Adjuvant Antibiotic Activity of Acidic Sophorolipids with Potential for Facilitating Wound Healing

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ABSTRACT

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

Introduction

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

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

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

Antimicrobial Agents and Chemotherapy

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 ≥5 mg mL^{-1} a clear inhibitory effect
87 was observed in growth of *Enterococcus faecalis*: colony formation was zero with 20 mg mL^{-}

[1] C18:1 NASL in two out of three experiments (Figure 1A). Inhibition of growth in *Pseudomonas aeruginosa* was also evident at concentrations of ≥5 mg mL^{-1} acidic sophorolipid; following treatment with 20 mg mL^{-1} C18:1 NASL colony formation was zero in one out of three experiments (Figure 1B).

Antibiotic adjuvant activity of Sophorolipid

In culture experiments, a 10 & 20 mg mL^{-1} solution of C18:1 NASL successfully inhibited growth of both bacterial species (as determined by OD_{600nm} measurements); therefore, for the adjuvant assay we tested sub-inhibitory concentrations of 2 and 4 mg mL^{-1} respectively. Addition of purified C18:1 NASL to bacterial cultures reduced the minimal inhibitory concentration (MIC) of kanamycin and cefotaxime in the majority of replicates, however the value of the MIC itself was difficult to determine due to inter-experimental variation (shown in Table 1). The highest concentration of C18:1 NASL was clearly the most effective at reducing antibiotic MIC for both strains and both drugs. In fact, 4 mg mL^{-1} C18:1 NASL alone effectively reduced growth of *Pseudomonas aeruginosa*. The adjuvant effect was most obvious for strains treated with 4 mg mL^{-1} C18:1 NASL together with cefotaxime. The effect of C18:1 NASL addition on kanamycin MIC was generally less marked than for cefotaxime. Interestingly, the MIC of kanamycin against *Pseudomonas aeruginosa* was increased with addition of 2 mg mL^{-1} C18:1 NASL.

In vitro cell viability assay

The addition of acidic C18:1 NASL to culture media in doses ranging from 0.01 – 500 µg mL^{-1} did not affect cell viability of HUVECs, HDMVECs or HaCaTs (Figure 2) as measured by the MTT assay.

In vivo wound healing assay

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

Histology of healing wounds

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

Discussion

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

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

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

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

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

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

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

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

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

Antimicrobial Agents and Chemotherapy

Materials and methods

Sophorolipids

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

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

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

Antibiotic adjuvant activity of Sophorolipid

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

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

Data and Statistical Analysis

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

The highest concentration of purified acidic sophorolipid applied in the adjuvant assay (4 $mgmL^{-1}$) invariably increased the optical density of broth (indicated visually and by Mann-Whitney comparisons of OD measurements), therefore raw OD data were adjusted taking into account the background from the relevant SL+ or SL- control. To determine adjuvant efficacy of purified C18:1 NASL, the minimal inhibitory concentration (MIC) of antibiotic was defined as the lowest concentration at which OD_{600nm} was not significantly different between a culture + antibiotic condition and the relevant blank control; and the minimal effective concentration (MEC) was defined as the lowest concentration of antibiotic at which OD600nm was significantly lower than the relevant culture control.

Tissue culture

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

In vitro cell viability assay

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

Animals and surgical procedure

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

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

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

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

Treatment and Monitoring

Mice were assigned to a treatment group of 20, 200 or 400mg kg^{-1} sophorolipid, or control (n=8 per group). Sophorolipid was prepared in phosphate buffered saline (PBS) then mixed with commercially available aqeuous (aq) cream in a 1:1 volume to weight ratio. The control group was treated with an aq cream similarly mixed with an equal volume of PBS. Animals were briefly anaesthetised with isoflurane to permit administration of treatment and consecutive photographs to be made of the wounds. Treatment was applied daily to the surface of the wounds for 7 days (Figure 1B), with photographs taken on day 1 (day of surgery), day 2 then every 2 days thereafter. The plunger of a 1mL syringe was used to smooth the edges of the cream around the wound edge if necessary. The dressing, if not detached already, was removed on day 3 post-surgery. Food and water intake was recorded periodically throughout the study. On day 21 animals were euthanized in a CO_2 atmosphere

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

Image and Statistical Analysis

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

Contributors

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

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Ethical statement

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

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Conflict of interest statement

Helen L. Lydon, Niki Baccile, Breedge Callaghan, Roger Marchant, Christopher A. Mitchell and Ibrahim M. Banat all declare that they have no conflict of interest.

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Antimicrobial Agents and Chemotherapy

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 (mm^2, 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)
Enterococcus faecalis		
+ 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
Pseudomonas aeruginosa		
+ 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.