. In contrast, in footrot samples, the bacterial load was highest in the
80 superficial (or cornified) epidermal layers and decreasing in the deeper layers but still
81 beyond follicular depth (10). This suggests that the infection allows for further
82 invasion from other species of bacteria to penetrate deeper into the interdigital tissue,
83 however these data were limited to presence of bacteria based on universal primers
84 not allowing to identify species.

85

86 There is also a lack of information regarding the host infection, how an immune
87 response is mounted and the species interactions. This area of investigation has
88 recently benefitted from the use of metatranscriptomics, a method of assessing host-
89 pathogen interactions based on associated gene expression changes (11). The use
90 of metatranscriptomics has been reviewed extensively (12), however, current
91 published methods are based on, or optimisations of, cell culture models as
92 developed in the original methods article (11). The use of metatranscriptomics in
93 natural polymicrobial infections is not as well reported. The first documented use was
94 in relation to the onset of paediatric asthma (13), and oral disease (14, 15). However,
95 its application to Bovine Digital Dermatitis (BDD) (16), which has a similar clinical
96 presentation and bacteria associated with footrot (17–20) highlighted its suitability to
97 further our understanding of the inter-cellular microbial populations associated with

98 agricultural diseases. Utilising this experimental design, we have been able to
99 determine the bacterial populations on the surface of the interdigital skin and within
100 the deeper infected tissue, identify the differential expression of the host transcripts
101 and elucidate interactions between the host and bacteria.

102 **Results**

103 ***Sequence data***

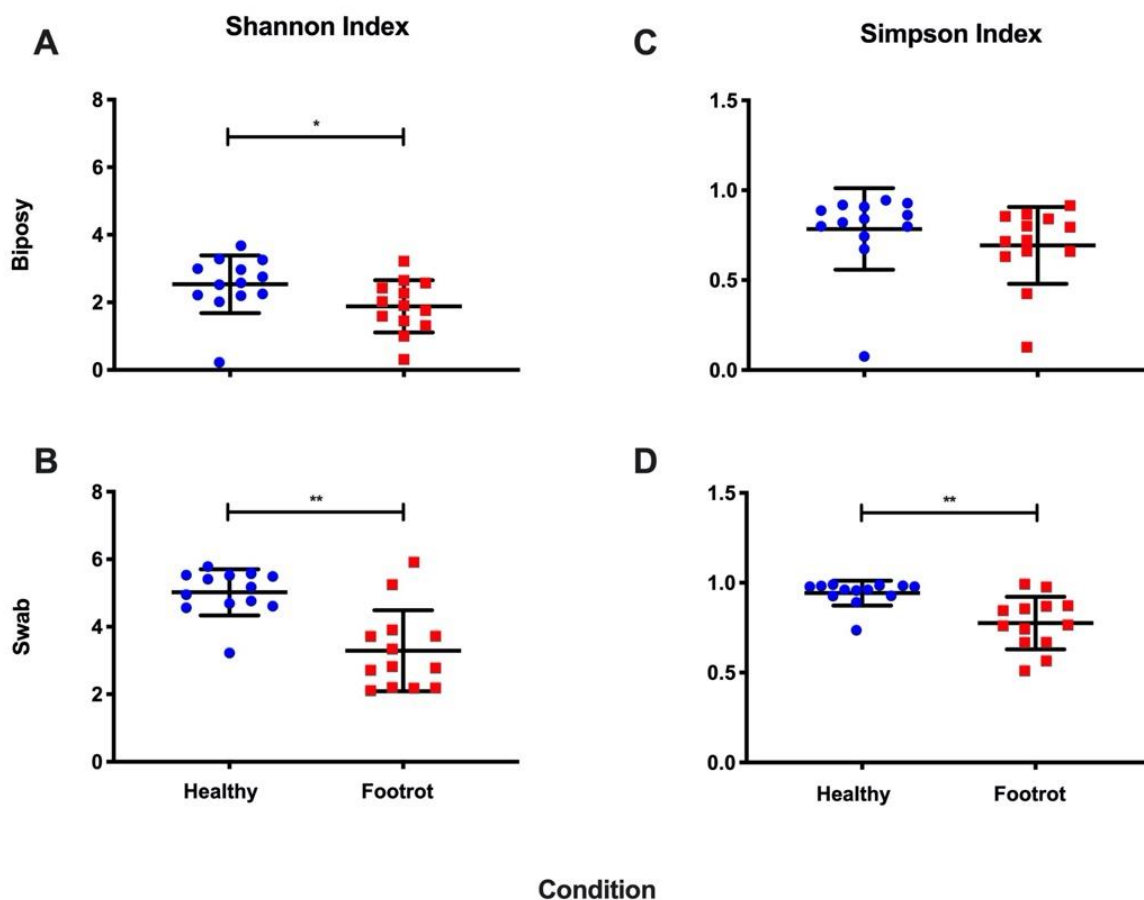
104 Foot swabs and whole thickness skin biopsies were collected from sheep post
105 slaughter that had at least one apparently healthy foot (n=13) and one with signs of
106 footrot (n=13) to obtain matching samples from the same sheep. After quality filtering
107 there was an average of 8.7 million discordant ovine reads per sample to be used for
108 bacterial taxonomic assignment from the foot biopsies, and 20.8 million discordant
109 ovine reads for the accompanying swabs. All reads had an average phred score of
110 40. Diversity statistics were calculated for each sample after assignment. Using the
111 Shannon indices and calculating an equitability score (natural log of the species
112 richness) representing a maximum diversity, revealed that healthy feet were highly
113 diverse but footrot feet showed a reduction in diversity (Table 1, Fig 1A). The
114 Simpson indices also indicated that there was more diversity in the healthy samples
115 with an average of 0.78 in healthy compared to 0.69 in the footrot biopsies. (Figure
116 1B). This was furthermore reflected in the swabs with an average of 0.94 in the
117 healthy samples compared to 0.77 in footrot (Figure 1). The Shannon index showed
118 a significant difference between the two conditions for both, biopsies ($p \leq 0.005$) and
119 swabs ($p \leq 0.05$), whereas only the swabs showed a significant difference for the
120 Simpson index ($p \leq 0.005$; Figure 1).

121

122 **Table 1 Comparison of average calculated and maximum diversity for each**
 123 **condition.** Demonstrating the overall reduction in bacterial community diversity for
 124 footrot affected individuals when compared to the calculated maximum diversity
 125 expected from the data.
 126
 127

Condition	Biopsy Equitability	Biopsy Shannon	Swab Equitability	Swab Shannon
Healthy	3.9	2.5	7.1	5.0
Footrot	3.3	1.9	7.1	3.2

128



129

130

131 **Figure 1 Diversity statistics for the biopsy and swab samples.** A) Shannon Index
 132 of biopsy samples, B) Shannon Index of swab samples, C) Simpson Index of biopsy
 133 samples, D) Simpson index of swab samples. Significant decreases were observed
 134 from footrot affected samples, for swabs using both Shannon and Simpson indices. A
 135 significant decrease in footrot affected samples was only observed for biopsies using
 136 the Shannon index. Statistical significance calculated using Mann Whitney U (*
 137 $p=0.05$, ** $p=0.005$, *** $p=0.0005$).

138 **Bacterial community**

139

140 Differences in abundance calculated between the two conditions were identified as
141 samples having a >2 log fold change, with an FDR (Benjamini-Hochberg) corrected
142 p-value <0.05 and where average counts had a difference greater than 10 (full
143 taxonomic assignments are available in Supplementary Table 1 for swabs and
144 Supplementary Table 2 for biopsies). In swabs, 20 species of bacteria were found in
145 significantly increased abundance in footrot samples. These included *T. pedis*, *T.*
146 *denticola*, *D. nodosus*, and *F. necrophorum*, all known to cause various foot diseases
147 in sheep. Among the bacterial species found in significantly reduced abundance in
148 footrot samples were ten species of *Staphylococcus* spp., *Bacillus licheniformis*,
149 *Parageobacillus thermoglucosidasius* and *Nocardiopsis alba*. All differential
150 abundance data for the swabs are available in Supplementary Table 3.

151

152 Applying the same criteria to biopsies, three species of bacteria were found in
153 differential abundance between the two conditions, namely *D. nodosus*, *Mycoplasma*
154 *fermentans* (*M. fermentans*) and *Porphyromonas asaccharolytica* (*P.*
155 *asaccharolytica*). *D. nodosus* had the most significant increase in footrot biopsies
156 with a log-fold change increase of 7.0 (p=1.89E-06), *M. fermentans* had a log-fold
157 change of 6.2 (p=2.59E-05) whilst *P. asaccharolytica* had a log-fold increase of 3.5
158 (p=0.018). No species were found to be significantly decreased between the two
159 conditions in the biopsies (all differential abundance data for the biopsies are
160 available in Supplementary Table 4). Although some archaea were identified in both
161 biopsy and swab samples, none were significantly more or less abundant in footrot
162 affected feet compared to healthy feet.

163

164 As short read sequencing has limitations in identifying bacteria to species level, the
165 most significantly increased abundant bacteria in footrot affected tissues were
166 confirmed to be *D. nodosus*, *M. fermentans* and *P. asaccharolytica*, by specific
167 qPCR, species specific PCR and PCR followed by sequencing, respectively. In
168 addition, *F. nucleatum* was identified as the only *Fusobacterium* species, however
169 qPCR demonstrated this as misidentification and *F. necrophorum* was present as
170 expected.

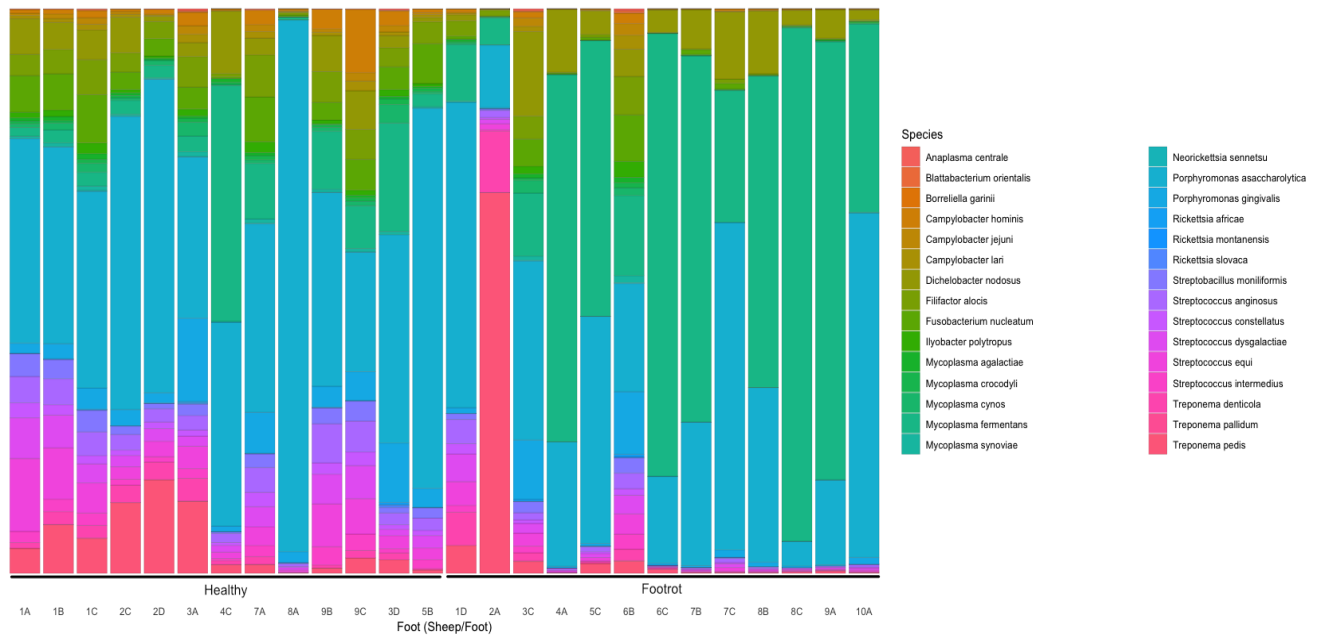
171

172 **Comparative analysis of in tissue and surface bacterial communities**

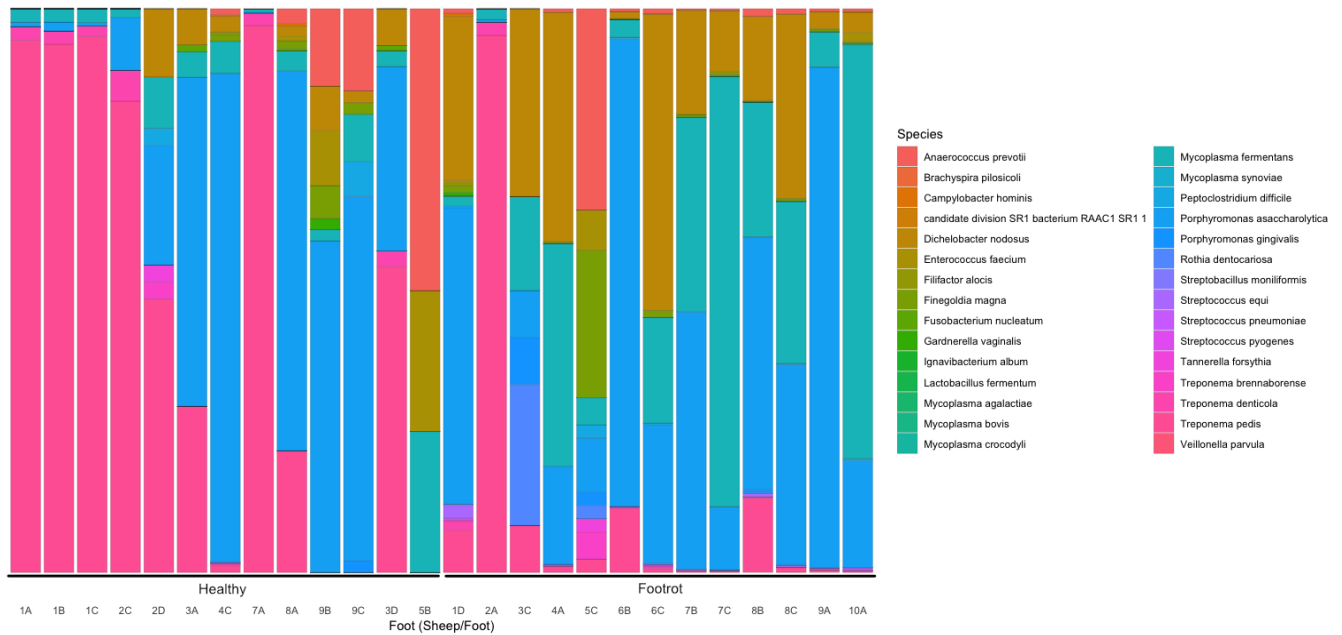
173 The taxonomic assignments from both, swab and biopsy data, were tested to
174 ascertain whether a clear relationship existed between taxonomic assignments for
175 the same sheep using both the correlation and similarity hypothesis tests outlined in
176 the methods section. Under the null hypothesis for the correlation test, there was no
177 correlation between swab and tissue samples. A p-value of 0.0301 was obtained,
178 providing strong evidence of a relationship. However, it should be noted that this is
179 evidence of a relationship in the presence of bacterial species between biopsy and
180 swab samples rather than them containing the same species.

181 To test the latter claim, the similarity test from the methods section was used. Here,
182 the null hypothesis was that biopsy and swab samples reveal the presence of the
183 same bacteria. This test produced a conservative p-value of $< 10^{-5}$, providing over
184 overwhelming evidence that swab and biopsy samples from the same sheep do not
185 contain the same species of bacteria. Specifically, two random biopsy samples will
186 have more species in common than a swab and biopsy from the same sheep.

A



B



187

188

189 **Figure 2 Species of bacteria identified as increased in abundance in footrot**
 190 **affected feet when compared to healthy feet. A) Shows the top 30 species of**
 191 **bacteria in swab samples and B) Shows the top 30 species of bacteria in biopsy**
 192 **samples.**

193

194 **Differential expression of pro-inflammatory mediators in healthy versus footrot**
195 **affected interdigital skin.**

196 Among the transcripts that showed increased expression in footrot-affected
197 interdigital skin were a large number of proteins important for barrier function. These
198 included proteins involved in collagen production and collagen binding (Procollagen
199 C-endopeptidase enhancer 2 [PCOLCE2], Collagen Type VI alpha 6 chain, Collagen
200 Type XXIII alpha 1 chain and keratocan/lumican [collagen-binding leucine-rich
201 proteoglycans widely distributed in interstitial connective tissues]; cell-cell adhesion
202 (cadherin[CDH]3, CDH19, pro[P]CDH10); maintenance of cell junctions (GJB4) and
203 long chain fatty acid synthesis (fatty acid elongase [ELOVL]7, ELOVL3, acyl-CoA
204 synthetase bubblegum family member [ACSBG]1), and acyl-CoA wax alcohol
205 acyltransferase [AWAT]1). In addition, transcripts involved in immunosurveillance
206 such as scavenger receptors SCARA5 and SSC5D were more highly expressed in
207 footrot affected samples (see Table 2 for top 25 transcripts, Supplementary Table 5
208 for all transcripts). In contrast, transcripts that showed lower expression compared to
209 healthy interdigital skin include cytokines involved in wound-healing (IL-19, IL-20)
210 and keratinocyte proliferation/differentiation (IL-6 and leukaemia inhibitory factor
211 [LIF]), epithelial cell-derived chemokines that recruit monocytes (CCL2), lymphocytes
212 (CCL20) and neutrophils (CXCL1, CXCL8) and prostaglandin-endoperoxide synthase
213 2 (PGE2/COX2), which is also involved in skin wound healing. Another group of
214 significantly decreased transcripts include matrix metalloproteases (MMP1, MMP3,
215 MMP9, MMP13, MMP20, (tenascin C) TNC, TIMP1) and their regulators (SERPINE1,
216 ADAMTS4, ADAMTS16) associated with chronic wounds and collagen turnover (see
217 Table 3 for top 25 transcripts, Supplementary Table 5 for all transcripts).

218 **Table 2: Top 25 differentially higher expressed genes in footrot affected skin**
 219 **when compared to healthy skin**

Gene name	Description	β -value	Standard error	q-value
ELOVL7	ELOVL fatty acid elongase 7	2.812237	0.884427	0.035516
MNT	MAX network transcriptional repressor	2.415885	0.773256	0.038156
FRAS1	Fraser extracellular matrix complex subunit 1	2.358302	0.738887	0.034774
CA6	carbonic anhydrase 6	2.273248	0.686033	0.029014
ATP13A4	ATPase 13A4	2.261712	0.648362	0.023149
PNPLA5	patatin like phospholipase domain containing 5	2.230351	0.627058	0.021215
ELOVL3	ELOVL fatty acid elongase 3	2.070317	0.598268	0.02425
STMN2	stathmin 2	2.021979	0.464736	0.007856
NOS1	nitric oxide synthase 1	2.004654	0.546986	0.018297
ACSBG1	acyl-CoA synthetase bubblegum family member 1	1.933404	0.590342	0.030742
AWAT1	acyl-CoA wax alcohol acyltransferase 1	1.849357	0.543633	0.02594
CCL26	C-C motif chemokine ligand 26	1.842727	0.621591	0.046882
CCDC155	coiled-coil domain containing 155	1.791928	0.584831	0.041351
PI16	peptidase inhibitor 16	1.765247	0.400402	0.007477
FAR2	fatty acyl-CoA reductase 2	1.711846	0.519782	0.02993
PTX4	pentraxin 4	1.681009	0.336588	0.004979
LRRC36	leucine rich repeat containing 36	1.669794	0.465845	0.020504
AGTR1	angiotensin II receptor type 1	1.661825	0.442673	0.016379
GALNT8	polypeptide N-acetylgalactosaminyltransferase 8	1.631845	0.457915	0.020974
CYP2F1	cytochrome P450 family 2 subfamily F member 1	1.55567	0.44436	0.022765
TOGARAM2	TOG array regulator of axonemal microtubules 2	1.530427	0.48021	0.035066
DNASE1L2	deoxyribonuclease 1 like 2	1.515543	0.406144	0.0169
FAM221A	family with sequence similarity 221 member A	1.506933	0.378682	0.01257
AQP9	aquaporin 9	1.506146	0.384521	0.013256
DGAT2L6	diacylglycerol O-acyltransferase 2 like 6	1.505104	0.480771	0.03797

220 qval: FDR adjusted p-value using Benjamini-Hochberg; β -value: bias estimator
 221 analogous to fold change

222 **Table 3: Top 25 lower expressed genes in footrot affected interdigital skin**
 223 **compared to healthy skin**

Gene name	Description	β -value	Standard error	q-value
IL-19	interleukin 19	-2.96775	0.804409	0.017781
PTGS2	prostaglandin-endoperoxide synthase 2 (PGE2/COX2)	-2.8683	0.75813	0.015736
MMP3	matrix metalloproteinase 3	-2.71059	0.616087	0.007597
IL-6	interleukin-6 precursor	-2.4267	0.568937	0.008635
IL-20	interleukin 20	-2.30205	0.758643	0.04305
A2ML1	alpha-2-macroglobulin like 1	-2.23879	0.765254	0.049443
CCL20	C-C motif chemokine ligand 20	-2.18479	0.501858	0.007856
CXCL8	Interleukin-8	-2.10659	0.685411	0.040981
SLPI	antileukoproteinase precursor	-2.04964	0.672506	0.042275
MGAM	maltase-glucoamylase	-2.02022	0.568845	0.021334
MARCKSL1	MARCKS like 1	-1.95955	0.631205	0.039119
MEFV	MEFV, pyrin innate immunity regulator	-1.90552	0.514121	0.017474
MMP13*	matrix metalloproteinase 13	-1.83676	0.523263	0.022523
ADAMTS16	ADAM metalloproteinase with thrombospondin type 1 motif 16	-1.80019	0.479709	0.016379
ACOD1	aconitate decarboxylase 1	-1.68652	0.563289	0.045291
PTX3	pentraxin 3	-1.66311	0.446328	0.016904
MMP13*	matrix metalloproteinase 13	-1.65481	0.561768	0.048097
ADAMTS4	ADAM metalloproteinase with thrombospondin type 1 motif 4	-1.59355	0.394111	0.011418
MMP1	matrix metalloproteinase 1	-1.55518	0.37913	0.01037
FOSL1	FOS like 1, AP-1 transcription factor subunit	-1.51685	0.383897	0.012932
MGAT3	mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase	-1.46074	0.377507	0.014131
CCL2	C-C motif chemokine ligand 2	-1.40425	0.325691	0.008232
CSF3	colony stimulating factor 3	-1.4015	0.473053	0.047061
PLAUR	urokinase plasminogen activator surface receptor precursor	-1.36363	0.322566	0.009128

224 qval: FDR adjusted p-value using Benjamini-Hochberg; β -value: bias estimator
 225 analogous to fold change

226 *Splice variants

227

228 **Biological process enrichment**

229 Using BCCC biclustering and associated GO biological processes, the genes and
230 conditions grouped into a total of 32 clusters. There were 2531 genes over 20
231 samples that clustered showing upregulated biological processes including significant
232 positive regulation. The top 15 were positive regulation of transcription (n=106,
233 p=2.94e-26), protein folding (n=34, p=3.5e-21), regulation of DNA templated
234 transcription (n=121, p=4.51e-21), metabolic process (n=61, p=1.44e-18), DNA
235 templated transcription (n=63, p=1.48e-18), rRNA processing (n=20, p=9.12e-15),
236 protein transport (n=32, p=2.08e-13), ribosome biogenesis (n=17, p=2.20e-13),
237 osteoblast differentiation (n=24, p=4.01e-12), transcription from RNA polymerase II
238 promotor (n=49, p=5.84e-12), negative regulation of transcript from RNA polymerase
239 II promotor (n=63, p=7.75e-12), negative regulation of apoptotic process (n=46,
240 p=1.91e-11), positive regulation of telomerase RNA localisation to cajal body (n=10,
241 p=9.34e-11), proteolysis (n=63, p=1.65e-10) and translational initiation (n=16,
242 p=1.74e-10).

243

244 There were 541 genes over 17 samples that clustered showing significant
245 downregulation of biological process in footrot affected samples. Cluster one showed
246 a decrease in epidermis development (n=17, p=2.3e-06), multicellular organismal
247 water homeostasis (n=8, p=1.8e-05), peptidoglycan catabolic processes (n=4,
248 p=4.6e-05), antimicrobial humoral response (n=7, p=9.6e-05), tissue development
249 (n=41, p=1.1e-04), monovalent inorganic cation homeostasis (n=8, p=8.2e-4),
250 defence response to bacteria (n=11, p=8.2e-04), fatty acid metabolic processes
251 (n=12, p=1.3e-03), polyol transport (n=3, p=2.7e-03), skin development (n=11,
252 p=2.7e-03), water transport (n=4, p=3.4e-03) and regulation of pH (n=6, p=3.4e-03).

253 Cluster two showed downregulation for neutrophil chemotaxis (n=7, p=4.3-e06),
254 myeloid leukocyte migration (n=9, p=7.2e-06), leukocyte migration (n=11, p=9.8e-
255 06), cell chemotaxis (n=10, p=2.6e-05), defence response (n=19, p=2.8e-05),
256 immune system processes (n=26, p=4.3e-05), chemotaxis (n=12, p=1.2e-04),
257 antimicrobial humoral response (n=5, p=1.2e-04), immune response (n=18, p=1.2e-
258 04) and response to external stimulus (n=23, p=1.5e-04). The third cluster showed
259 downregulation for S-adenosylhomocysteine catabolic process (n=2, p=5.7e-04)
260 alone.

261
262 Investigating KEGG pathway enrichment also identified cytokine-cytokine receptor
263 interaction (n=26, p=4.5e-6), IL-17 signalling pathway (n=13, p=7.7e-08), TNF
264 signalling pathway (n=12, p=7.7e-05) to be downregulated in the footrot affected
265 samples. Whilst steroid hormone biosynthesis (n=7, p=2.4e-04) and ribosome
266 biogenesis (n=31, p=4.07e-08) were upregulated.

267

268 **Putative Host pathogen interactions**

269 The bacterial RNA reads were aligned against the bacterial transcriptomes that were
270 identified as those traditionally associated with ovine foot disease (*D. nodosus*, *F.*
271 *necrophorum*, *T. pedis* and *T. denticola*) and those additionally found to be the most
272 differentially abundant in the footrot biopsy samples (*M. fermentans* and *P.*
273 *asaccharolytica*). These data were then used, along with the host expression data to
274 understand correlations and host and pathogen interactions (Supplementary Table
275 6).

276

277 The interactions were calculated with an FDR adjusted p-value using the Benjamini-
278 Hochberg procedure. Due to the stringency of this multiple test adjustment, no

279 significance was determined. However, the raw p-values were low (in some cases <
280 0.00005), therefore these data were investigated further but only as an indicative
281 positive bacteria/gene correlation. Based on a raw p-value of <0.00005 there were
282 four sheep transcripts that were associated with five *D. nodosus* genes (Table 4).
283 From *D. nodosus* aminoacyl-histidine dipeptidase, acidic extracellular subtilisin-like
284 protease precursor (AprV5), outer membrane protein 1E, Bacterial extracellular
285 solute-binding protein and aminoacyl-histidine dipeptidase were identified to correlate
286 with small nucleolar RNA, C/D box, U6 spliceosomal RNA, synapsin and U6
287 spliceosomal RNA from *Ovis aries*. There were more correlations between *M.*
288 *fermentans* and *Ovis aries* with a total of 15 bacterial transcripts associated with four
289 host transcripts where raw p=0.0005. The bacterial transcripts were shown to be
290 overwhelmingly responsible for cellular transport on both the host and pathogen side.
291 There were a further three bacterial and sheep interactions in *T. pedis* (p=0.00005)
292 which suggested the bacterial flagellin and host membrane protein, and a bacterial
293 hypothetical protein and putative lipoprotein correlates with a sheep miRNA (Table
294 4).

295

296 The correlations between the sheep transcripts and the bacterial transcripts from *F.*
297 *necrophorum*, *P. asaccharolytica* and *T. denticola* had raw p-values of <0.0005,
298 <0.001 and <0.004, respectively. Although low, p-values with the number of tests
299 being performed they were not investigated any further (full data is available in
300 Supplementary Table 6).

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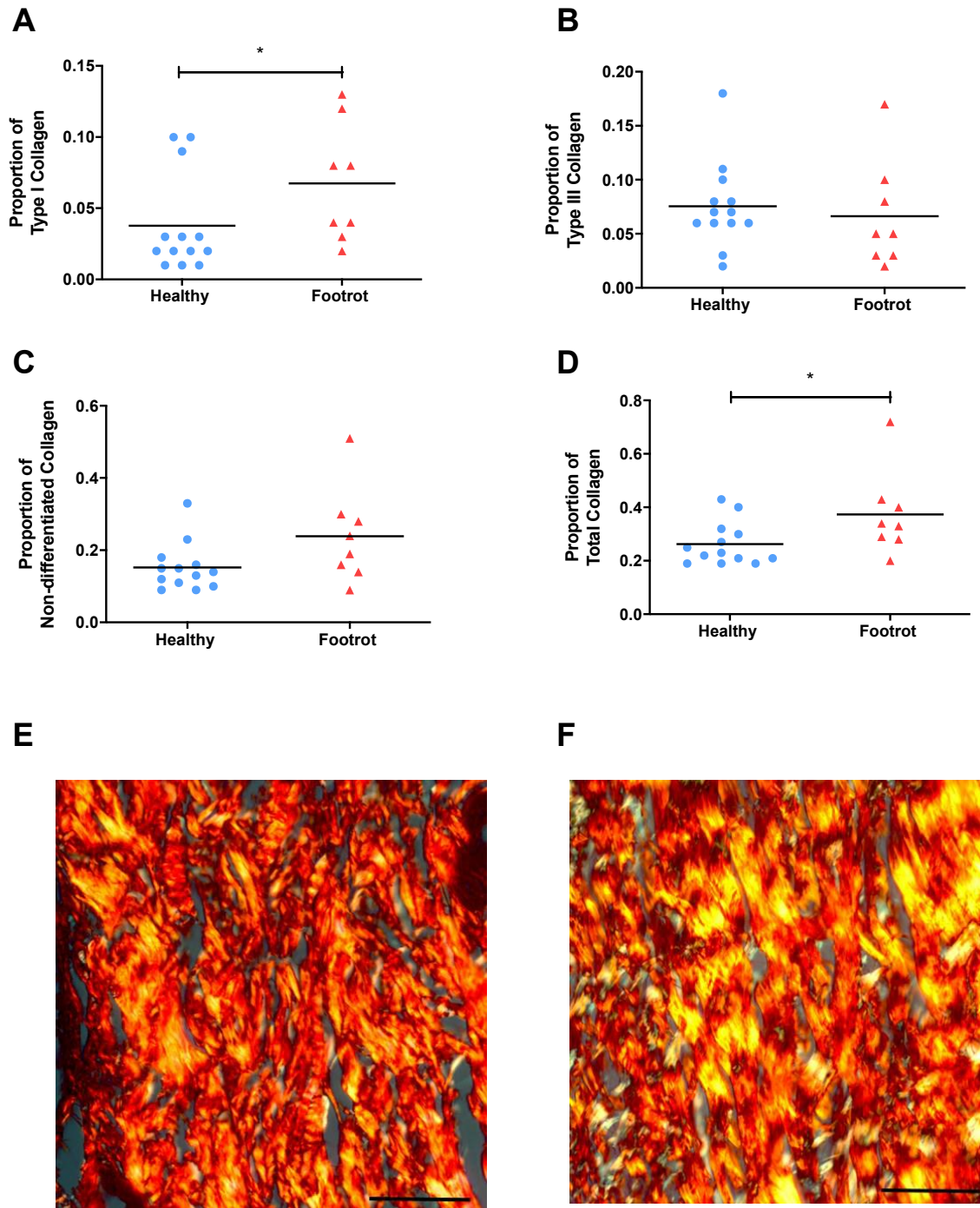
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303 **Table 4. Correlations between bacterial and host gene expression.**

Bacterial Species	Protein Accession	Function	<i>Ovis aries</i> Transcript accession	Function	p-value		
D. nodosus	ABQ13122.1	aminoacyl-histidine dipeptidase	ENSOART00000026163	small nucleolar RNA, C/D box	1.66E-05		
D. nodosus	ABQ13667.1	acidic extracellular subtilisin-like protease precursor (AprV5)	ENSOART00000023789	U6 spliceosomal RNA	7.10E-05		
D. nodosus	ABQ13351.1	outer membrane protein 1E	ENSOART00000014155	synapsin	7.49E-05		
D. nodosus	ABQ13881.1	Bacterial extracellular solute-binding protein			7.49E-05		
D. nodosus	ABQ13122.1	aminoacyl-histidine dipeptidase	ENSOART00000023789	U6 spliceosomal RNA	7.49E-05		
M. fermentans	WP_013526775.1	Protein translocase subunit	ENSOART00000015973	Pro-platelet basic protein	2.88E-05		
M. fermentans	WP_013526734.1	Putative Oligopeptide ABC transporter, ATP-binding protein	ENSOART00000022767	Novel Transcript	4.71E-05		
M. fermentans	WP_013526734.1	DNA-directed RNA polymerase subunit beta	ENSOART00000013696	Potassium voltage-gated channel	7.04E-05		
M. fermentans	WP_013526778.1	ATP synthase subunit beta	ENSOART00000017736	Novel Transcript	7.04E-05		
M. fermentans	ADV34079.1	MgpA like protein	ENSOART00000013889	CXXC Type Zinc Finger 1 CPG Binding PHD Finger	9.28E-05		
M. fermentans	WP_013354336.1	bifunctional oligoribonuclease/PAP phosphatase			9.28E-05		
M. fermentans	WP_013526633.1	Adenine phosphoribosyltransferase			9.28E-05		
M. fermentans	ADV34286.1	Oligopeptide ABC transporter permease protein			9.28E-05		
M. fermentans	WP_013354483.1	ABC transporter permease			9.28E-05		
M. fermentans	WP_013354556.1	sugar ABC transporter permease			9.28E-05		
M. fermentans	ADV34629.1	NADPH flavin oxidoreductase			9.28E-05		
M. fermentans	WP_013354747.1	nitroreductase family protein			9.28E-05		
M. fermentans	ADV34954.1	Transcription antitermination protein			9.28E-05		
M. fermentans	WP_013527166.1	ABC transporter ATP-binding protein			9.28E-05		
M. fermentans	WP_013527168.1	ABC transporter permease			2.88E-05		
T. pedis	WP_024465740.1	Flagellin			ENSOART00000022912	Novel Membrane Protein	5.38E-05
T. pedis	WP_051150643.1	Hypothetical Protein			ENSOART00000026139	ncRNA (MiRNA)	5.47E-05
T. pedis	AGT42887.1	Putative Lipoprotein	5.47E-05				

305 **Collagen composition differs in the dermis of healthy and footrot tissues**

306 Since collagen composition changes in scar tissue formation, picrosirius stained
307 tissue sections were used to differentiate collagen type I and III from each other and
308 other collagen types. To investigate whether there were any differences in the
309 collagen composition of the dermis, the proportions of type I, type III, non-
310 differentiated and total collagen were calculated (Fig 4). The proportions of total and
311 type I collagen were significantly increased in the dermis of footrot samples
312 compared to healthy samples ($p=0.04$ and 0.042 , respectively, Fig 4 A, D). The
313 proportions of non-differentiated and type III collagen were not significantly different
314 in the dermis layers of healthy and footrot-affected tissues (Fig 4, B-C).



315

316 **Figure 4: Collagen expression in healthy and footrot ovine interdigital skin**

317 **dermis.** Picosirius histological staining was used to differentiate and quantify

318 collagens in healthy (n=13) and footrot (n=8) samples. Proportions of type I collagen

319 (A), type III collagen (B), undifferentiated collagens (C) and total proportion of

320 collagen (D). Representative photomicrographs showing picosirius staining under

321 phase microscopy from healthy (E) and footrot (F) samples, type I collagen stained

322 yellow, type III collagen stained green, undifferentiated collagen stained red. Scale

323 bars represent 50µm. Significance is designated by asterisk on a straight line with the

324 T-test result defined as * = $p \leq 0.05$.

325

326 **Discussion**

327 *D. nodosus*, was established as the causative bacterium of ovine footrot in the
328 1940's, and it has long been accepted that *F. necrophorum* plays a role in the
329 disease aetiology (4). However, in our work we have identified additional common
330 core species that are also associated with footrot lesions (*M. fermentans* and *P.*
331 *asaccharolytica*) and we have shown putative interactions with the molecular host
332 defence systems during infection in bacterial species identified as highly abundant in
333 footrot and even without a significant difference in abundance between healthy and
334 footrot affected such as *T. pedis*. Using paired biopsies collected from footrot
335 affected sheep at point of slaughter we were able to comprehensively show that skin
336 swabs are a poor proxy for identifying what bacteria are present in the tissue. This is
337 potentially due to the bacterial contamination present from environmental sources
338 such as faeces and soil collected during transport and grazing. However, we have
339 shown that biopsies provide an intradermal approach to reproducibly assess
340 differences between individual animals in an invasive infection like footrot.

341

342 ***The bacterial community structure of footrot***

343 Previous studies have shown the ovine interdigital bacterial community structure
344 using 16S rDNA, with predominant bacterial genera identified as *Mycoplasma spp*,
345 *Corynebacterium spp*, *Psychobacter spp*, *Treponema spp*, *Staphylococcus spp*,
346 *Peptostreptococcus spp* and *Dichelobacter spp* (6, 7). The results from this study are
347 highly congruent with what has previously been identified, however due to the greater
348 taxonomic sensitivity afforded by metagenomics and metatranscriptomics we have
349 been able to classify those bacterial genera to a species level. Those with differential
350 abundance associated with footrot found on the skin surface were identified as *T.*

351 *pedis*, *T. denticola*, *D. nodosus*, and *F. necrophorum*. These species are commonly
352 found with other ovine foot diseases, contagious ovine digital dermatitis (CODD) (21)
353 and interdigital dermatitis (ID) (7) and the bovine foot disease bovine digital
354 dermatitis (BDD) (18). The species with differential abundance associated with
355 footrot intradermally were *D. nodosus*, *M. fermentans* and *P. asaccharolytica*. Given
356 that *D. nodosus* is a poor pathogen and often requires tissue damage and the
357 presence of other bacteria to infect, it stands to assume *P. asaccharolytica* and *M.*
358 *fermentans* may also have an important role in disease susceptibility. These
359 differences between healthy and footrot affected feet also extended beyond presence
360 and absence of species to the overall bacterial diversity, with a significant drop in
361 footrot samples. This reduction in diversity has been mirrored in CODD (21).

362

363 The benefits of using metagenomics compared to 16S rDNA studies include that the
364 scope can be extended to incorporate archaeal and DNA virus discovery. In the
365 current study there was a lack of correlation between either and the disease state,
366 however, this may be unsurprising without an enrichment step or optimal DNA
367 extraction to make them specifically more suitable for viral or archaeal identification,
368 resulting in a poor representation for those species identified.

369

370 ***Host response and pathogen interactions***

371 Investigating the host pathogen interactions through correlation analysis has
372 identified some interesting associations which warrant further investigations. The
373 most promising appears to be the association between virulence gene *aprV5* and the
374 *Ovis aries* transcript U6 spliceosomal RNA. This particular non-coding small nuclear
375 RNA (snRNA) is responsible for catalysing the excision of introns and is a major

376 aspect of post translation modifications, with the ability to alter the structure, function
377 and stability of the translated protein. In the case of infections, some species of
378 bacteria have been implicated in hijacking the host splicing machinery and altering
379 the splicing pattern leading to the perturbation of the host response (22, 23). Despite
380 the lack of knowledge around the mechanism, there is evidence that certain *Listeria*,
381 *Salmonella* and *Mycobacterium* species have the ability to produce factors that have
382 a direct or indirect impact on the regulation of alternative splicing (23–25). Alternative
383 splicing from the U6 spliceosomal RNA can interfere with the normal activation of T
384 cell and B lymphocytes and the regulation of the signalling in several TLR's (TLR2,
385 TLR3 and TLR4) (26), which could tie in with certain pathways (monovalent inorganic
386 cation homeostasis, defence response to bacteria, skin development, neutrophil
387 chemotaxis, leukocyte migration, defence response, immune system processes,
388 immune response) which were identified as being downregulated in these data.

389

390 The acidic extracellular protease *aprV5* is associated with the correct cleavage of the
391 other proteases secreted by *D. nodosus*, *AprV2* and *BprV*, to their mature active form
392 (Han et al., 2012). Whereas the closely related *AprV2* acidic protease is a known
393 virulence factor responsible for elastase activity and its degradation of the host extra
394 cellular matrix (28), the role of *AprV5* in footrot is unclear. The abundance of isolates
395 with *aprV5* has been shown to be around 25% from clinically affected farms and
396 lacks any clear delineation between disease severity (29). However, as viral-
397 mediated proteases have been implicated in the degradation of host small non-
398 coding ribonucleal proteins (snRNP) (30) and other bacteria possess other
399 mechanisms of action on snRNP's it may be an interesting focus of future studies.

400

401 ***Sheep interdigital skin microbiota and scar tissue formation in footrot***

402 The host response to the skin microbiota has to be carefully regulated as innocuous
403 microbes and the host surveillance at epithelial barriers are in constant close
404 proximity. In healthy tissues bacteria are located predominantly in the epidermis
405 while tissue damage and invasive bacteria such as *D. nodosus* allow access of
406 bacteria into deeper dermal tissue layers (10). The ovine host response to footrot
407 demonstrated through differential expression of a range of transcripts involved in
408 proinflammatory mediation (cytokines; IL-19, IL-20, IL-6, LIF, chemokines; CCL2,
409 CCL20, CXCL1, CXCL8 and prostaglandin-endoperoxide synthase 2; PGE2/COX2),
410 of matrix metalloproteases (MMP1, MMP3, MMP9, MMP13, TNC, TIMP1) and
411 interestingly their regulators (SERPINE1, ADAMTS4, ADAMTS16) during footrot, all
412 of which are associated with wound healing, collagen turnover and scar tissue
413 formation. Collagen I was detected significantly more in diseased dermis than in non-
414 infected dermal tissue leading to the conclusion that infection or co-infection clearly
415 indicates current, or ongoing, scar formation in the dermis.

416

417 The process of second intention wound healing with scar formation is classically
418 divided into three main overlapping phases: inflammation, proliferation, and
419 remodelling. Localised inflammation is the first response to any breach of
420 haemostasis with the initiation of cytokine and chemokine production leading to
421 neutrophil and macrophage recruitment to the site of inflammation (31). The
422 cytokines and chemokines IL-6, CCL2, CXCL1, CXCL8 identified as differentially
423 expressed in response to footrot, are associated with acute inflammation in response
424 to tissue injury (31, 32). In normal skin wound healing, the inflammation usually lasts
425 for 2–5 days and ceases once the harmful stimuli have been removed. The IL-20

426 cytokine family (IL-19, IL-20, IL-22, IL-24, IL-26) contribute to various stages of this
427 wound healing process: they are primarily secreted by infiltrating innate immune cells
428 and lymphocytes shortly after an injury. Initially released by infiltrating macrophages,
429 they preferentially stimulate keratinocytes to secrete antimicrobial peptides and
430 chemokines, in order to reduce infection and accelerate inflammation, and to produce
431 increased levels of vascular endothelial growth factor A (VEGFA), which in turn
432 promotes angiogenesis. IL- 20 subfamily cytokines directly stimulate keratinocyte
433 proliferation and migration, and indirectly support the proliferation of keratinocytes by
434 enhancing the production of epidermal growth factor (EGF) and keratinocyte growth
435 factor (KGF) (33). Surprisingly, we observed significantly reduced expressed IL-19
436 and IL-20 transcripts in footrot samples. This was accompanied by reduced
437 expression of secretory leucocyte protease inhibitor 1 (SLP1), a protein essential for
438 optimal wound healing due to its antimicrobial and anti-inflammatory properties (34).
439 Macrophages, initially producing pro-inflammatory mediators, transition in response
440 to local immune signals to an anti-inflammatory phenotype. This promotes the
441 resolution of inflammation and a transition to the proliferation phase of second
442 intention wound healing focusing on re-epithelialisation through migration and
443 proliferations of keratinocytes, deposition of type III collagen, and angiogenesis (31).
444 Matrix metalloproteases (MMPs) are crucial to this phase as extracellular matrix
445 degradation and deposition is essential for wound re-epithelialisation and also during
446 tissue remodelling. MMP expression and activity are tightly controlled during wound
447 healing, at the expression levels and through endogenous tissue inhibitors of metallo
448 proteases (TIMPs); specific MMPs are confined to particular locations in the wound
449 and to specific stages of wound repair (35). MMP-1, MMP-3, and MMP-9 are the
450 major chemokine regulators during wound healing, degrading chemokines by

451 proteolysis and promoting the transition to the proliferation phase. MMPs-1, 8, 9 and
452 13 are transiently upregulated to remodel the fibrin clot and replacing it with new
453 extracellular matrix. In addition, they are fundamentally stimulating the migration of
454 keratinocytes into the wound bed. During re-epithelialisation, keratinocytes migrate
455 from the surrounding epithelium and proliferate to achieve wound closure. This is
456 accompanied by a decreased expression of pro-migratory MMPs (MMP-1 & 2) and
457 an increased tissue remodelling MMP-3 expression (35, 36).

458 The dysregulation of MMPs leads to prolonged inflammation and delayed wound
459 healing (37). In footrot affected tissues we observed differential expression of the
460 collagenases MMP-1 and 13, the gelatinase MMP-9 and the stromalysin MMP-3. It is
461 well established that high levels of MMP-1 lead to defective re-epithelialisation, with
462 MMP-13, expressed deeper in the tissues, leading to granulation tissue formation
463 (37). Increased MMP-9 levels are consistent with chronic wounds, leading to the
464 reduced expression of the growth factors required for the healing process while
465 prolonging the inflammatory phase (37). The stromalysin MMP-3 is expressed by
466 proliferating keratinocytes at the distal end of the wound and is essential for wound
467 healing (37). However, we observed reduced expression compared to uninfected
468 interdigital skin tissue, which would impact on the ability of infected interdigital skin
469 tissue to heal. Long chain fatty acids and collagens are essential for skin barrier
470 function (36, 38). The increase in expression of fatty acid elongases (ELOVL7,
471 ELOVL3, ACSBG1) and of proteins involved in collagen production and collagen
472 binding in response to footrot suggests some level of skin regeneration is ongoing.

473

474 One of the bacteria significantly increased in abundance on footrot-infected lesions,
475 *M fermentans*, might affect the ability of host skin cells to respond to bacterial

476 infection. Chronic infections of monocytes and macrophages with intracellular low
477 pathogenic *Mycoplasma* spp. of have been shown to impair their inflammatory
478 response to live bacteria and bacterial products (39, 40). That we see higher
479 transcript levels of outwardly healthy interdigital skin is in contrast to the lack of
480 detection of MMP RNA in healthy human or murine skin (41). However, this is
481 consistent with a marked expression of the inflammatory cytokines/chemokine IL1 β ,
482 IL6 and CXCL8 in outwardly healthy ovine interdigital skin, which might be due to the
483 constant environmental changes and pressures impacting on interdigital skin or might
484 be associated with subclinical disease that may have developed into ID and footrot in
485 the future (7). During the remodelling phase of scar tissue formation, initially
486 deposited collagen-III molecules are gradually replaced by type I collagen and their
487 orientation becomes more organised (36). Mature cutaneous scars consist of 80-90%
488 type I collagen arranged in parallel bundles (42). This particular orientation as well as
489 less pronounced or missing rete ridges weaken the strength of the scar tissue
490 compared to normal skin in humans to only 70-80% (36). This renders the tissue
491 more susceptible to injury and trauma which are suspected predisposing factors of
492 footrot. The latter might also contribute to the frequently observed relapses and
493 underlines the not only polymicrobial but rather multifactorial aetiology of footrot. For
494 BDD a dysfunctional skin barrier and disturbed tissue integrity is hypothesised to be
495 an essential prerequisite for infection altogether since experimental disease models
496 without skin maceration prior to infection fail to mirror naturally occurring BDD lesions
497 appropriately (43).

498

499 Currently there is conflicting evidence of the impact of the microbiome on wound
500 healing, with some evidence of host commensal interactions promoting wound

501 healing while colonisation of pathogenic bacteria may invade deeper into tissues or
502 lead to chronic infections and biofilm formation (44, 45). In the context of footrot, we
503 identified another bacterial species in addition to *D. nodosus* that is associated with
504 footrot and also known to be a synergistic wound pathogen, *P. asaccharolytica*.
505 When present in combination with anaerobic and aerobic bacteria such as *Prevotella*
506 *melaninogenicus*, *Peptostreptococcus micro* and *Klebsiella pneumoniae*, *P.*
507 *asacharolytica* exacerbates the disease process (46–48). While antibiotic injections
508 will affect indiscriminately on commensal and pathogenic bacteria, the effectiveness
509 of parenteral antibiotics in footrot demonstrate their high impact on the pathogenic
510 bacteria leading to swift recovery in most cases (49). Interestingly, resistance genes
511 against tetracycline, the most commonly used antibiotic against footrot have so far
512 not been identified in *D. nodosus* genome sequences, suggesting that the antibiotic
513 treatment mainly affects other microbes of that polymicrobial infection enabling host
514 immune system to eliminate *D. nodosus*.

515

516 **Conclusion**

517 Ovine footrot is a complex polymicrobial disease and there is a clear need to further
518 elucidate the intricate host microbial interactions. We aimed to investigate the host
519 response as well as the microbial taxa in tissues and their intra-tissue expression
520 levels using metatranscriptomics in naturally infected tissues. It is well published that
521 skin damage is required to allow *D. nodosus* infection to establish (2, 50). As
522 expected, the host response in footrot is characterized by differential expression of
523 proteins with roles in wound healing and chronic wounds. As in the absence of *D.*
524 *nodosus*, interdigital dermatitis resolves, the presence of *D. nodosus* may be
525 essential to allow the establishment of the microbes associated with under running
526 footrot, including *P. asaccharolytica*. In these later stages of disease, the presence of

527 those bacteria, such as *M. fermentans*, may contribute to a dampening of the
528 immune response unable to remove the invading bacterial pathogens leading to
529 chronic infection.

530

531 **Materials and Methods**

532

533 ***Sample collection***

534 Sheep were assessed post slaughter for foot health. Any individual animals showing
535 signs of footrot were selected for sample collection. Debris was removed from all the
536 feet and cleaned using purified water. Sterile nylon flock swabs (E-swabs 480CE,
537 Copan U.S.A.) were taken from the interdigital space and stored in liquid Amies
538 media at 5°C overnight. The foot was then washed with a chlorohexidine solution
539 (National Veterinary Services, U.K.). Any hair was removed from the feet with
540 scissors, prior to the collection of an 8mm biopsy using a punch (National Veterinary
541 Services, U.K.). Biopsies were placed in RNALater (Sigma Aldrich, U.K.), stored at
542 5°C overnight before being frozen at -80°C.

543

544 ***DNA Extraction from swabs***

545 The interdigital space swabs were placed on a MixMate (ThermoFisher, U.K.) for 5
546 minutes at 800rpm to thoroughly disperse the bacteria in the amies solution from the
547 swab. The liquid was transferred into a low-bind 1.5ml tube and centrifuged at 12,000
548 rpm for 5 minutes. The supernatant was removed, and the pellets were resuspended
549 in 200µl of RNase-free molecular biology grade water (Thermo Fisher, U.K.) (51).
550 DNA was isolated using the Qiagen Cador Pathogen Mini Kit, following the

551 manufacturer's guidelines, eluted in 60µl of elution buffer. The DNA samples were
552 quantified using the Qubit 3.0 and dsDNA high sensitivity dye (Qiagen).

553

554 ***RNA Extraction***

555 Biopsies were thawed on ice before being cut into approximately 30mg sections. One
556 section was added to a MACs M tube (Miltenyi Biotech, U.K.) containing 1ml Qiazol
557 (Qiagen, U.K.) and dissociated on a GentleMACs (Miltenyi Biotech, U.K.) using the
558 manufacturer's RNA settings. The sample was centrifuged and incubated at room
559 temperature (RT) for 5 minutes before transferring the lysate to a 1.5ml centrifuge
560 tube. Proteinase K (20µl) was added to the sample before being incubated at 56°C
561 for an hour. Chloroform (200µl) was added and shaken vigorously for 15 seconds.
562 The sample was then incubated at RT for 2 minutes before being centrifuged at
563 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh
564 1.5ml centrifuge tube before the addition of 1x volume of 70% ethanol. The sample
565 (up to 700µl) was added to an RNeasy Mini Spin column (Qiagen, U.K.) and
566 centrifuged at RT at 8000xg for 30 seconds. Any remaining sample was also passed
567 through the column. All remaining steps followed the manufacturer's guidelines with
568 elution in 30µl of RNase-free molecular biology grade water (Thermo Fisher, U.K.).

569

570 ***Dual RNA Sequencing***

571 The extracted RNA was quantified using the Agilent Bioanalyser RNA Nano 6000 kit.
572 Healthy foot sample RNA with a RIN score of ≥ 7 and footrot sample RNA with a
573 DV200 > 85 was chosen for sequencing. The samples were treated using the
574 RiboZero Gold (epidemiology) ribosomal depletion kit (Illumina, U.S.A.) and prepared
575 for sequencing using Illumina TruSeq library preparation (Illumina, U.S.A.). The

576 samples were sequenced on a HiSeq 3000 using 150bp paired end chemistry (Leeds
577 Institute of Molecular Medicine, sequencing facility) at 6 libraries per lane over 14
578 lanes giving approximately 75 million reads per sample.

579

580 **Data Analysis**

581 All analysis was carried out using default settings unless stated. Raw reads were
582 analysed for quality and adaptor removal using Skewer (52). An initial step for the
583 RNASeq data consisted of aligning the reads with HISAT2 (53) against the sheep
584 genome (Oar_v3.1, downloaded 21/07/2017) (54) to separate the ovine and potential
585 bacterial transcripts. The sheep reads were then parsed for transcript alignment
586 using Kallisto (55) and the sheep genome (Oar_v3.1, downloaded 21/07/2017) (54).
587 Differential analysis was calculated with Sleuth (56). The differentially expressed
588 genes were imported in R (57) and clustered using BiClust and the Block Correlated
589 Coupled Clustering (58). The reads which did not align to the sheep genome and the
590 metagenomic reads were used as input for taxonomic assignment and bacterial
591 populations were determined with Kraken (59), false positives were identified with
592 KrakenUniq (60) and results were filtered with MAG_TaxaAssigner
593 (<https://github.com/shekas3/BinTaxaAssigner>).

594

595

596 **Confirmation of selected bacterial species by PCR or qPCR**

597 Parallel tissue samples from the same foot as for RNA isolation were used. Tissue
598 homogenisation and DNA extractions were performed as described previously using
599 QIAamp Cador kit (Qiagen) (61). The DNA samples were quantified using the Qubit
600 3.0 and dsDNA high sensitivity dye (Qiagen). Bacterial load was quantified using
601 real-time PCR based on 16S rRNA gene for eubacteria (62) and *D. nodosus* (63), *F.*

602 *necrophorum* subspecies *necrophorum* primers targeted the *gyrB* gene (51). *M.*
603 *fermentans* sequences were amplified by species specific PCR for 16S rRNA (64)
604 and *P. asaccharolytica* sequences were amplified by PCR (65) followed by
605 sequencing (Eurofins Genomics).

606

607 **Correlation Testing**

608 Both tests were performed using the same dataset. For each of the samples (biopsy,
609 swab and host), each of the bacteria species were labelled as either present or
610 absent in each sample. A difference score was prescribed if species was present in
611 one of the swab or biopsy samples from a sheep but, not both (i.e., the swab and
612 biopsy gave different results for the presence of the species). If present in both or
613 neither the biopsy nor swab samples for then. A t-test statistic was calculated by
614 taking the sum of all differences across all sheep. To perform the randomisation
615 procedure the labels of the original 52 samples (sheep and sample type) were
616 randomly reassigned and the test statistic recalculated. This procedure was repeated
617 10 000 times, giving randomised test statistics.

618

619 **Host Pathogen Interactions**

620 To identify putative host pathogen interactions the correlation script PHnder was
621 used (<https://github.com/addyblanch/PHnder>). Briefly, samples with zero
622 assignments were removed, and a minimum presence value was set to one. A new
623 data matrix was formed and the hypergeometric distribution was calculated using
624 phyper
625 (<https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/Hypergeometri>
626 [c](#)) and a probability, producing a significance (p) value and adjusted p value.

627 ***Tissue staining, image capture and analysis***

628 Biopsies were processed in ethanol and xylene, mounted in paraffin and 7 µm thick
629 serial sections collected throughout the tissue onto polysilinated microscope slides.
630 The paraffin from each tissue section was melted at 60°C for 5-10min, followed by
631 immersed in xylene twice for 5min each to remove the paraffin. Tissue sections were
632 then rehydrated in 100% ethanol, 90% ethanol, 70 % ethanol and distilled water for
633 5min each. Picrosirius red (PS) stain was used to differentiate and quantify collagen
634 types I, III and undifferentiated collagens using the picrosirius stain kit (Polysciences,
635 Inc., Pennsylvania, USA). The observer was blinded to the sample identification to
636 avoid subconscious bias. Images were captured using a Leica CTR500 microscope
637 (Leica Microsystems, Germany) with or without polarised light. For each sample,
638 three sections approximately 400 µm apart were analysed. At 40x magnification, five
639 non-overlapping photos were taken from each section from the dermis of PS stained
640 sections (330 photomicrographs analysed).

641
642 For 13 healthy and 8 footrot samples, 15 photomicrographs per sample were
643 captured (systematic random sampling) and analysed using Image-Pro Plus (Media
644 Cybernetics, Inc, Pennsylvania, USA) to quantify the area of collagen in each image,
645 with separate measurements for type III (green), type I (yellow) and undifferentiated
646 (red) collagen (315 images analysed in total). Total collagen proportion was
647 calculated as the sum of type III, type I and undifferentiated collagen proportions.

648

649 ***Statistical analysis***

650 The taxonomic count data were analysed for statistically significant differences in R
651 (57) using the edgeR wrapper (66) as part of the Phyloseq package (67). Diversity

652 statistics were calculated using vegan (68) and differences were calculated using
653 Mann-Whitney U tests in Prism 8.01 (GraphPad Software Inc. USA).

654

655 Statistical analyses of histology images were performed on GraphPad Prism version
656 6 for windows. Resulting data were presented as frequencies and percentages and
657 were analysed by student T-test or Kruskal Wallis test, dependent on data
658 distribution. Analysis was taken as significant when $p \leq 0.05$.

659

660 **Author Contributions**

661 AMB and ST developed the idea, designed, and supervised the experiments and
662 alongside SW, CMB, JKM and GE have written the manuscript. AMB completed the
663 bioinformatics analysis. CES, CMB and JKM carried out the lab work, NN performed
664 and analysed bacterial qPCR. CR and CB performed and analysed the collagen
665 assays. LS performed additional statistical and mathematical assessment of the data.
666 All authors have read the manuscript.

667

668 **Ethical Statement**

669 This study was reviewed and approved by the University of Nottingham, School of
670 Veterinary Medicine and Science ethical review committee ERN: 1144 140506 (Non
671 ASPA).

672

673 **Funding**

674 This work was supported by the Biotechnology and Biological Sciences Research
675 Council [grant number BB/M012085/1] (BBSRC) Animal Health Research Club 2014
676 and the University of Nottingham. NB was funded by BBSRC STARs scheme (2017-

677 2019). GE & SRW were also funded by the Scottish Government Rural and
678 Environment Science and Analytical Services (RESAS) GE and SRW also received
679 funding from the European Union's Horizon 2020 research and innovation
680 programme under grant agreement No. 731014 (VetBioNet). The funders had no role
681 in the design of the study, collection, analysis, interpretation of the data nor in the
682 writing of the manuscript.

683

684 **Competing Financial Interests**

685 All the authors state that there is no competing financial interest in the production of
686 this manuscript.

687

688 **Acknowledgements**

689 The authors would like to thank Professor Christoph Mülling for helping facilitate this
690 research collaboration with the Faculty of Veterinary Medicine, Leipzig University.

691 The authors would also like to thank Dr Ian Carr and Ummey Hany at The Leeds
692 Institute for Molecular Medicine Sequencing Facility for all their experimental
693 suggestions and sequencing support.

694

695 **Open Access Data**

696 All sequence data generated for this study is held in the NCBI SRA under the
697 accession number PRJNA725378.

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