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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

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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⁻¹. 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⁻¹. 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 mg mL^{-1}

88 ¹ C18:1 NASL in two out of three experiments (Figure 1A). Inhibition of growth in
89 *Pseudomonas aeruginosa* was also evident at concentrations of ≥ 5 mg mL⁻¹ acidic
90 sophorolipid; following treatment with 20 mg mL⁻¹ 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⁻¹ solution of C18:1 NASL successfully inhibited
95 growth of both bacterial species (as determined by OD_{600nm} measurements); therefore, for the
96 adjuvant assay we tested sub-inhibitory concentrations of 2 and 4 mg mL⁻¹ 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⁻¹ 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⁻¹ 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⁻¹ 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 μ g
110 mL⁻¹ 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 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⁻¹ [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 (OD₆₀₀) of 0.05 (~1 x 10⁸ c.f.u./mL), then diluted further into broth
228 containing sophorolipid (20 mg mL⁻¹ 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 (100µL) 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⁻¹ C18:1
250 NASL). The control inoculum was dispensed (100µL) into one half of the plate (antibiotic +
251 culture only; n = 4 wells) and the SL-inoculum was dispensed (100µL) into the other half of
252 the plate (antibiotic + culture + SL at 2 or 4 mgmL⁻¹; 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⁻¹) 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_{600nm} 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_{600nm} 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⁻¹ 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 μM), 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 mg mL^{-1} and the plates
293 incubated for a further 4 h. Media were then removed and DMSO (200 μL) 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 mg kg^{-1}
304 and 10 mg kg^{-1} respectively) and provided with sub-cutaneous administered pre-emptive pain
305 relief (2 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⁻¹ 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 CO₂ 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⁻¹) 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 (mm², 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.





