Microbiology of equine wounds and evidence of bacterial biofilms

S.J. Westgate, S.L. Percival, D.C. Knottenbelt, P.D. Clegg, C.A. Cochrane

Accepted Manuscript

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Authors: S.J. Westgate, S.L. Percival, D.C. Knottenbelt, P.D. Clegg, C.A. Cochrane

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3 **Westgate[1], S.J.*, Percival[2], S. L., Knottenbelt[1], D.C., Clegg[1], P.D. and Cochrane[1], C. A.**

4 [1] University of Liverpool, School of Veterinary Sciences, Faculty of Health and Life

5 Sciences, Leahurst, Neston, South Wirral, United Kingdom, CH64 7TE.

6 E-mail:s.j.westgate@liv.ac.uk

7 Telephone: 01517946089

8

9 [2] Department of Pathology, School of Medicine, West Virginia University, Morgantown,

10 West Virginia.

11

12 **Abstract**

13

14 Horse wounds have a high risk of becoming infected due to their environment. Infected

15 wounds harbour diverse populations of microorganisms, however in some cases these

16 microorganisms can be difficult to identify and fail to respond to antibiotic treatment,

17 resulting in chronic non-healing wounds. In human wounds this has been attributed to the

18 ability of bacteria to survive in a biofilm phenotypic state. Biofilms are known to delay

19 wound healing, principally due to their recalcitrance towards antimicrobial therapies and

20 components of the innate immune response. This study describes the presence of bacterial

21 biofilms within equine wounds. Thirteen eight millimetre diameter tissue samples were

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22   collected from (n=18) chronic wounds. Following histological staining, samples were

23   observed for evidence of biofilms. Fifty one wounds and control skin sites were sampled

24   using sterile swabs. Control skin sites were on the uninjured side of the horse at the same

25   anatomical location as the wound. The isolated bacteria were cultured aerobically and

26   anaerobically. The biofilm forming potential of all the isolated bacteria was determined using

27   a standard crystal violet microtitre plate assay. Stained tissue samples provided evidence of

28   biofilms within 61.5% (8 out of 13) equine wounds. In total 340 bacterial isolates were

29   identified from all the equine wound and skin samples. *Pseudomonas aeruginosa* and

30   *Enterococcus faecium* were the most predominantly isolated bacterial species from equine

31   wound and skin samples respectively. *Staphylococcus* was the most commonly isolated

32   genus in both environments. Bacteria cultured from chronic and acute wounds showed

33   significantly ($P<0.05$) higher biofilm forming potential than bacteria isolated from skin. This

34   paper highlights preliminary evidence supporting the presence of biofilms and a high

35   microbial diversity in equine chronic wounds. The presence of biofilms in equine wounds

36   partly explains the reluctance of many lower limb wounds to heal. Non-healing limb wounds

37   in horses are a well documented welfare and economic concern. This knowledge can be used

38   to shape future treatments in order to increase the healing rate and decrease the costs and

39   suffering associate with equine wounds.

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Introduction

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47 The incidence and prevalence of trauma wounds in horses is considered to be high (Singer et

48 al., 2003) and when these wounds fail to heal they are referred to as chronic wounds (Adam

49 and Southwood, 2006). Human and equine chronic wounds are considered to be

50 pathologically similar (Cochrane, 2003) but unlike human chronic wounds the microbiology

51 of equine wounds has not been studied in any great detail, either in terms of the number of

52 inhabiting bacteria, the types of inhabiting bacteria or the ability of bacteria to form

53 protective biofilms. In a number of human wound studies there is significant evidence

54 suggesting that chronic wounds have a delayed healing response due to the presence of a

55 polymicrobial biofilm (Davis et al., 2008). In these cases, wounds may appear to be

56 uninfected, but resist healing due to a persistent bacterial population residing within

57 protective biofilm.

58

59 A biofilm is the name given to a community of microorganisms attached to a surface, or each

60 other, and encased within a three dimensional matrix of extracellular polymeric substances

61 (EPS) (Mertz, 2003). Biofilm formation has been linked to an increased inherent resistance

62 to antimicrobial agents and the host's immune response (Stewart and Costerton, 2001). For

63 example *Staphylococcus aureus* when growing within a biofilm has been reported to be up to

64 100 times more resistant to antimicrobials when compared to their free floating or planktonic

65 counterparts (Leid et al., 2000). Consequently biofilms are known to be a causative agent in

66 persistent mammalian infections (Costerton et al., 2003) including reoccurring leg ulcers

67 (Bjarnsholt et al., 2008). Recently, preliminary evidence proposing the presence of biofilms

68 in chronic equine wounds was reported (Freeman et al., 2009).

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69

70 The purpose of this study was two-fold. Firstly it aimed to highlight evidence that biofilms

71 exist in equine wounds; by examining tissue sections for evidence of biofilms and by

72 comparing the virulence of bacteria isolated from chronic and acute equine wounds, and from

73 equine skin. Virulence was assessed by determining the biofilm forming potential (BFP) of

74 the isolates. Using skin cultures as a control, the study also aimed to investigate the microbial

75 richness of equine wounds, in order to highlight differences in the species that were prevalent

76 in chronic wounds, acute wounds and equine skin.

77

78 **Material and methods**

79

80 **Experimental design**

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82 Between November 2007 and May 2008, 51 of the horses and ponies at the Philip

83 Leverhulme Equine Hospital (PLEH), Neston, UK that presented with acute or chronic

84 wounds were selected for this study. All 41 trauma wound cases admitted to PLEH within the

85 study period were included in the study. Ten healing surgical wound cases were randomly

86 selected for inclusion within the study in order to present a wound control group. The

87 selection criteria for surgical wounds entailed sampling 1 clean surgical wound for every 4

88 wound cases. The surgical case was selected on the basis of being admitted to the hospital at

89 the same time as the fourth wound case (or every 4 thereafter). Breeds incorporated into the

90 study included 16 Thoroughbred/Thoroughbred cross, eight Cobs, four Arab/Anglo Arabs,

91 four Warm bloods, four Welsh ponies one Hunter type, one Clydesdale, one American

92 quarter horse, one shire and nine horses and two ponies of unknown breed. The horses were

93 aged between two and 22. There were 33 colts/ geldings and 18 fillies/ mares. Informed

4

94   consent was obtained from the owners of the animals participating in this study. Prior to

95   sample collection all the horses included in this study had received systemic antibiotics,

96   topical antibiotics or both; however information regarding the types and duration of antibiotic

97   treatment were not recorded. No antimicrobial dressings such as Silver dressings had been

98   used prior to sample collection. Of the 51 wounds analysed, 10 were of surgical origin, and

99   41 were the result of trauma. Surgical wounds were the result of elective surgery and, trauma

100 wounds were the result of an injury caused by a foreign object. The wounds were categorised

101 as chronic or acute by the attending veterinary surgeon. Acute wounds (including all surgical

102 wounds) were seen to progress at a normal rate through each stage of the healing process, and

103 did not show signs of local or systemic infection. Wounds were classified as chronic if the

104 case description involved a non-healing wound, or if the wound showed two or more chronic

105 signs, including; bright red colouration, slimy surface, discharge, fetid odour, and/or a lack of

106 healthy granulation tissue (Goodrich, 2006). Of the 51 wounds sampled, 33 were categorised

107 as acute and 18 as chronic. A skin swab was taken from uninjured skin at the same

108 anatomical site of the horse as the wound, but on the uninjured side of the horse, for example

109 if the wound site was the left front carpus, the skin site was the right front carpus. Wounds

110 included in this study were a mixture of thoracic and limb wounds. All swab samples were

111 analysed microbiologically for aerobic and anaerobic bacteria, using traditional

112 microbiological techniques as described below. The biofilm forming potential for each isolate

113 was determined using crystal violet stained microtitre plate assays. As part of the normal

114 wound treatment, debrided tissue samples that would usually be disposed of as surgical waste

115 were removed from the edge of 5 wounds as described below. These samples underwent

116 histological staining in order to look for evidence of bacterial biofilms.

117

118 **Tissue sampling**

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120 As part of the normal wound debridement treatment, tissue samples that were classified as

121 surgical waste were collected from the edge of the wound. Samples of the debrided tissue

122 were sectioned using a disposable eight mm punch biopsy (Amcor flexible, UK). The tissue

123 was then immediately stored in formalin (VWR, Leicestershire, UK) for 24h, and then

124 sectioned and stained with three histological stains; Gram stain, haematoxylin and eosin (H

125 and E) and Masson's trichrome (MT) stains. All Stains were made from individual reagents,

126 sourced from H.D Supplies, Buckingham, UK, V.W.R International, Leicestershire, UK,

127 Fisher Scientific UK Ltd Leicestershire, UK and Sigma-Aldrich Company Ltd, Dorset, UK.

128 The stained samples were examined under light microscopy (Nikon Eclipse 80i with digital

129 sight capture) at a magnification of x1000.

130

131 **Bacterial sample collection using swabs**

132

133 Standard microbiological protocols as described below were utilised for the sampling of the

134 wound and skin sites. All sampling procedures were performed within one to three days of

135 the horse or pony being admitted to PLEH. Wound and skin sampling sites were washed with

136 sterile saline prior to swabbing in order to remove surface debris. Swabbing involved holding

137 the sterile cotton tipped swab between the thumb and fore finger and rubbing it across a $1cm^2$

138 area for five seconds, as described in the Levine technique (Levine et al., 1976). Surgical

139 swabs were taken from the surface of the wound which had been closed by primary intention.

140 All wound and skin sites were swabbed in triplicate. The first swab was stored in Stuart's

141 transport medium (Oxoid Limited, Hampshire, UK) and used to isolate aerobic bacteria, the

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142 second was added to Robertson's Cooked Meat Broth (Oxoid Limited, Hampshire, UK), for

143 the enrichment of anaerobic bacteria, and the third was stored in an eppendorf tube and

144 frozen at -20°C for molecular analysis.

145

146 **Bacterial isolation and identification**

147

148 Aerobic swabs were stored at 4ºC and processed within 24h. Aerobic samples were

149 inoculated onto the enrichment agar Tryptone soy agar (TSA) and the selective agars;

150 MaConkey agar (MCA), Columbian blood agar (CBA), and Cysteine lactose electrolyte

151 deficient (CLED) agar (Lab M, Bury, UK).   All agar plates were incubated for 24 hours at

152 37ºC (+/-2^{0}c). Swabs which had been stored in Robertson's cooked meat broth were

153 incubated at 37ºC for 24 hours, and then at room temperature for 1 week. At 48 hour intervals

154 samples of this media were added to fastidious anaerobic agar (FAA) (Lab M, Bury, UK), for

155 the culture of anaerobic bacteria. The prolonged incubation period was used to enhance the

156 recovery of slow growing anaerobic species. Isolates grown in anaerobic conditions were also

157 sub-cultured onto CBA and incubated overnight in aerobic conditions at 37 ºC. Isolates that

158 grew in both aerobic and anaerobic conditions were identified following the aerobic protocol.

159

160 Following incubation, all the phenotypically distinct bacterial colonies were sub cultured onto

161 TSA (aerobic isolates) or FAA (anaerobic isolates). Pure bacterial colonies were identified

162 using Gram staining, oxidase testing, catalase testing, coagulase testing and biochemical

163 analysis. API identification strips (Oxoid Ltd, Hampshire, UK), Vitek identification cards

164 (BioMerieux, Hampshire, UK) and the Microseq 3130 Microbial Identification System

165 (Applied Biosystems, UK) were utilised for further species identification.

166

167 **Microtitre plate assay for the assessment of biofilm formation**

168

169 The biofilm forming potential of all the bacteria identified throughout this study was

170 measured using a published method (Wakimoto et al., 2004). In brief, colonies of individual

171 purified bacteria were inoculated into Muller Hinton Broth (MHB), and incubated overnight

172 at 37°C in an orbital incubator (50rpm). Following incubation, all the bacterial suspensions

173 were adjusted to 10^{8} cfu ml^{-1} using sterile MHB. The concentration was confirmed using a 0.5

174 McFarland standard (StepanoviĆ et al., 2007). Then 250μl of each inoculum was transferred

175 into 3 wells of the Costar 96 well microtiter plates (Corning Costar, NY, USA). The 96 well

176 plates were incubated at 37°C for 24 h at 50 rpm in an orbital incubator. Following

177 incubation, each well was washed (x3) with sterile saline. Attached bacteria were then fixed

178 in 96% pure ethanol and then stained with 2% crystal violet. Excess crystal violet was then

179 aspirated from the wells, and the wells were washed (x3) with sterile distilled water to

180 remove all excess dye and planktonic bacteria. Each well was then filled with 200μl of 33%

181 glacial acetic acid to remove the stained biofilm. The optical density (OD) of each well was

182 measured at 570nm using an Opsys MR microtiter plate reader (DYNEX Technologies,

183 Worthing, UK). The average OD of the control wells (containing MHB only) was subtracted

184 from the OD of the test wells. All isolates and controls were tested in triplicate.

185

186 **Statistical analysis**

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188 A Wilcoxon signed rank test was used to investigate differences in the genus and species of

189 bacteria present at wound sites compared to control sites. A Fisher's exact Chi Squared test

190 was used to assess the different kinds of bacteria found in chronic wounds compared to acute

191 wounds and in surgical wounds compared to traumatic wounds. A Mann Whitney U test was

192 used to compare the presence of different bacterial genus at each sample site, and also to

193 compare the biofilm forming ability of each bacterial genus, and the biofilms forming ability

194 of the bacteria isolated from different wound types. All statistical analysis was carried out

195 using the statistical package Minitab 15.

196

197 **Results**

198

199 **Tissue samples**

200

201 Bacterial clustering was seen in the stained tissue samples, suggesting evidence of a bacterial

202 biofilm, based on published observations and biomarkers (Parsek and Singh, 2003). Infected

203 tissue contained extensive 'thumb-print' like aggregates and microcolonies of Gram positive

204 cocci and Gram negative rods, at the epidermal surface of the debrided tissue (Figure 1).

205 Although Gram positive and Gram negative bacteria occupied the same tissue regions, more

206 often than not, they appeared to cluster in discrete segregated microcolonies. The staining

207 also highlighted extensive evidence of inflammatory cells throughout the chronic wound

208 tissue. In un-infected tissue, little or no evidence of bacteria was seen (Figure 2). This was

209 supported by a culture negative swab sample.

9

210

Bacterial isolation

212

213 From 51 equine skin and wound swab samples, 340 bacterial isolates were cultured (Table 2)

214 and 338 were identified to the species level. The most common bacterial species isolated

215 from wounds was *P. aeruginosa* (5.6%) and the most commonly isolated species from skin

216 was *E. faecium* (5.6%). *Staphylococcus sp* were isolated more often than most other genus

217 (P<0.05) and there was significant difference in the genera isolated from wound and skin

218 sites (P<0.05).

219

220 The wound swabs were further divided into wounds that were the result of surgery or a

221 traumatic incident, and wounds that were deemed chronic or acute (Figure 3). The average

222 number of bacterial species identified from trauma wound swabs was 3.02 ±1.65 (range 0-8)

223 and the average number of species per surgical wound swab was 3.8 ±3.82 (range 1-14).

224 Fourteen isolates were identified from only one surgical wound. At a genus level, the number

225 of isolates identified per swab for trauma and surgical wounds was 2.51 ±1.36 (range 0-6)

226 and 2.8 ±1.87 (range 1-7) respectively. Again the mean number of surgical wound isolates

227 obtained per swab was increased due to the inclusion of the surgical wound containing 14

228 isolates.

229

230 The species most often isolated from traumatic wound swabs and surgical swabs was *P.*

231 *aeruginosa*, where *P. aeruginosa* made up 4.84% and 7.89% respectively, of the total

232 identified species. However the most commonly isolated genus in both traumatic and surgical

10

233   wounds was *Staphylococcus*, which respectively made up 22.58% and 18.42% of the total

234   identified bacterial load. The *Staphylococcus* species cultured from equine wounds included;

235   *S. aureus,S. auricularis, S. epidermidis, S. xylosus, S. Sciuri, S. simulans, S. warneri, S.*

236   *equorum, S. hominis,* and *S. pasteuuri*. *Proteus* sp, *Acinetobacter* sp, *Salmonella* sp,

237   *Alcaligenes* sp, *Propionibacterium* sp, *Bordetella* sp, *Cellumonas* sp, were found exclusively

238   in trauma wounds. Furthermore the prevelence of *Staphylococcus sp* in traumatic wounds

239   was greater than the prevelence of *Micrococcus sp*, *Enterococcus sp*, *E. coli sp*, *Enterobacter*

240   *sp*, *Alcaligenes sp*, *Clostridium sp*, *Propionibacterium sp*, *Bordetella sp*, and *Cellulomonas*

241   *sp* (P<0.05).

242

243   Of the 162 identified wound isolates, 32.72% were of chronic wound origin, and 67.28%

244   were isolated from acute wounds. *Pseudomonas* spp were significantly (P<0.05) more

245   prevelant in chronic wounds than acute wounds. This kind of variation may be occuring

246   between other genus such as *Proteus* and *Enterobacter*, but this difference was not significant

247   with the current sample size. The genera *Aerococcus, Micrococcus, Alcaligenes,* and isolates

248   that could not be identified, were isolated exclusively from acute wounds, but no significant

249   difference was seen in the data (Figure 4).

250

251   In chronic wounds, *Pseudomonas* spp were identified significantly more often than

252   *Aerococcus* spp, *Micrococcus* spp, *Pasturella* spp, *Corynebacterium* spp, *Alcalgenes* spp,

253   *Bordetella* spp, and *Cellumonas* spp (P<0.01) and to a lesser extent (P<0.05), *Bacillus* spp,

254   *Acinobacter* spp, *Salmonella* spp, *E.coli, Clostridium* spp. and *Propinobacterium* spp. No

255   significant difference was seen in the isolation prevelence of the other identified bacterial

256 genus within chronic wounds, and there was no significant difference in the prevelence of

257 isolates cultured from acute wounds.

258

259 Obligate anaerobic isolates were recoverd from swabs and made up 6.8% and 7.3 % of the

260 total wound and skin isolates respectively. There was no significant difference in the amount

261 of times obligate anaerobes were identified from wound and skin swabs or between the

262 anaerobic recovery rate from the swabs used to sample different wound types.

263

264 **Microtitre plate assay for the assessment of biofilm formation**

265

266 The species *Aerococcus viridans 3, S. warneri, B. licheniformis, B. pumilus,* and

267 *Acinetobacter spp.* all showed significantly stronger biofilm formation when they were

268 isolated from wounds compared to skin (P<0.05). *Enterococcus durans* was the only species

269 to show significantly higher biofilm formation (BFP) when isolated from skin than from

270 wounds (P<0.05). Collectively the bacteria isolated from wounds displayed a significantly

271 (P<0.05) higher BFP than those isolated from skin. Wound isolates had an average BFP value

272 of 0.50 ± 0.35 and skin isolates had an average value of 0.32 ± 0.30. An optical density

273 above 0.2 was indicative of bacteria that showed a level of adherence linked to biofilm

274 formation (Table 1) (Stepanovic et al., 1999).

275 At a genus level, the wound isolates *Pseudomonas, Proteus, Bacillus* and *Aerococcus*

276 displayed significantly stronger attachment, indicative of biofilm formation, than the

277 equivalent skin isolates (P<0.05) (Figure 5). *Pseudomonas* and *Proteus* spp. wound isolates

278 displayed strong adherence (OD > 0.4) and *Bacillus* and *Aerococcus* spp. displayed moderate

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279   adherence (OD between 0.2 and 0.4). The genus *Moraxella* produced the highest wound

280   isolate OD (OD = 2.17±1.33), but the largest OD recorded was from skin isolates of

281   *Myroides* bacteria (OD = 2.46±0.05). These values were suggestive of extremely strong

282   adherence and thus a strong BFP. The genera *Burkholderia* sp*, Shigella* sp*, Yersinia* sp*,*

283   *Rhizobium* sp*, Sphingomonas* sp*, Pasteurella* sp and *Aeromonas* sp were isolated exclusively

284   from skin swabs. These isolates had ODs of between 0.01 and 0.37.

285   The BFP of Gram negative trauma wound isolates was significantly (P<0.01) greater than

286   those of Gram negative surgical wound isolates. However there was no significant difference

287   in the BFP of Gram positive isolates sampled from trauma and surgical wounds. At a species

288   level the species *S. epidermidis, S. xylosus, B. subtilis, Enterococcus faecalis* and

289   *Enterobacter cloacae* all displayed a stronger BFP when isolated from surgical wounds than

290   when they were isolated from trauma wounds. Similarly, *Enterobacter cloacae* and

291   *Bordetella petrii* both displayed a stronger biofilm forming potential when they were isolated

292   from acute wounds compared to chronic wounds.

293

294   There were significant differences (P<0.05) in the BFP of different isolates within trauma

295   wounds. The Gram positive isolates *Staphylococcus* spp. *Micrococcus* spp. *Bacillus* spp.

296   and *Enterococcus* spp. and the Gram negative *Pseudomonas* isolates all showed significantly

297   (P<0.05) greater attachment than *Enterobacter* spp.

298

299   **Discussion**

300

13

301 Tissue samples taken from chronic equine wounds displayed morphologically similar

302 bacterial clustering to those seen in human biofilm infected wounds (Costerton et al., 1994).

303 Bacteria displayed a greater BFP when they were isolated from wounds compared to skin,

304 suggesting that equine wounds harbour bacterial pathogens which display the biofilm

305 phenotype more commonly than the equivalent skin isolates. Chronic equine wounds did not

306 harbour bacteria that displayed a stronger BFP than acute wound. An explanation for this is

307 that culture results are heavily weighted towards easily cultured organisms (Dowd et al.,

308 2008). If biofilm forming isolates in chronic wounds were encased within a bacterial biofilm,

309 as suggested by the tissue sections, then the chance of a false negative culture may have been

310 increased (Costerton et al., 2003).

311

312 Equine wounds were polymicrobial and contained pathogens which were significantly more

313 prevalent in chronic wounds than acute wounds. *Aerococcus viridians, S. warneri* and

314 *Acinetobacter* Spp. demonstrated greater BFP when they were isolated from equine wounds

315 compared to when they were isolated from equine skin. In human studies *Aerococcus*

316 *viridians* has been linked to leg ulcers (Cooper et al., 2009) and infected surgical wounds

317 (Huljev and Kucisec-Tepes, 2005), *S. warneri* has been linked to osteomyelitis (Bryan et al.,

318 1987) and endocarditis (Wood et al., 1989) infections, and *Acinetobacter* Spp. have been

319 repeatedly linked to chronic wounds (Gjodsbol et al., 2006; Hill et al., 2003; James et al.,

320 2008). These pathogens may have contributed to the healing retardation of the chronic

321 wounds in this study. Interestingly *B. licheniformis* and *B. pumilus* also demonstrated a

322 greater BFP when isolated from wound sites compared to skin sites but previous literature has

323 highlighted their ability to disrupt *S. aureus* (Rivardo et al., 2009) and *Pseudomonas* (Nithya

324 et al., 2010) biofilms.

14

325

326   This study used bacterial attachment as a measure of BFP. Most of the species displayed

327   some ability to attach to the polystyrene surface suggesting that the adaptive mechanism of

328   biofilm formation is exhibited to some degree by the majority of wound colonising bacteria.

329   The degree to which different bacterial species attached to the polystyrene surface of the

330   microtitre plate wells was variable, reflecting the fact that not all organisms have an equal

331   ability to attach to polystyrene (Harrison et al., 2010). As such, *in vitro* attachment may not

332   accurately reflect *in vivo* attachment. Variations in the *in vivo* attachment are also likely to

333   reflect the synergistic interactions of the colonising/ infecting populations (Cooper et al.,

334   2009). All the horses in this study were receiving antibiotic treatment. The antibiotic

335   challenge may have favoured the biofilm forming phenotype (Dancer, 2008) however this

336   accurately reflects the clinical situation in which biofilms are problematic.

337

338   The OD of bacteria attached to the 96 well plates, ranged from 0 to 2.46. These values

339   concurred with previously presented data for 24 hour biofilms (Djordjevic et al., 2002).

340   However the range in this study was slightly larger than the range reported in some previous

341   studies. Previous data was obtained for single species biofilms for example *Staphylococcus*

342   biofilms, *E coli* biofilms and *Listeria monocytogenes* biofilms were assessed by Stepanovic *et*

343   *al* (1999), Wakimoto *et al* (2004) and Djordevic *et al* (2002) respectively. The multispecies

344   nature of this investigation explains the large range of OD values obtained in this study.

345   Knowledge about the BFP of different bacterial species allows researchers and clinicians to

346   recognize the isolates most likely to form a biofilm. Further work would be required in order

347   to advise appropriate treatment and prevention strategies for the removal of bacterial biofilms

348   from equine wounds, however if their aetiology is assumed to be similar to that of human

15

349   diabetic ulcers, thorough debridement coupled with appropriate antibiotic treatment would be

350   proposed (Davis et al., 2006).

351

352   The isolation of multiple bacterial species per swab supports the theory that equine *in vivo*

353   wound environments harbour a polymicrobial community of microorganisms (Freeman et al.,

354   2009). However it has been well reported that the culture of isolates using a swabbing

355   technique can results in an overrepresentation of surface bacteria such as *S. aureus* and an

356   underrepresentation of isolates that reside more deeply within the wound (Fazli et al., 2009).

357   Despite this observation, these authors deemed swabbing to be the most appropriate sampling

358   technique for this study due to its un-invasive nature. In human diabetic foot ulcers the

359   synergistic interactions of bacteria are thought to promote an environment that supports the

360   development and maintenance of a chronic wound (Dowd et al., 2008). The evidence

361   presented in this paper suggests that this scenario is also likely in chronic equine wounds,

362   adding weight to the theory that equine wounds provide a good model for human chronic

363   wounds (Cochrane, 2003).

364

365   Microbial diversity varies between acute and chronic wounds, for example *Pseudomonas* spp.

366   were more common in chronic wounds than acute wounds ($P<0.05$). The opportunistic

367   pathogenesis of isolates such as *Pseudomonas* have been well documented (MacDonald et al.,

368   1994). The possibility that opportunistic pathogens are resisting standard treatments by

369   residing in a biofilm is of great medical importance, in terms of individual cases and

370   antibiotic usage in veterinary medicine (Van den Eede et al., 2009). *Staphylococcus* and

371   *Enterococcus* spp. were also seen to colonise chronic wounds, supporting earlier work which

16

372 highlighted their presence in traumatic (Orsini et al., 2004) and in surgical equine wounds

373 (MacDonald et al., 1994).

374

375 Recognising and understanding biofilms in equine wounds would help to guide appropriate

376 management strategies for the treatment of recalcitrant wounds in horses. The use of

377 appropriate anti-biofilm therapies in equine wounds may help to improve equine welfare by

378 increasing healing rates and help to decrease the emergence of antibiotic resistant bacterial

379 strains in response to inappropriate and ineffective antibiotics.

380

381 **Conclusion**

382

383 This paper has highlighted preliminary evidence supporting the presence of biofilms in

384 equine chronic wounds. The presence of these biofilms could partly explain the reluctance of

385 many lower limb equine wounds to heal in a timely manner, despite the use of traditional

386 wound management strategies.

387

388 Multiple bacterial species can be isolated from equine skin and wounds providing evidence of

389 a microbial community that is not removed by standard wound cleansing. Although wound

390 colonisation does not necessarily prevent wound closure, the presence of multiple bacterial

391 species capable of biofilm formation suggests that these bacteria may be surviving and

392 proliferating within a biofilm and subsequently hindering wound closure.

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17

394   **Acknowledgements**

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495 **Table 1.** The interpretation of optical density values. These values were obtained from

496 bacteria which had attached to the 96 well plate and been stained with crystal violet

497 (Stepanovic *et al*, 1999)

498

499 **Table 2.** The number of times each genus was identified from wound and skin swabs. The

500 number shown in brackets is the total number of species identified in that genus. When a

501 genus could not be identified to the species level it was reported as Spp. Spp was counted as

502 1 species.

503

504 **Figure 1.** Gram stained debrided tissue taken from a chronic, non healing equine lower limb

505 wound, showing evidence of Gram negative (arrow heads) and Gram positive (long arrows)

506 and individual colonies (elbow arrows). The epidermal edge of the wound is to the left of the

507 image. (x1000 magnification).

508

509 **Figure 2.** Gram stained debrided tissue taken from a chronic, non healing equine lower limb

510 wound, showing an area of uninfected tissue. The epidermal edge of the wound is visible on

511 the bottom right of the image. (x1000 magnification) (Bar +100μm).

512

513 **Figure 3.** Hierarchical flow diagram showing the number of each wound type included in the

514 study. Control swabs were also taken from the uninjured skin of all the 51 cases.

515

22

516   **Figure 4.** Percentage representation of bacterial genera isolated from chronic wounds (black

517   bar) and acute wounds (grey bar) (n=51). * - significant difference between chronic and acute

518   wounds $P<0.05$.

519

520   **Figure 5.** A comparison between the OD of wound (Black bars) and Skin (Grey bars)

521   isolates. The upper graph shows Gram positive isolates and the lower graph shows Gram

522   negative isolates. * show significant differences between wound and skin OD values * -

523     $P<0.05$, ** - $P<0.01$.

23

Table 1 ACCEPTED MANUSCRIPT

The interpretation of the optical density values obtained from the crystal violet assays

Optical Density	Interpretation
0	Non-adherent
≤ 0.2	Weakly adherent
$> 0.2 - \leq 0.4$	Moderately adherent
> 0.4	Strongly adherent

Table 1. The interpretation of OD values obtained from the attachment of bacteria grown on

96 well plates as part of a crystal violet assay. Stepanovic *et al*, 1999.

Table 2

Genus	Wound samples (N = 51)		Skin samples (N = 51)	
	Number of times the genus was isolated	Number of species represented	Number of times the genus was isolated	Number of species represented
Staphylococcus (**14**)	35	10	34	14
Bacillus (**8**)	16	6	17	7
Enterococcus (**6**)	16	5	16	5
Pseudomonas (**6**)	15	5	10	5
Uncultured bacterium	13	*	12	*
Clostridium (**3**)	6	2	11	3
Escherichia (**1**)	4	1	7	1
Acinetobacter (**4**)	5	2	5	4
Propionibacterium (**1**)	5	1	4	1
Micrococcus (**2**)	3	2	4	1
Proteus (**2**)	4	2	2	1
Enterobacter (**2**)	4	1	2	1
Bordetella (**1**)	4	1	2	1

Aerococcus (**4**)	3	2	2	2
Pasteurella (**2**)	0	*	5	2
Cellulomonas (**1**)	2	1	3	1
Aeromonas (**1**)	0	*	4	1
Salmonella (**2**)	2	1	2	2
Corynebacterium (**1**)	0	0	4	1
Alcaligenes (**2**)	2	2	2	2
Others (**33**)	23	18	27	22
No Identification	1	*	2	*

Table 2. The number of times each genus was identified from wound and skin swabs. The number shown in brackets is the total number of species identified in that genus. When a genus could not be identified to the species level it was reported as Spp. Spp was counted as 1 species.

Figure 1 on line publication only   ACCEPTED MANUSCRIPT

Figure 1. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing evidence of Gram negative (arrow heads) and Gram positive (long arrows) and individual colonies (elbow arrows). The epidermal edge of the wound is to the left of the image. (x1000 magnification).
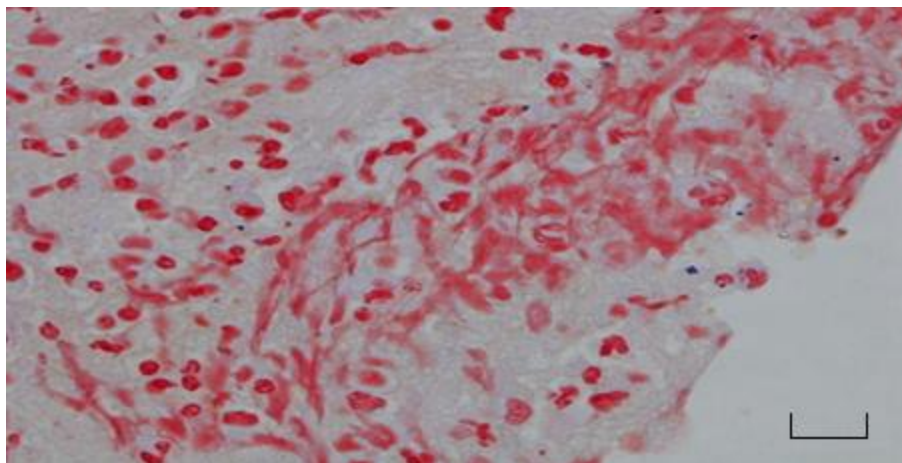
Figure 2 on line publication only   ACCEPTED MANUSCRIPT

Figure 2. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing an area of uninfected tissue. The epidermal edge of the wound is visible on the bottom right of the image. (x1000 magnification) (Bar +100μm).

Figure 3 on line publication only   ACCEPTED MANUSCRIPT

Figure 3. Hierarchical flow diagram showing the number of each wound type included in the study. Control swabs were also taken from the uninjured skin of all the 51 cases.

Figure 4

Figure 4. Percentage representation of bacterial genera isolated from chronic wounds (black bar) and acute wounds (grey bar) (n=51). * - significant difference between chronic and acute wounds P<0.05.
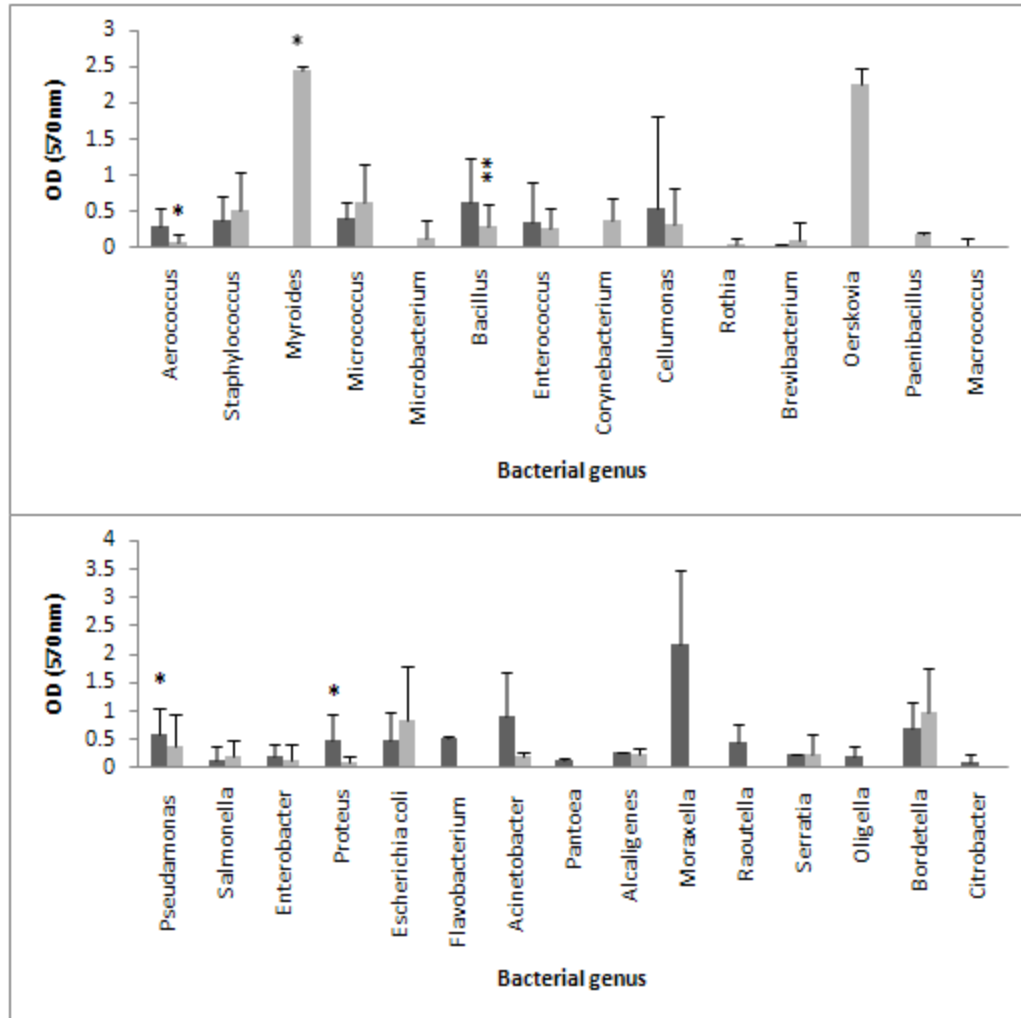
Figure 5　　　　　ACCEPTED MANUSCRIPT

Figure 5. A comparison between the OD of wound (Black bars) and Skin (Grey bars) isolates.

The upper graph shows Gram positive isolates and the lower graph shows Gram negative

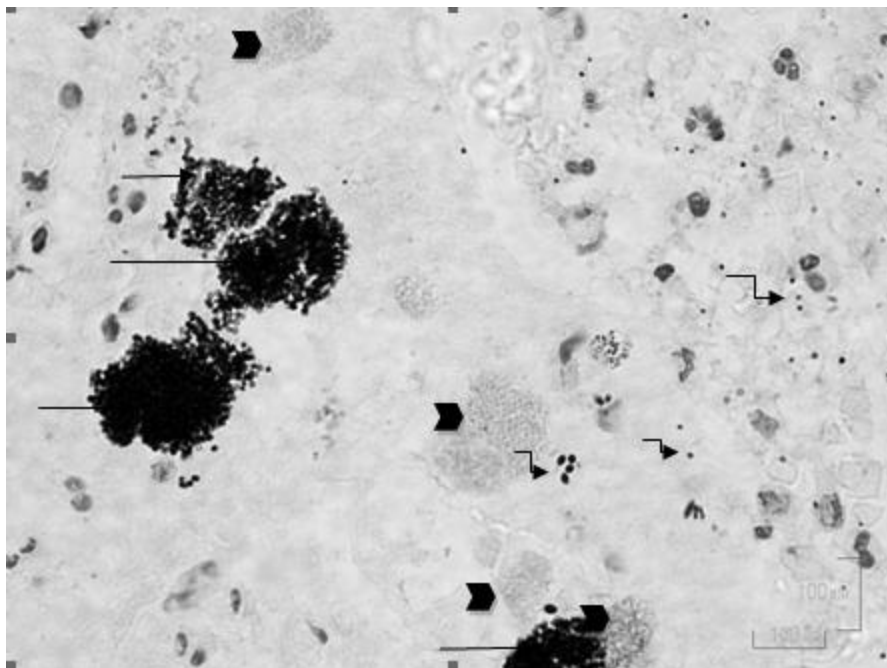isolates. * show significant differences between wound and skin OD values * - P<0.05, ** -

P<0.01.

Figure 1 printed publication ACCEPTED MANUSCRIPT

Figure 1. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing evidence of Gram negative (arrow heads) and Gram positive (long arrows) and individual colonies (elbow arrows). The epidermal edge of the wound is to the left of the image. (x1000 magnification).
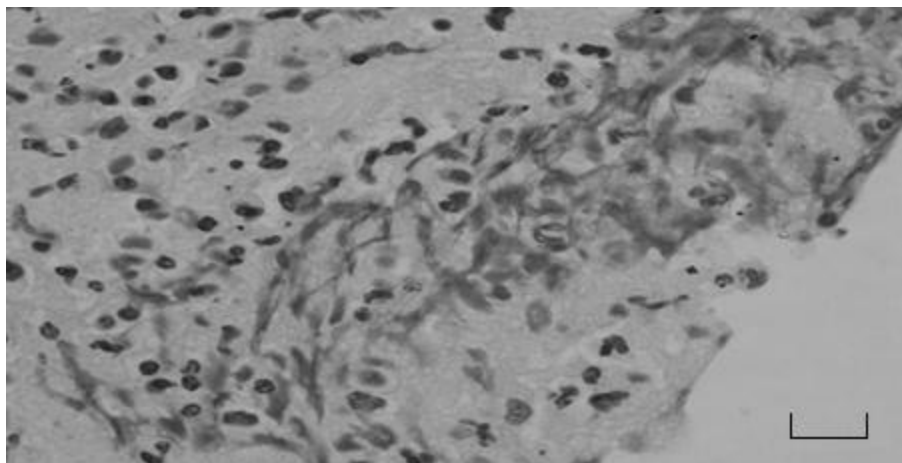
Figure 2 printed publication

Figure 2. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing an area of uninfected tissue. The epidermal edge of the wound is visible on the bottom right of the image. (x1000 magnification) (Bar +100μm).
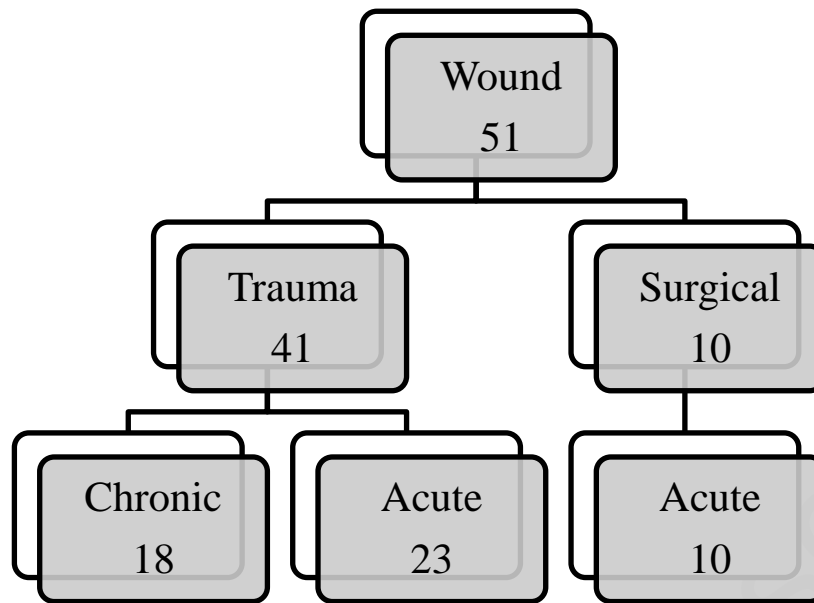
Figure 3 printed publication ACCEPTED MANUSCRIPT

Figure 3. Hierarchical flow diagram showing the number of each wound type included in the study. Control swabs were also taken from the uninjured skin of all the 51 cases.