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1 **Adjuvant antibiotic activity of acidic sophorolipids with potential for**  
2 **facilitating wound healing**

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9

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12

13 **Keywords:** acidic sophorolipids, microbial pathogens, growth inhibition, endothelium, keratinocytes, *in vivo*,  
14 **wound healing**

## 15 ABSTRACT

16 The sophorolipid class of biosurfactants are finding increasing use in personal care as well as  
17 pharmaceutical products and have the potential to disrupt biofilm formation and inhibit  
18 growth in a variety of clinically relevant organisms. In order to investigate potential  
19 biomedical applications of sophorolipids derived from non-pathogenic organisms, we  
20 fractionated and purified glycolipid biosurfactant sophorolipids produced by the yeast  
21 *Starmerella bombicola*, which yielded both acidic C 18-1 acetylated and non-acetylated  
22 congeners that were essentially free from other contaminants (>95% purity). These acidic  
23 sophorolipids have antimicrobial activities against the nosocomial infective agents  
24 *Enterococcus faecalis* and *Pseudomonas aeruginosa* with significant reduction in c.f.u.'s at  
25 concentrations as low as 5 mgmL<sup>-1</sup>. In addition, the sophorolipid showed similar effects  
26 when combined with kanamycin or cefotaxime against the same two bacterial strains. As a  
27 potential use of these sophorolipids is as a component of topically applied creams for  
28 treatment of wound infections, it is clear that they must have no demonstrable adverse effect  
29 on wound healing. To assess this we evaluated mammalian cell toxicity *in vitro* using  
30 viability tests and revealed no adverse effect on either endothelial or keratinocyte derived cell  
31 lines with sophorolipid concentrations <0.5 mgmL<sup>-1</sup>. In addition, *in vivo* experiments using a  
32 mouse skin wounding assay revealed that the time-course in healing wounds was unaffected  
33 by application of sophorolipid containing creams and histological examination of regenerated  
34 skin tissue confirmed that the healing process was similar to that observed in control animals,  
35 with no evidence of inflammation. These results are consistent with the suggestion that acidic  
36 sophorolipids can be used as a component of anti-microbial creams to reduce the risk of  
37 wound infection during healing.

38

39 **Introduction**

40 Microbial contamination is a major obstacle to tissue healing, with cutaneous wounds in  
41 particular representing one of the major routes of exposure to pathogenic bacterial strains. A  
42 systematic review [1] recently identified *Pseudomonas aeruginosa* as the most commonly  
43 reported burn wound infective pathogen. Multiple strains of multidrug resistant *P.*  
44 *aeruginosa* have been isolated from burn patients in India [2] and Enterococcal in addition to  
45 Pseudomonad species have been identified in cases of diabetic foot ulcer infection [3]. Many  
46 medical and surgical procedures carry a significant risk of microbial infection [4, 5], with a  
47 significant proportion of these cases demonstrating bacterial drug resistance, making the  
48 search for alternative approaches to treatment a clinical imperative.

49

50 Surfactants are a diverse group of amphiphilic compounds commonly used in detergents and  
51 products for human consumption or application (such as the food industry or in cosmetic or  
52 medical creams for example), although their production is heavily reliant on the  
53 petrochemical industry and associated raw materials. In contrast, biosurfactants are a diverse  
54 group of surfactants produced by certain species of yeast or bacteria which represent a more  
55 sustainable and perceived environmentally-friendly alternative to traditional surfactants.  
56 There is increasing evidence that microbial biosurfactants as well as possessing industrially  
57 valuable properties of detergency, emulsification and foaming also have significant  
58 bioactivities, including inhibitory or antibacterial-adjuvant activities against various  
59 microorganisms [6-8; 10-19] and specific anti-cancer activity [9].

60 Sophorolipids (SLs) are a diverse group of glycolipid biosurfactants, characterised by a  
61 sophorose molecule attached to a variable length fatty acid chain, that can be produced in  
62 significant quantities by the yeast *Starmerella bombicola*. The sophorose molecule may be  
63 acetylated in two positions, the carbon chain between 16 and 18 atoms in length and contain

64 none, one or more double bonds. The arrangement of the fatty acid chain accounts for the  
65 two major SL subtypes: acidic SLs terminate in a carboxylic acid group (open chain) whilst  
66 the chain in the lactonic type is re-attached to the sophorose molecule, forming a closed ring  
67 structure. The antimicrobial effects of SLs are dependent on the SL structure and class of  
68 bacteria examined, yet SLs are predominantly produced and used as mixtures with very few  
69 studies examining the effects of SL samples containing well-defined and purified single  
70 congeners. In addition, sophorolipids have virucidal and antibiotic-adjuvant characteristics  
71 [6, 7], and may have potential applications in infection, wound healing or anti-inflammatory  
72 contexts [8, 9]. However, the widely variable methods of production, extraction and  
73 purification, coupled with the diversity of possible SL structures and a lack of sufficient  
74 characterization of preparations, makes comparison between published *in vitro* and *in vivo*  
75 studies difficult to interpret.

76 The purpose of our studies was to use a highly purified preparation of acidic sophorolipid that  
77 contained only the C18 congener (predominantly non-acetylated) and ascertain if it could act  
78 as an antimicrobial agent or antibiotic-adjuvant against two common nosocomial infection-  
79 causing bacteria: *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Furthermore, we also  
80 explored whether these acidic sophorolipids inhibited the growth of mammalian cells  
81 (endothelium, fibroblasts and keratinocytes) that are relevant to the wound healing process *in*  
82 *vitro* and are compatibility with healing of uncomplicated wound *in vivo* .

83

## 84 **Results**

### 85 *Analysis of bacterial growth with purified non-acetylated acidic sophorolipid treatment*

86 Following treatment with doses of acidic sophorolipids  $\geq 5 \text{ mg mL}^{-1}$  a clear inhibitory effect  
87 was observed in growth of *Enterococcus faecalis*: colony formation was zero with  $20 \text{ mg mL}^{-1}$

88 <sup>1</sup> C18:1 NASL in two out of three experiments (Figure 1A). Inhibition of growth in  
89 *Pseudomonas aeruginosa* was also evident at concentrations of  $\geq 5$  mg mL<sup>-1</sup> acidic  
90 sophorolipid; following treatment with 20 mg mL<sup>-1</sup> C18:1 NASL colony formation was zero  
91 in one out of three experiments (Figure 1B).

92

### 93 *Antibiotic adjuvant activity of Sophorolipid*

94 In culture experiments, a 10 & 20 mg mL<sup>-1</sup> solution of C18:1 NASL successfully inhibited  
95 growth of both bacterial species (as determined by OD<sub>600nm</sub> measurements); therefore, for the  
96 adjuvant assay we tested sub-inhibitory concentrations of 2 and 4 mg mL<sup>-1</sup> respectively.

97 Addition of purified C18:1 NASL to bacterial cultures reduced the minimal inhibitory  
98 concentration (MIC) of kanamycin and cefotaxime in the majority of replicates, however the  
99 value of the MIC itself was difficult to determine due to inter-experimental variation (shown  
100 in Table 1). The highest concentration of C18:1 NASL was clearly the most effective at  
101 reducing antibiotic MIC for both strains and both drugs. In fact, 4 mg mL<sup>-1</sup> C18:1 NASL  
102 alone effectively reduced growth of *Pseudomonas aeruginosa*. The adjuvant effect was most  
103 obvious for strains treated with 4 mg mL<sup>-1</sup> C18:1 NASL together with cefotaxime. The effect  
104 of C18:1 NASL addition on kanamycin MIC was generally less marked than for cefotaxime.  
105 Interestingly, the MIC of kanamycin against *Pseudomonas aeruginosa* was increased with  
106 addition of 2 mg mL<sup>-1</sup> C18:1 NASL.

107

### 108 *In vitro cell viability assay*

109 The addition of acidic C18:1 NASL to culture media in doses ranging from 0.01 – 500  $\mu$ g  
110 mL<sup>-1</sup> did not affect cell viability of HUVECs, HDMVECs or HaCaTs (Figure 2) as measured  
111 by the MTT assay.

112

113 *In vivo wound healing assay*

114 All wounds were completely closed with no remaining residual coagulum covering by day 16  
115 of the study. Treatment with cream containing C18:1 acidic sophorolipid did not significantly  
116 affect wound size on the monitored days in comparison to the control group (Fig. 3 &  
117 supplementary material). Wound size in the vehicle group was initially larger than controls  
118 on day 4 ( $p<0.05$ ), but was smaller than controls by day 8 of the study ( $p<0.001$ :  
119 supplementary material).

120 *Histology of healing wounds*

121 Qualitative assessment of light microscopic images from samples taken at 21 days after  
122 injury, revealed typical morphological features of murine skin samples including a thin  
123 cornified epithelial layer, a dermal layer with numerous obliquely sectioned hair follicles and  
124 a deep 3-5 cell layers thick *panniculus carnosus* (skeletal muscle) layer (supplementary  
125 Figure 2). Morphological features of tissue structure were consistent across all treatment  
126 groups with no evidence of fibrosis or lymphocytic infiltration noted.

127

128 **Discussion**

129 To our knowledge, the work presented here details the first investigation on the application of  
130 purified sophorolipids in the context of healing wounds. In addition to our observations of  
131 consistent antimicrobial and antibiotic-adjuvant activities of this purified sophorolipid  
132 product, we show that C18:1 NASL does not affect cell viability in human endothelial  
133 (HUVEC & HDMVEC) and keratinocytes (HaCaT) *in vitro* or the rate of wound healing in a  
134 murine model of longitudinal wound healing *in vivo*. Taken together, this data provides  
135 evidence that purified sophorolipid application is compatible with healing wounds and could  
136 be beneficial in the context of wound contamination or infection with opportunistic bacterial  
137 pathogens.

138 In terms of the effects of sophorolipids on cultured cells, much of the published data focuses  
139 on relatively impure preparations and these studies are primarily directed at anti-migratory or  
140 cytotoxic effects on phenotypically invasive cell lines in the context of anti-cancer therapy  
141 [10-15] rather than the compatibility with and low toxicity to normal (non-transformed) cells.  
142 Our observations are consistent with the suggestion of continuing research into possible  
143 therapeutic applications. More detailed investigations of the interactions between  
144 sophorolipids and cultured human cells, such as assays of membrane integrity, biomarkers of  
145 irritancy or inflammation for example, will be vital to inform the progression of these agents  
146 into clinical application.

147 A range of sophorolipid preparations, including mixtures containing both acidic and lactonic  
148 structures, were shown to be ineffective against Gram positive and Gram negative bacteria  
149 (*Escherichia coli* and *Staphylococcus aureus*) with MICs above  $512 \mu\text{g mL}^{-1}$ ; similar to the  
150 sophorolipid concentrations used to demonstrate adjuvant activity in our study [16].  
151 However, when ethanol was employed as a vehicle inhibition of *E. coli* and *S. aureus* was  
152 observed at concentrations  $>128 \mu\text{g mL}^{-1}$ . A study of a “natural SL” mixture (approximately  
153 75% lactonic and 25% acidic structures), was effective against *E. coli* at  $1 \text{ mg mL}^{-1}$  and *S.*  
154 *aureus* at  $15\text{-}150 \mu\text{g mL}^{-1}$  respectively [6]; approximately 5-10-fold lower than the  
155 concentrations applied in our antimicrobial studies. The mixture previously reported [6] also  
156 displayed adjuvant activity at lower concentrations  $<1 \text{ mg mL}^{-1}$  than those we have reported  
157 here. Another study of natural mixture SLs with a variety of sugar head groups reported  
158 antimicrobial activity against a range of bacteria, which were predominantly Gram positive  
159 [17] and evident at 100-1000-fold lower concentrations than we tested. These differences in  
160 antimicrobial activities may be associated with the presence of lactonic structures in natural  
161 mixture SLs. The range of activities presented by sophorolipid structures is further  
162 highlighted by the observation that acidic SL structures are virucidal against HIV *in vitro* at



163 doses of 3 mg mL<sup>-1</sup> [7]; similar to the doses we observed which exert an adjuvant effect in  
164 bacterial pathogenic strains. The dosages employed during our *in vitro* antimicrobial studies  
165 that were effective in bacterial killing are consistent with those that were administered  
166 intravenously *in vivo* (1-1.2 mg dose of natural mixture SL) and subsequently shown to  
167 prevent lethal septic shock in two distinct rat models of peritonitis [8, 9].

168

169 While research activity in the fields of antimicrobial, antiviral, anti-adhesion or adjuvant  
170 activities of various types of biosurfactants are increasing, their applications in the context of  
171 wound healing are rare. The di-rhamnolipid (“BAC-3”) produced by *Pseudomonas*  
172 *aeruginosa* is well tolerated and promoted faster healing in studies examining burn wounds  
173 in mice and rats; these studies led to its’ successful application in a single clinical case of  
174 chronic decubitus ulcer [18, 19]. However, differences in wound type, size, animal model,  
175 surfactant type and biosurfactant mixtures, make comparisons between these respective  
176 studies difficult. In order to improve our understanding of the potential benefits or risks of  
177 this diverse group of compounds in wound healing, information on the composition of  
178 biosurfactant mixtures and standardization of the experimental wound healing model that is  
179 employed will be crucial.

180 In comparison to the control group, only addition of cream that contained the vehicle-only  
181 solution resulted in a significant change to wound size, with no histological differences being  
182 detected between treatment groups. We interpreted these results as indicating that topical  
183 creams containing C18:1 NASL play a passive role in the process of uncomplicated, non-  
184 infected, wound healing. Factors affecting percutaneous penetration of sophorolipids, such as  
185 molecular weight and lipophilicity may be important in choosing an appropriate delivery  
186 mechanism or vehicle. Investigating the roles of acidic sophorolipid preparations on critical

187 micelle concentration and dissociation characteristics from suitable creams or their extent of  
188 percutaneous penetration, may thus be beneficial to determine their efficacy for antibiotic  
189 prophylaxis of wounded skin.

190 Whilst the majority of studies report that the lactonic sophorolipids are more effective in  
191 terms of antimicrobial effects or adjuvant activity, the C18:1 NASL sample tested was  
192 selected for evaluation in cultured cells *in vitro* and *in vivo* because of its purity (verified at  
193 source by NMR) and lyophilised state. The relative solubility of our NASL stock solutions  
194 varied according to the vehicle used in each assay. In this study, we used the highest final  
195 concentrations possible in both microbiological assay (PBS: 4 mg/ml) and mammalian cell  
196 culture assay (culture media; 0.5 mg/ml [500 mg/ml]). We were unable to achieve higher  
197 doses in either of these cases as it led to precipitation in the growth media and thus an  
198 inability to calculate accurate final concentrations. This is not ideal as there is no overlap in  
199 dosage between the anti-microbial effect we observed (maximal at 4 mg/ml) and the highest  
200 dose used for cell toxicity testing (0.5 mg/ml). However, we believe that observations on full-  
201 thickness skin healing *in vivo* following topical application of high dosage (200 & 400  
202 mg/kg; well above the equivalent dose in tissue culture) is consistent with a lack of toxicity  
203 for purified NASL.

204 Furthermore, *in vitro* cytotoxic effects of purified lactonic sophorolipids observed by our  
205 group [20] extended to control cell lines as well as cancer cell lines – this was not the case for  
206 similarly tested purified acidic sophorolipid. Overall, we conclude that topical application of  
207 purified acidic sophorolipid did not impair wound healing *in vivo*, and together with evidence  
208 of antibacterial, antibiofilm and antibiotic adjuvant activity, supports the future evaluation of  
209 sophorolipids as novel wound healing agents, particularly in the contexts of acutely or  
210 chronically infected wounds.

211

212 **Materials and methods**213 *Sophorolipids*

214 A purified acidic sophorolipid sample was produced from the yeast *Starmerella bombicola*  
215 using the culture and purification methods outlined by Van Bogaert et al. [21] and comprised  
216 >90% non-acetylated C18:1 acidic sophorolipid (C18:1 NASL) by NMR spectroscopy (at  
217 point of purification). Other congeners present include a mixture of sophorolipid structures  
218 with chain lengths C16-18 containing from 0 - 2 double bonds. Sophorolipid solutions of  
219 various concentrations (by mass) were prepared in the relevant culture media for *in vitro*  
220 experiments or sterile phosphate buffered saline (PBS) for *in vivo* studies.

221

222 *Analysis of bacterial growth with purified non-acetylated acidic sophorolipid treatment*

223 *Enterococcus faecalis* (ATCC 29212) and *Pseudomonas aeruginosa* (PAO1) were obtained  
224 from Ulster University Microbiology Research Group stock. Nutrient broth (5mL; Oxoid,  
225 Basingstoke, UK) was inoculated with a single colony, then transferred to a shaking  
226 incubator overnight (16-18 h). Each overnight culture was adjusted, under sterile conditions,  
227 to an optical density (OD<sub>600</sub>) of 0.05 (~1 x 10<sup>8</sup> c.f.u./mL), then diluted further into broth  
228 containing sophorolipid (20 mg mL<sup>-1</sup> C18:1 NASL), such that the final concentration of  
229 culture was 1/100th of the 0.05 OD-adjusted inoculum. Inoculated broth (100μL) ± C18:1  
230 NASL was loaded, alongside blank controls, into 96-well microtitre plates (Nunc,  
231 ThermoFisher, UK) which were incubated at 37°C for 12 h with no agitation. Cultures were  
232 then appropriately diluted and spread onto nutrient agar plates (Oxoid) and incubated at  
233 37°C. Following 12 h incubation, agar plates were photographed for colony counting by  
234 ImageJ software (v1.50b, NIH). The above procedure was performed three times in total for  
235 each strain, starting with overnight cultures of separate colonies.

236

237 *Antibiotic adjuvant activity of Sophorolipid*

238 Antibiotics (cefotaxime and kanamycin) were purchased from Sigma (Dorset, UK). Stock  
239 antibiotic solutions were prepared in sterile water and filter sterilised as required.  
240 Appropriate dilutions of antibiotic (<512 mg/L) were prepared in sterile nutrient broth  
241 (Oxoid, Basingstoke, UK) before being loaded (100 $\mu$ L) into separate columns of a 96-well  
242 plate. Each plate contained a two-fold dilution series of antibiotic (n= 8 per concentration).  
243 Purified acidic sophorolipid (SL) solutions were prepared in nutrient broth. Diluted  
244 sophorolipid samples and loaded microtitre plates were stored at 4°C and used within 5 days  
245 of preparation.

246 Initial bacterial cultures were prepared by inoculating 5mL nutrient broth with a single  
247 colony from an agar plate. The inoculum was placed in a shaking incubator (37°C) for 6-8 h  
248 and then adjusted to an optical density (600nm) of 0.05. Cultures were then diluted (1/50)  
249 into nutrient broth alone (control) or containing purified acidic SL (4 or 8 mgmL<sup>-1</sup> C18:1  
250 NASL). The control inoculum was dispensed (100 $\mu$ L) into one half of the plate (antibiotic +  
251 culture only; n = 4 wells) and the SL-inoculum was dispensed (100 $\mu$ L) into the other half of  
252 the plate (antibiotic + culture + SL at 2 or 4 mgmL<sup>-1</sup>; n = 4 wells). Nutrient broth containing  
253 the appropriate concentration of sophorolipid or no SL was included as a control (n=4 wells).  
254 The plates were sealed with Parafilm® and incubated in a shaking incubator at 37°C for 12 h,  
255 at which point optical density (600nm) was recorded and the plates visually inspected for  
256 growth, indicated by opacity. Each experiment was performed three times.

257

258 *Data and Statistical Analysis*

259 Statistical analyses were performed using GraphPad Prism (v6.01). Datasets were analysed  
260 by comparing the mean endpoint for each test group to the relevant control group by one-way  
261 ANOVA with Holm-Sidak's multiple comparison tests. Any value differing by more than 0.2  
262 OD units from *at least 2* other values in the group were omitted from analysis for all assays.  
263 This step was informed by previous experience, whereby air bubbles, excessive precipitation  
264 or contamination was linked to intra-group variation of this magnitude, up to the detection  
265 limits of the equipment (approximately 1 OD unit). This approach was not applied where  
266 mean values were above 1 OD unit which was classed as “positive”.

267 The highest concentration of purified acidic sophorolipid applied in the adjuvant assay (4  
268 mgmL<sup>-1</sup>) invariably increased the optical density of broth (indicated visually and by Mann-  
269 Whitney comparisons of OD measurements), therefore raw OD data were adjusted taking  
270 into account the background from the relevant SL+ or SL- control. To determine adjuvant  
271 efficacy of purified C18:1 NASL, the minimal inhibitory concentration (MIC) of antibiotic  
272 was defined as the lowest concentration at which OD<sub>600nm</sub> was not significantly different  
273 between a culture + antibiotic condition and the relevant blank control; and the minimal  
274 effective concentration (MEC) was defined as the lowest concentration of antibiotic at which  
275 OD<sub>600nm</sub> was significantly lower than the relevant culture control.

276 *Tissue culture*

277 Human dermal microvascular endothelial cells (HDMVECs); human umbilical vein  
278 endothelial cells (HUVECs; Caltag Medsystems, Cambridgeshire, UK) were cultured in  
279 M200 phenol red-free (PRF) culture media supplemented with low serum growth supplement  
280 (LSGS, giving a final serum concentration of 2%; Gibco, UK). Human keratinocytes  
281 (HaCaT) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM;  
282 4.5gL<sup>-1</sup> glucose) supplemented with 10% foetal bovine serum (FBS; Gibco, UK).

283

284 *In vitro cell viability assay*

285 Cells were trypsinised as normal, counted using trypan blue, and dispensed at varying  
286 concentrations into 96-well plates. HaCaT cells were washed with PBS after adhering to  
287 plates and incubated overnight (16-18 hours) in serum-free media, whereas endothelial cells  
288 remained in LSGS-supplemented media. The media was then removed and the wells washed  
289 once more in PBS before applying fresh complete media containing purified acidic  
290 sophorolipid (0.01-500 $\mu\text{g mL}^{-1}$ ), etoposide (5 $\mu\text{M}$ ), or media with a low (2%) or high (10%)  
291 fetal calf serum content. Plates were subsequently incubated for 22 h, after which MTT was  
292 added (in low light conditions) to a final concentration of 0.5  $\text{mg mL}^{-1}$  and the plates  
293 incubated for a further 4 h. Media were then removed and DMSO (200 $\mu\text{L}$ ) dispensed into  
294 each test well with gentle mixing before recording optical density at 570nm.

295

296 *Animals and surgical procedure*

297 Adult (8-14 week old) male C57Bl/6J mice were obtained from Harlan, UK. Animals were  
298 housed singly with freely available food and water and provided with soft adsorbent pads as  
299 bedding for at least one week prior to surgery, with their body weights being measured during  
300 this time. In order to reduce the risk of infection associated with contaminated bedding and  
301 reduce stress, animals were placed into fresh cages the day before surgery and a clean  
302 bedding pad was placed on top of the previous pad on the day of the experiment.

303 Mice were anaesthetised by intraperitoneal injection with ketamine and xylazine (100 $\text{mg kg}^{-1}$   
304 and 10 $\text{mg kg}^{-1}$  respectively) and provided with sub-cutaneous administered pre-emptive pain  
305 relief (2  $\text{mg kg}^{-1}$  metakam). Following appropriate anaesthesia, the dorsal surface was  
306 shaved with electric clippers and hair removed by application of a commercially available  
307 depilatory cream. The skin was prepared for surgery by wiping with three repeat applications

308 of gauze soaked in warmed hibiscrub followed by warmed 70% IMS . Animals were then  
309 placed on a warming mat for the duration of the surgical procedure.

310 Circular dressings, 2 cm diameter, were cut from sheets of Opsite™ Flexigrid™ transparent  
311 adhesive dressings (Smith & Nephew, Hull, UK). The mouse was placed in ventral  
312 recumbancy, then the dressings were placed onto the depilated dorsal skin, centrally across  
313 the midline and between the forelimbs and allowed to adhere for a few moments. The mouse  
314 was then placed into flank recumbency and the dressing was used to create a fold of the  
315 dorsal skin surface. A uniform, circular wound was created using an 8mm dermal biopsy  
316 punch, by applying half the blade of the punch to the folded skin (Figure 1A), while the  
317 dressing remained in place. Animals were placed into a warming cabinet, with moistened  
318 food in the cage, until they recovered from anaesthesia.

319

#### 320 *Treatment and Monitoring*

321 Mice were assigned to a treatment group of 20, 200 or 400mg kg<sup>-1</sup> sophorolipid, or control  
322 (n=8 per group). Sophorolipid was prepared in phosphate buffered saline (PBS) then mixed  
323 with commercially available aqueous (aq) cream in a 1:1 volume to weight ratio. The control  
324 group was treated with an aq cream similarly mixed with an equal volume of PBS. Animals  
325 were briefly anaesthetised with isoflurane to permit administration of treatment and  
326 consecutive photographs to be made of the wounds. Treatment was applied daily to the  
327 surface of the wounds for 7 days (Figure 1B), with photographs taken on day 1 (day of  
328 surgery), day 2 then every 2 days thereafter. The plunger of a 1mL syringe was used to  
329 smooth the edges of the cream around the wound edge if necessary. The dressing, if not  
330 detached already, was removed on day 3 post-surgery. Food and water intake was recorded  
331 periodically throughout the study. On day 21 animals were euthanized in a CO<sub>2</sub> atmosphere

332 and the skin surrounding the wound area was collected, laid flat onto moistened filter paper  
333 and placed flat into 10% neutral buffered formalin. Following fixation at 4°C, skin samples  
334 were processed using an auto-processor, embedded in paraffin blocks, sectioned at 5µm and  
335 stained with haematoxylin & eosin according to conventional protocols.

336

#### 337 *Image and Statistical Analysis*

338 Statistical analyses for all assays were performed with the aid of GraphPad Prism (v6.01).  
339 Wound size in the *in vitro* and *in vivo* assays was measured using ImageJ software [22]. *In*  
340 *vitro* datasets were analysed by comparing the mean endpoint for each test group to the  
341 control group by Kruskal-Wallis with Dunn's multiple comparison tests. For *in vivo* assays,  
342 mean wound size on each monitoring day was compared between each treatment group and  
343 the control group as above.

344

#### 345 **Contributors**

346 HLL carried out the animal experimental work and the microbiological testing, BC was  
347 responsible for the histological results and NB provided the purified samples of sophorolipid.  
348 CAM, RM and IMB planned experiments, reviewed data and were responsible for  
349 management of the project. RM and CAM were responsible for assembly and review of this  
350 manuscript. All authors have read, edited and approved the final manuscript.

351

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354 Behavioural Research Unit for their assistance with *in vivo* studies.

#### 355 **Ethical statement**



356 All relevant international, national, and/or institutional guidelines for the care and use of  
357 animals were followed. Specifically approval from the local (Ulster University) ethics  
358 committee was granted and experiments conducted according to the guidelines provided in  
359 UK Animals (Scientific Procedures) Act 1986 and following appropriate national ethical  
360 approval from the UK Home Office.

361

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365

366

367 **Conflict of interest statement**

368 Helen L. Lydon, Niki Baccile, Breedge Callaghan, Roger Marchant, Christopher A.

369 Mitchell and Ibrahim M. Banat all declare that they have no conflict of interest.

370

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432

433 **Figure 1.** Growth (colony forming units; c.f.u. mL<sup>-1</sup>) of (A) *Enterococcus faecalis* and (B)  
434 *Pseudomonas aeruginosa* was significantly reduced by exposure to 0.5-2% purified acidic  
435 sophorolipid (C18:1 NASL). Representative data from one of three experiments is presented  
436 (mean ± s.d. n=4 technical replicates; \*\*\*\*p<0.0001).

437

438 **Figure 2** Reduction of formazide salt (absorbance at 570nm, mean ± standard deviation;  
439 n=6) to formazan crystals in (A) HUVEC, (B) HDMVEC and (C) HaCaT cells *in vitro* was  
440 not affected by addition of acidic C18:1 sophorolipid (SL). Low serum concentration (2%)  
441 serves as the control condition for endothelial cells (A &B) and high serum (10%) is the  
442 control condition for HaCaT cells (C). Representative data from two repeat assays is shown.

443

444 **Figure 3.** Size of wounds (mm<sup>2</sup>, mean ± standard deviation; n≤8) 8 days after excision  
445 created on depilated dorsal skin of male C57 mice and treated with aqueous cream with PBS  
446 (vehicle; n=8) or varying doses of purified C18:1 NASL in aqueous cream for 7 days or left  
447 untreated (control). Asterisks indicate a significant difference in wound size between a test  
448 group and the control group (\*p<0.05; \*\*\*p<0.001).

449

	MIC (mg/L)	MEC (mg/L)
<i>Enterococcus faecalis</i>		
+ kanamycin	4-16+	2-4
+2mgmL +C18:1 NASL	2	2-4
+4mgmL + C18:1 NASL	2-4	not determined
+ cefotaxime	0.5-2	0.5-2
+2mgmL +C18:1 NASL	0.5-1	0.25-1
+4mgmL +C18:1 NASL	<0.016-1	<0.062-1
<i>Pseudomonas aeruginosa</i>		
+ kanamycin	8	8
+2mgmL +C18:1 NASL	8-16	8
+4mgmL +C18:1 NASL	<0.25	<0.25
+ cefotaxime	0.5-4	0.5-4
+2mgmL +C18:1 NASL	0.5	<0.62
+4mgmL +C18:1 NASL	<0.062	<0.062

**Table 1.** Minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) of kanamycin or cefotaxime against *Enterococcus faecalis* or *Pseudomonas aeruginosa*, alone or in combination with purified C18:1 NASL.





