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1 **Cytotoxicity Evaluation of Microbial Sophorolipids and** 2 **Glucolipids using Normal Human Dermal Fibroblasts (NHDF)** 3 ***in vitro***

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5
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11 **Abstract**

12 Fibroblasts are considered a key player in the wound healing process. Although this
13 cellular family is constituted by several distinct subtypes, dermal fibroblasts are crucial
14 thanks to their ability to secrete pro-regenerative growth factors, extracellular matrix
15 (ECM) proteins and their immune and anti-inflammatory role. Sophorolipids (SL),
16 sophorosides (SS) and glucolipids (G), mono-unsaturated (C18:1) or saturated (C18:0),
17 glycolipids derived from microbial fermentation of wild type or engineered yeast
18 *Starmerella bombicola*, constitute a novel sustainable class of bio-based chemicals with
19 interesting physicochemical characteristics, which allow them to form soft diverse
20 structures from hydrogels to vesicles, micelles or complex coacervates with potential
21 interest in skin regeneration applications. In this study, we first tested the
22 cytocompatibility of a broad set of molecules from this family on normal human dermal
23 fibroblasts (NHDF). Our results show that, up to an upper threshold (0.1 % w/v), the
24 selected microbial glycolipids (SL-C18:1, G-C18:1, SS_{bola}-C18:1, SL-C18:0 and G-C18:0)
25 were able to sustain cell growth. Furthermore, we studied their potential to promote
26 wound healing by measuring the gene expression of several key skin regeneration
27 markers (i.e. collagen, elastin, transforming growth factor β , fibroblast growth factor ...)
28 using qPCR. Unfortunately, none of these glycolipids modulated the gene expression of
29 molecules involved in tissue repair. However, this study aims to encourage the
30 community to test this novel class of molecules for novel high-end biomedical
31 applications.

33 **Importance**

34 Biosurfactants prepared by microbial fermentation are natural amphiphiles of growing
35 importance, with the goal of replacing synthetic surfactants in commercial formulations.
36 However, their cytotoxicity profile is still poorly known, especially for new molecules like
37 single-glucose lipids or bolaform sophorolipids. This wants to contribute to all those
38 applications, which could be developed with biosurfactants in contact with the skin
39 (cosmetics, wound healing). We test the cytotoxicity of five structurally-related molecules
40 (C18:1 and C18:0 sophorolipids, C18:1 and C18:0 single-glucose lipids, C18:1 di-
41 sophoroside) against normal human dermal fibroblasts (NHDF) and evaluate the

42 metabolic activity of the least toxic among them. To the best of our knowledge,
43 cytotoxicity of these molecules, and of microbial biosurfactants in general, was never
44 tested against NHDF.
45

46 **Keywords:**

47 Fibroblasts, NHDF, sophorolipids, sophorosides, cytotoxicity, biosurfactants

48 **Introduction**

49 Skin wound healing is the most intensive commercial area where tissue engineering and
50 regenerative medicine (TERM) solutions are being developed. This is due to the high
51 socioeconomic impact imposed, especially due to aging populations [1]. For instance, the
52 total spending on wound care alone has been estimated in 10 billion EUR per year just in
53 France [2]. Most of the current cell therapies are focused on the use of stem cells and
54 their secretome. However, most of the clinical trials have been performed with
55 biomaterials such as soft hydrogels such as alginate, fibrin or collagen, encompassing
56 bioactive molecules and possessing smart properties to maintain the wettability, combat
57 bacterial infections, or increase the pro-regenerative environment [3, 4].
58

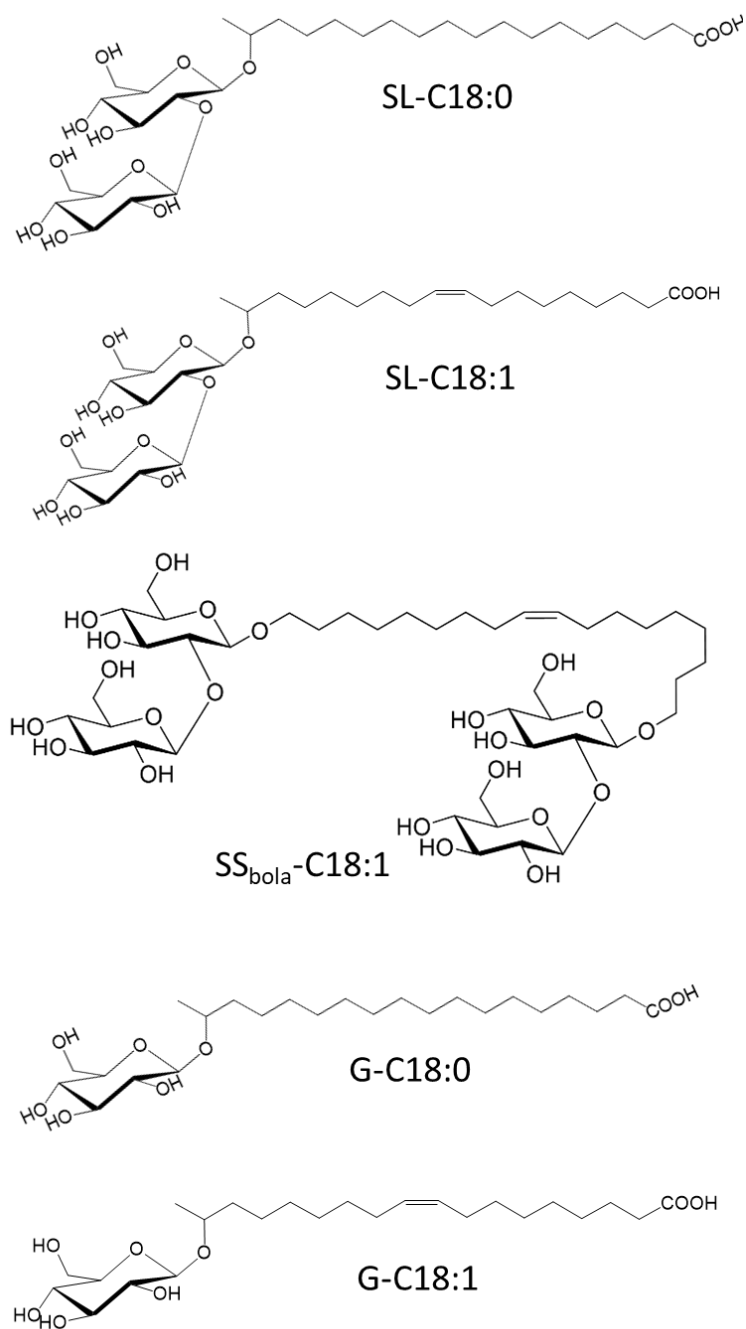
59 Dermal fibroblasts are fundamental during skin wound healing thanks to their ability to
60 synthesize *de novo* tissue matrix, to promote epidermalization and their immunoactivity
61 role [5]. In this sense, they are responsible for the production of key pro-regenerative
62 growth factors (i.e. transforming growth factor- β , platelet-derived growth factor,
63 fibroblast growth factor), inflammatory intermediates and immunomodulators (i.e.
64 interleukins and tumour necrosis factor- α), extracellular matrix (ECM) molecules and
65 enzymes involved in matrix remodelling (collagens, fibronectin, actin, elastin, matrix
66 metalloproteinases). Furthermore, dermal fibroblasts play a central role in cell-cell
67 communication processes with the other cell types involved in tissue repair (mast cells,
68 macrophages, keratinocytes, endothelial cells or mesenchymal stem cells) [6-8]. Last, they
69 increase tissue-resident cell migration and proliferation, modulate lymphocytic activity
70 and the activation of macrophages, and secrete key chemotactic factors for development
71 of neovasculature [9].
72

73 Biological amphiphiles, such as sophorolipids, sophorosides or glucolipids, are amphiphilic
74 molecules, also known as biosurfactants, synthesised by microorganisms which have
75 been previously used in the biomedical field due to their antimicrobial properties [10, 11].
76 While these molecules have been extensively studied for industrial applications such as
77 oil remediation, biosustainable surfactants or the production of personal healthcare
78 products [12-14], little is known about their cell biocompatibility and their possible use in
79 TERM applications. Their stunning self-assembly, in particular their hydrogel-forming
80 ability [15, 16], could be exploited to prepare soft scaffolds for TERM. This idea is gaining
81 particular interest as it has been recently disclosed in the pioneering work of the Banat
82 team at the University of Ulster, U.K. [17, 18].
83

84 The underlying hypothesis is the improved safety of biosurfactants towards mammalian
85 cells as compared to synthetic surfactants. If this assumption is most likely reasonable,
86 considering the biobased origin and chemical nature of many biosurfactants, the actual
87 body of data is relatively scarce and scattered across many different molecules. Recently,
88 Adu et al. [19] have reported that purified acidic sophorolipids, differently than sodium
89 lauryl ether sulphate (SLES), have no deleterious effect on a 3D *in vitro* skin model
90 colonized with *S. epidermidis*. However, they also state they do not test mammalian cells
91 and they state that their conditions of work should be broadened for a better
92 understanding. In a concomitant work [20], the same team shows the human keratinocyte
93 (HaCaT) cell viability upon exposure to several biosurfactants and results are less
94 straightforward. If acidic sophorolipids have no impact on cell viability up to 500 $\mu\text{g mL}^{-1}$,
95 di-rhamnolipids decrease the cell viability to less than 30 % above 70 $\mu\text{g mL}^{-1}$ while
96 lactonic sophorolipids have comparable cytotoxicity to SLES, cell viability is none above
97 about 70 $\mu\text{g mL}^{-1}$. These results are actually not surprising and demonstrate that this topic
98 should be treated with rigor. As a matter of fact, if *in vitro* and *in vivo* studies are still rare,
99 the interactions between biosurfactants and model lipid membranes are studied since
100 decades [21-23] and recently reviewed [24]. The current knowledge shows that molecules
101 like rhamnolipids or surfactin can modify biophysical properties of phosphatidylcholine
102 bilayer membranes like the gel to liquid crystalline phase transition temperature, the
103 interlamellar periodicity [22] and even their shape [23]. Further studies are then crucial
104 to better apprehend the impact of biosurfactants on mammalian cells for health-related
105 applications.

106
107 In an effort to understand their biological activity, we opted to treat human dermal
108 fibroblasts with solutions of selected acidic sophorolipid standard as well as hydrogel-
109 forming sophorolipids (SL) [25], sophorosides (SS) [26], and glucolipids (G) [27-29] to
110 determine whether or not they could increase regenerative profiles for applications in
111 skin regeneration and wound healing. For this reason, we studied the cytotoxicity of five
112 structurally-related microbial amphiphiles, all non-acetylated in their open form (Figure
113 1): a) sophorolipids C18:1 (SL-C18:1) and C18:0 (SL-C18:0) contain a di-glucose
114 (sophorose) and a carboxylic acid at the other side of the aliphatic chain; b) glucolipids
115 C18:1 (G-C18:1) and C18:0 (G-C18:0) contain a single glucose headgroup and also an
116 opposite carboxylic acid; c) bolaform sophoroside (SS_{bola}-C18:1), structurally similar to SL-
117 C18:1, replaces the COOH group by a second sophorose headgroup. Known for their
118 antimicrobial properties, [30] the amphiphilic character of microbial amphiphiles also
119 suggests toxicity towards human cells, today essentially known for acidic SL, lactonic SL
120 and rhamnolipids on human keratinocytes [18, 19]. The structural diversity in relationship
121 to the complementary solution self-assembly properties [26, 31] of the molecules chosen
122 for this work will then help better understanding their cytotoxicity on normal human
123 dermal fibroblasts (NHDF) using metabolic activity evaluation. We further studied the
124 effect of the selected molecules, based on cytotoxicity, on the upregulation or
125 downregulation of several key target genes in the wound healing process using
126 quantitative gene expression techniques.

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Figure 1 – Chemical structure of the microbial glycolipids used in this work.

137 **2. Materials and Methods**

138 **2.1. Glycolipids**

139 All glycolipids used in this work are provided by Amphistar, Belgium, and used as such:
140 Non-acetylated acidic C18:1 sophorolipids (SL-C18:1), non-acetylated acidic C18:1
141 glucolipids (G-C18:1), non-acetylated bolaform sophorolipids (SS_{bola}-C18:1). The saturated
142 form of sophorolipids (SL-C18:0) and glucolipids (G-C18:0) was prepared from the mono-
143 unsaturated compounds. All compounds were studied and used in previous works: SL-
144 C18:1 [32], G-C18:1 [28], SS_{bola}-C18:1 [33]. SL-C18:0 and G-C18:0 were prepared by
145 catalytic hydrogenation of SL-C18:1 and G-C18:1 according to a recently-published
146 protocol [34]. The chemical structure of all compounds is given in Figure 1.

147

148 **2.2. Preparation of the different glycolipid solutions**

149 Solutions with concentrations ranging from 3 % (w/v) to 0.0001 % (w/v) were prepared
150 from the microbial glycolipids. For this, solutions were prepared in falcon tubes where
151 warm complete cell culture medium and the appropriate mass of each molecule were
152 vortexed. To ensure the complete dissolution, the pH was adjusted to 8.0 using small
153 volumes of 1 M NaOH or 1 M HCl solutions. Then, solutions were vigorously vortexed
154 again to ensure complete homogenisation and warmed up to 37 °C to be further sterilised
155 by passing them through a 0.22 µm filter. Each solution was fabricated
156 extemporaneously.

157

158 **2.3. Normal human dermal fibroblast (NHDF) cell culture**

159 NHDF were purchased from Merck® and stored in liquid nitrogen according to
160 manufacturer's recommendations (Promocell). NHDF cells were cultured in complete cell
161 culture medium (Glutamax™ Dulbecco's Modified Eagle's Medium (DMEM)
162 supplemented with 10 % (v/v) foetal bovine serum, 100 U/mL penicillin, 100 µg/mL
163 streptomycin, 0.25 µg/mL Fungizone for expansion under normal culture conditions
164 (37°C, 5% CO₂). NHDF were exposed to Phosphate Buffered Saline (DPBS) solution for 5
165 minutes, trypsinized to detach them and centrifuged at 1,000 RPM for 5 minutes. Cells
166 were resuspended in complete culture medium and seeded into wells of 6-well plates at
167 a density of 30,000 cells/well. NHDF were cultivated for 3 days in normal culture
168 conditions before being exposed to the different sophorolipids. All chemicals were
169 purchased from Merck® unless otherwise specified.

170

171 **2.4. Cytotoxicity evaluation**

172 Cytotoxicity evaluation was performed 24 and 48 hours after treating NHDF cells (group
173 size= 3, N-value= 3) with the different glycolipid solutions, using the Alamar Blue assay.
174 Glycolipid solutions were removed and cells were carefully washed two times with warm
175 phenol-free DMEM. Following, cells were incubated at 37 °C with 1 mL of a 10 µg/mL
176 resazurin solution for 4 hours. For this, a stock solution of resazurin at 100 µg/mL was
177 diluted 1:10 in phenol-free and serum-free DMEM culture medium. The supernatant was
178 then collected from each well and distributed in 96-well plates in triplicates. Absorbance
179 was measured at λ = 560 nm and λ = 590 nm using a Varioskan™ LUX multimode plate
180 reader (ThermoFisher Scientific). Cells were carefully washed twice with phenol-free

181 DMEM to remove excess resazurin solution and initial treatment solution were applied to
182 further culture cells for the next time point. The percentage of resazurin reduction was
183 calculated following manufacturer's instructions. Untreated NHDF cells at each timepoint
184 were used as controls with an arbitrary value of 100 %. Results were expressed as a
185 percentage compared to the control (untreated cells). Three samples per condition and
186 timepoint were analysed.

187

188 **2.5. RNA purification**

189 Total RNA was extracted by scratching adherent NHDF cells from treated and untreated
190 control conditions and preserved using 1 mL TRIzol® reagent. Following, phase separation
191 was performed by adding 0.2 mL of chloroform and a centrifugation step at 10,000 *g* for
192 15 min. After collecting the aqueous phase, RNA purification was carried out using the
193 RNeasy kit (Qiagen) according to manufacturer's instructions. Last, RNA concentration
194 and purity was determined by UV spectrophotometry using the Varioskan™ LUX
195 multiplate reader.

196

197 **2.6. Reverse transcription into cDNA**

198 1 µL of random primers (200 µM, Invitrogen) and 1 µL deoxyribonucleoside triphosphates
199 (10 mM, Invitrogen) were added to 10 µL purified RNA aliquots (around 150-300 ng).
200 Following denaturation of the secondary structure at 65 °C and random primer binding, 4
201 µL of 5x reaction buffer, 1 µL dithiothreitol (0.2 M) and 2 µL reverse transcriptase from
202 Moloney Murine Leukaemia Virus (M-MLV) (Invitrogen) were added to the reaction mix.
203 After 60 min at 37 °C, the reaction was stopped by heating at 70 °C for 10 min. The
204 resulting cDNAs were stored at -20 °C until further use.

205

206 **2.7. Real-time polymerase chain reaction (PCR)**

207 Gene expression of BAX, COL1A1, COL3A1, FN-1, ACTA-1, ELN, TGFβ-1, FGF-2, CXCL-12,
208 TIMP-1, MMP-14 and KGF was evaluated, after 24 hours in culture in presence of the
209 different glycolipids, using reverse transcription quantitative PCR (qPCR). The
210 quantification was performed using the Light Cycler FastStart DNA Mater plus SYBR Green
211 I kit (Roche) in a Light Cycler 480 system (Roche). Appropriate primers (Thermofischer
212 Scientific) are listed in Table 1. For cycling conditions, the initial Taq polymerase activation
213 at 95 °C for 5 min was followed by 40 cycles. Each cycle consisted of 10 s denaturation at
214 95 °C, 15 s annealing at 60 °C and 15 s elongation at 72 °C. Then, a melting curve was
215 generated by increasing the temperature from 60 °C to 95 °C at a rate of 0.1 °C/s to assess
216 the reaction specificity. The results were analysed using a relative quantification following
217 the Pfaffl method [35]. The efficiencies of the target and reference primer pairs were
218 measured by producing a standard curve based on the amplification of a serial dilution of
219 cDNAs. The mRNA transcript level of each target gene was normalised with the 18s
220 housekeeping gene. Fold changes in gene expression were calculated for each target gene
221 relative to a calibration point, which is the normalised gene expression of this target gene
222 for the untreated control NHDF cells after 24 hours in culture. The value 1 was arbitrarily
223 given to this calibration point. Four samples per condition were analysed and each PCR

224 reaction was performed in triplicate. The minimum information for publication of
 225 quantitative real-time PCR experiments (MIQE) guidelines were followed.

226

227

228

229

Table 1. Selected reverse and forward sequences from selected gene targets.

| TARGET | FORWARD (5'→3') | REVERSE (5'→3') |
|---------|--------------------------|----------------------------|
| BAX | GATGATTGCCGCCGTGGACACAGA | GGAGGAAGTCCAATGTCCAGCCCA |
| COL1A1 | TGGTGTGATGGGATTCCCTGGACC | CCTGAGCTCCAGCCTCTCCATCTT |
| COL3A1 | GGTGCTCGAGGCAGTGATGGTCAA | GGCACCATTTGAACCAGGAGACCC |
| TGFB1 | GGAGTTGTGCGGCAGTGGTT | GCCGGTAGTGAACCCGTTGATG |
| FGF-2 | AGTGTGTGCTAACCGTTACCTGGC | GCCCAGTTCGTTTCAGTGCCACA |
| CXCL-12 | CCATGTTGCCAGAGCCAACGTC | GAGTGGGTCTAGCGGAAAGTCC |
| FN1 | TACCCACACGGTCCGGGACTCAAT | GCCTGTCAGAGTGGCACTGGTAGA |
| ACTA1 | AATACTCGGTGTGGATCGGCGGCT | TGAGAAGTCGCGTGCTGGAGGT |
| 18S | TTACAGGGCCTCGAAAGAGT | TGAGAAACGGCTACCACATC |
| ELN | CTTCCCCGCAGTTACCTTTC | TGTGGTGTAGGGAGTCCAT |
| MMP14 | TCGCTGCCATGCAGAAGTTTTA | CTGGATGCAGAAAGTGATTTTCATTA |
| KGF | AAGGCTCAAGTTGCACCAGGCAG | GTGTGTCGCTCAGGGCTGGAAC |
| TIMP1 | GGCATCCTGTTGTTGCTGTGGC | CCCACGAACTTGCCCTGATGA |

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236 **2.8. Statistical analysis**

237 All experiments were carried out at least twice and results were expressed as mean values
238 \pm standard deviation (SD). Cytotoxicity difference between treatments were analysed for
239 each selected timepoint using the two-way ANOVA test followed by Turkey's test to
240 ensure statistical significance. Differences in gene expression between treatments were
241 analysed for each selected timepoint using the Kruskal-Wallis test followed by Dunn's test
242 as a *post-hoc* test to ensure statistical significance. A *p* value < 0.05 was considered to be
243 significant.
244

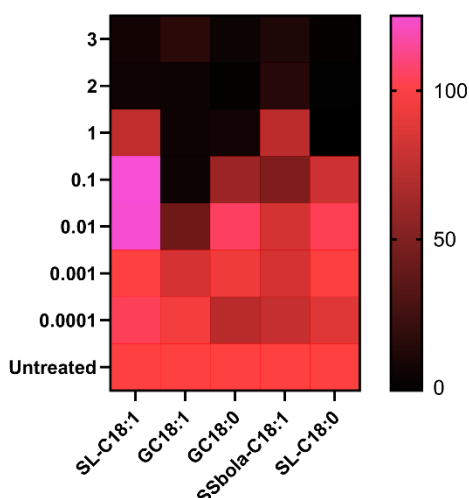
245 **3. Results and Discussion**

246

247 **3.1. Cytotoxicity evaluation**

248 Cytotoxicity evaluation is one of the key biological characterisations to be done when
249 using novel molecules for biomedical applications. The importance of healthy dermal
250 fibroblasts in skin regeneration has been well discussed [36-39]. Also, the glycolipids
251 chosen for this study have shown different interesting physicochemical characteristics,
252 such as their ability to form hydrogels, vesicles or micelles [15, 26, 40]. Considering also
253 novel results obtained by the Banat team from the University of Ulster [17, 18], these
254 molecules are interesting candidates in the field of drug delivery and TERM [12, 41]. As
255 per our knowledge, there has been no cytotoxicity evaluation of these molecules on
256 human fibroblasts. Initial cytotoxicity studies were performed. As shown in the heatmap
257 in **Figure 2a**, significant cytotoxicity was appreciated, regardless the treatment, for
258 concentrations higher than 1 % (w/v) if compared with untreated conditions after just 24
259 hours culture (****, $p < 0.0001$). On the contrary, cytotoxicity values from concentrations
260 lower than 0.01 % (w/v) were similar to those obtained for untreated conditions (ns,
261 $p > 0.1$). In the middle concentration range (1-0.01 % w/v), significant cytotoxicity was
262 observed for G-C18:1, G-C18:0 and SL-C18:0. In contrast, in this same interval, SL-C18:1
263 increased NHDF cell metabolic activity when compared to untreated conditions (i.e., SL-
264 C18:1 vs. Untreated 0.1 % w/v, ***, $p < 0.001$). In this context, SL-C18:1, SS_{bola}-C18:1 and
265 SL-C18:0 were selected for further experimentation, and **Figure 2b** shows the cytotoxicity
266 values of each of the selected molecules in the 1-0.01 % w/v concentration range.
267

a) Initial cytotoxicity evaluation



b)

| | | Cytotoxicity values | | | | Untreated |
|---------------|---------------------------|---------------------|----------------|----------------|-----|-----------|
| | | [molecule] (% w/v) | | | | |
| Molecule name | SL-C18:1 | 1 | 0.1 | 0.01 | 100 | |
| | | 69.18 ± 19.07 | 125.2 ± 44.98 | 124.45 ± 36.69 | 100 | |
| | SS _{bola} -C18:1 | 68.14 ± 46.13 | 42.86 ± 20.43 | 78.52 ± 17.68 | 100 | |
| SL-C18:0 | -1.03 ± 1.11 | 75.42 ± 9.8 | 104.79 ± 17.09 | 100 | | |

268
 269 **Figure 2:** Cytotoxicity evaluation of glycolipids on NHDF cells after 24 hours culture (group
 270 size= 3, N-value= 3). a) Heatmap representing cytotoxicity as the percentage difference in
 271 resazurin reduction between treated and untreated conditions. b) Cytotoxicity values for
 272 SL-C18:1, SS_{bola}-C18:1 and SL-C18:0, the molecules selected for further examination in the
 273 1-0.01 % w/v concentration range. Values are shown as mean percentage ± SD.

274
 275 Further cytotoxicity evaluation was performed on NHDF cells treated with SL-C18:1, SS_{bola}-
 276 C18:1 and SL-C18:0 for up to 48 hours. In general, metabolic activity levels were constant
 277 over the 48 hours culture. As seen in **Figure 3**, metabolic activity levels were lower after
 278 24 hours culture. In this first period, cells are usually under stress due to exposure to
 279 chemicals in solution and activity levels tend to be lower. However, there was a sharp
 280 decline when 1 % w/v treatments were used (****, $p < 0.0001$), indicating that these
 281 molecules are cytotoxic for NHDF cells at high concentration levels. Metabolic activity
 282 levels were comparable to those for untreated cells after 48 hours for SL-C18:1 and SL-
 283 C18:0 treatments, particularly for low concentrations (0.001 % w/v). However,
 284 interestingly, metabolic activity was significantly reduced to approximately 50 % when
 285 using SS_{bola}-C18:1 treatment for both timepoints at 0.1 % w/v concentration (****,
 286 $p < 0.0001$).

287
 288 Understanding the structure-cytotoxicity relationship between the selected molecule and
 289 NHDF is not straightforward, simply due to the lack of data in the literature concerning all
 290 of these molecules. Broadly speaking, a number of microbial glycolipids (sophorolipids,
 291 rhamnolipids, trehalolipids) were shown to interact with bilayer models membranes
 292 [42,43] but also with human keratinocytes [18, 20]. On a qualitative level, the higher
 293 toxicity of G-C18:1 and G-C18:0 after 24h could be explained by their ability to self-
 294 assemble into liquid crystalline membranes themselves [31], probably explaining their
 295 affinity towards phospholipid bilayers. Unpublished small X-ray scattering data from our
 296 group shows the permeability of model phospholipid (DOPC) bilayer membranes towards
 297 G-C18:1, while recent work demonstrates that G-C18:1 and G-C18:0 have a higher

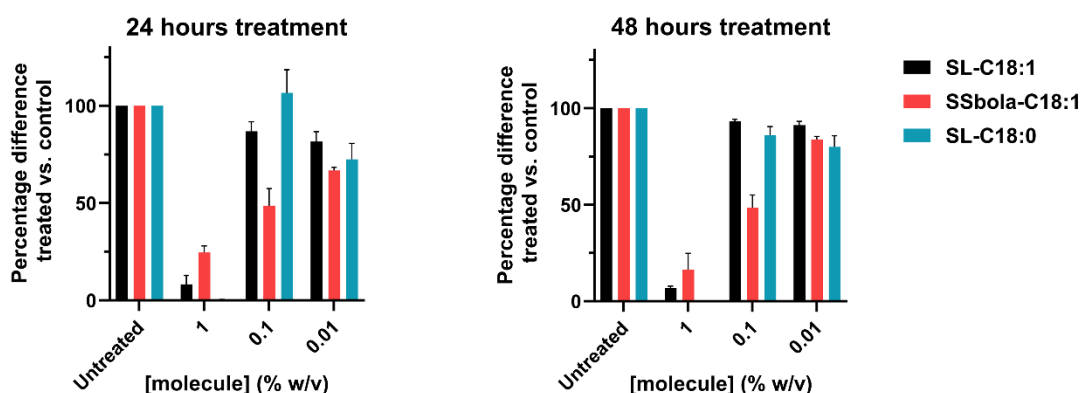
298 inhibitory action towards specific bacteria when compared to acidic sophorolipids (C18:1,
 299 C18:0) and sophorosides. Pala et al. [43] shows that the minimum inhibitory
 300 concentration (MIC) for both G-C18:1 (5a in [43] and G-C18:0 (9a in [43] is often lower
 301 than for SL-C18:1, SL-C18:0 and SS_{bola}-C18:1 (2, 6 and 5a in [43], respectively) for various
 302 pathogens. Cui et al. [44] have found similar results by comparing G-C18:1 (higher
 303 inhibition for most pathogens) and SL-C18:1, but they propose that the actual MIC may
 304 depend on pH, where lower MICs, promoting higher inhibition, are found at more acidic
 305 pH. However, it was not clear whether or not the effect on the MIC could actually be
 306 attributed to the pH.

307

308 As for possible differences between SL-C18:1 and SL-C18:0, the effect of unsaturation on
 309 the cytotoxicity is less clear and more prone to speculation. This being said, the results in
 310 this work show a comparable cytotoxicity and these agree with Pala et al. [43], who did
 311 not experience major inhibitory differences between the two compounds. This could be
 312 probably explained by the fact that both molecules have a similar anionic surfactant
 313 behaviour assembling into micelles at pH above 7.4 [31], which is the physiological pH at
 314 which toxicity experiments are generally conducted. It is then not unreasonable that both
 315 compounds have comparable cytotoxicity. Concerning SS_{bola}-C18:1, it seems that this
 316 compound has a comparable cytotoxicity to acidic sophorolipids, however, it is unclear
 317 the reason why its cytotoxicity after 48h is more pronounced. Given the practically
 318 inexistent data existing for this molecule, we prefer not to disclose any speculative
 319 interpretation.

320

321



322

323 **Figure 3:** Cytotoxicity evaluation of SL-C18:1, SS_{bola}-C18:1 and SL-C18:0 on NHDF cells
 324 (group size= 3, N-value= 3) showing metabolic activity data over a 48-hour treatment.
 325 Statistical significance evaluated using two-way ANOVA test followed by Turkey's test (not
 326 shown in the figure).

327

328 The cytotoxicity data constitute a precious database pour further applications, however
 329 translating them from isolated cells to an *in vivo* context is a complex challenge. In the
 330 field of skin tissue engineering, hydrogels are commonly used as a formulation. Their
 331 primary advantage lies in maintaining hydration of the wound bed, which promotes

332 wound healing. Typically, such hydrogels are prepared from biopolymers at
 333 concentrations ranging from 0.05 wt% to 4 wt% [45-47]. Hydrogels prepared from
 334 microbial amphiphiles, like SL-C18:0, G-C18:1 or SSbola-C18:1, require concentrations
 335 between at least 1 wt% and 4 wt% [16, 26, 28] a range which is lethal for NHDF cells and
 336 which may pose risks to tissues, depending on the hydrogel's stability and the release of
 337 soluble molecules. As an alternative, microbial glycolipids could be combined with other
 338 biopolymers, such as collagen, alginate, chitosan, or silk fibroin, [48-51] to create
 339 composite hydrogels with potentially enhanced properties.

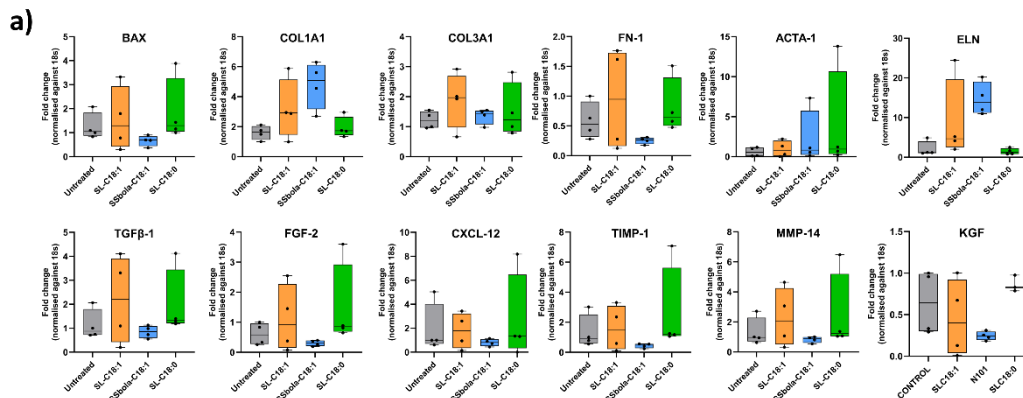
340

341 3.2. Gene expression analysis on key markers for dermal regeneration

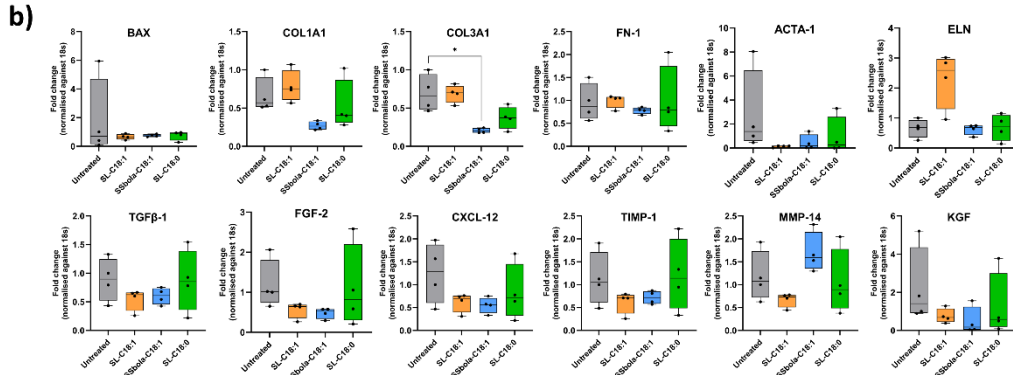
342 Fibroblasts are a key cellular component for skin wound healing [52] due to their capacity
 343 to secrete pro-regenerative growth factors, ECM proteins, and immunomodulators [9, 53-
 344 57]. Cytotoxicity evaluation results were able to exclude treatments at high
 345 concentrations; therefore, we focused this analysis on SL-C18:1, SS_{bola}-C18:1 and SL-C18:0
 346 treatments with 0.1-0.01 % w/v concentrations after culturing NHDF cells for 24 hours.
 347 Regardless of the conditions, the total RNA extracted was similar with a mean value of
 348 237.6 ± 45 ng/ μ L. We measured the gene expression of NHDF subjected to the
 349 aforementioned treatments to investigate differences in cell apoptosis (BAX), ECM
 350 proteins (COL1A1, COL3A1, FN-1, ACTA-1, ELN), pro-regenerative growth factors (TGF β -1,
 351 FGF-2, CXCL-12, KGF) and matrix remodellers (TIMP-1 and MMP-14). As seen in **figure 4a**
 352 for treatments at 0.1 % w/v concentrations and **figure 4b** for treatments at 0.01 % w/v
 353 concentrations, results showed no significant variations in gene fold changes compared
 354 to control (ns, $p > 0.01$).

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Figure 4: Gene expression analysis after 24h SL-C18:1, SS_{bola}-C18:1 and SL-C18:0 on NHDF cells showing fold changes for a) treatments with concentration at 0.1 % w/v and b) treatments with concentrations at 0.01 % w/v. Statistical significance evaluated using Kruskal-Wallis test followed by Dunn's test.

364 Conclusions

365 Dermal fibroblasts are key players governing the skin wound healing process due to their
366 capacity to secrete ECM proteins and pro-regenerative growth factors. Microbial
367 glycolipids, here soporolipids, sophorosides and glycolipids with different unsaturation,
368 are novel sustainable molecules obtained by microbial fermentation from several yeasts
369 and bacteria. Due to their physicochemical characteristics, it has been shown that by
370 modulating their pH and/or temperature, for example, it is possible to form soft
371 hydrogels, with possible output in the field of tissue regeneration. In this study we have
372 shown that, up to a certain concentration, several of these molecules were not toxic for
373 NHDF: out of all tested molecules, we were able to show that SL-C18:1, SS_{bola}-C18:1 and
374 SL-C18:0 sustained NHDF cell growth *in vitro* for concentrations up to approximately 0.1
375 % w/v for 48 hours continuous exposure. G-C18:1 and G-C18:0 were excluded due to their
376 higher cytotoxicity, probably explained by their higher tendency to behave as membrane-
377 forming lipids, thus showing a pronounced tendency to integrate and disrupt cell
378 membranes. For this reason, we studied changes in gene expression for the key elements
379 of the regenerative milieu (mainly growth factors and ECM proteins and remodellers) for
380 SL-C18:1, SS_{bola}-C18:1 and SL-C18:0 only. However, we found no significant gene
381 expression changes. This work might encourage researchers in the field to advance
382 towards finding different biomedical applications for this class of molecules.

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388 Conflict of interest

389 Authors declare no conflicts of interest.

391 **References**

- 392 [1] R. A. Fayne, L. J. Borda, A. N. Egger, and M. Tomic-Canic, "The Potential Impact of Social
393 Genomics on Wound Healing," *Adv. Wound Care*, vol. 9, no. 6, pp. 325–331, 2020, doi:
394 10.1089/wound.2019.1095.
- 395 [2] D. Queen and K. Harding, "What's the true costs of wounds faced by different healthcare
396 systems around the world?," *Int. Wound J.*, vol. 20, no. 10, pp. 3935–3938, 2023, doi:
397 10.1111/iwj.14491.
- 398 [3] Y. Shimizu, E. H. Ntege, and H. Sunami, "Current regenerative medicine-based
399 approaches for skin regeneration: A review of literature and a report on clinical
400 applications in Japan," *Regen. Ther.*, vol. 21, pp. 73–80, 2022, doi:
401 10.1016/j.reth.2022.05.008.
- 402 [4] M. H. Norahan, S. C. Pedroza-González, M. G. Sánchez-Salazar, M. M. Álvarez, and G.
403 Trujillo de Santiago, "Structural and biological engineering of 3D hydrogels for wound
404 healing," *Bioact. Mater.*, vol. 24, pp. 197–235, 2023, doi:
405 10.1016/j.bioactmat.2022.11.019.
- 406 [5] F. Cialdai, C. Risaliti, and M. Monici, "Role of fibroblasts in wound healing and tissue
407 remodeling on Earth and in space," *Front. Bioeng. Biotechnol.*, vol. 10, pp. 1–18, 2022,
408 doi: 10.3389/fbioe.2022.958381.
- 409 [6] N. Amiri, A. P. Golin, R. B. Jalili, and A. Ghahary, "Roles of cutaneous cell-cell
410 communication in wound healing outcome: An emphasis on keratinocyte-fibroblast
411 crosstalk," *Exp. Dermatol.*, vol. 31, no. 4, pp. 475–484, 2022, doi: 10.1111/exd.14516.
- 412 [7] M. Rodrigues, N. Kosaric, C. A. Bonham, and G. C. Gurtner, "Wound healing: A cellular
413 perspective," *Physiol. Rev.*, vol. 99, no. 1, pp. 665–706, 2019, doi:
414 10.1152/physrev.00067.2017.
- 415 [8] M. Fernández-Guarino, M. L. Hernández-Bule, and S. Bacci, "Cellular and Molecular
416 Processes in Wound Healing," *Biomedicines*, vol. 11, no. 9, 2023, doi:
417 10.3390/biomedicines11092526.
- 418 [9] A. Stunova and L. Vistejnova, "Dermal fibroblasts—A heterogeneous population with
419 regulatory function in wound healing," *Cytokine Growth Factor Rev.*, vol. 39, pp. 137–
420 150, 2018, doi: 10.1016/j.cytogfr.2018.01.003.
- 421 [10] J. D. Desai and I. M. Banat, "Microbial production of surfactants and their commercial
422 potential," *Microbiol. Mol. Biol. Rev.*, vol. 61, no. 1, pp. 47–64, 1997, doi:
423 10.1128/mnbr.61.1.47-64.1997.
- 424 [11] L. R. Rodrigues-, L. R. Rodrigues, and J. A. Teixeira, "Biomedical and Therapeutic
425 Applications of A Biosurfactants," *Adv. Exp. Med. Biol.*, vol. 672, pp. 75–87, 2010.
- 426 [12] W. Y. Cho, J. F. Ng, and W. H. Yap, "Sphorolipids — Bio-Based Antimicrobial Formulating
427 Agents," *Molecules*, vol. 27, no. 5556, pp. 1–24, 2022.
- 428 [13] S. Pal, N. Chatterjee, A. K. Das, D. J. McClements, and P. Dhar, "Sphorolipids: A
429 comprehensive review on properties and applications," *Adv. Colloid Interface Sci.*, vol.
430 313, p. 102856, 2023, doi: 10.1016/j.cis.2023.102856.
- 431 [14] M. R. Oliveira, A. Magri, C. Baldo, D. CAmiliou-Neto, T. Minucelli, and M. A. P. C. Celligoi,
432 "Review: Sphorolipids A Promising Biosurfactant and it's Applications," *Int. J. Adv.
433 Biotechnol. Res.*, vol. 6, no. 2, pp. 161–174, 2015.
- 434
435
436

- 437 [15] N. Baccile, C. Seyrig, A. Poirier, S. Alonso-De Castro, S. L. K. W. Roelants, and S. Abel,
438 "Self-assembly, interfacial properties, interactions with macromolecules and molecular
439 modelling and simulation of microbial bio-based amphiphiles (biosurfactants). A tutorial
440 review," *Green Chem.*, vol. 23, no. 11, pp. 3842–3944, 2021, doi: 10.1039/d1gc00097g.
- 441 [16] G. Ben Messaoud, "Viscoelastic systems from glycolipid biosurfactants," *Curr. Opin.*
442 *Colloid Interface Sci.*, vol. 71, p. 101805, 2024, doi: 10.1016/j.cocis.2024.101805.
- 443 [17] H. L. Lydon, N. Baccile, B. Callaghan, R. Marchant, C. A. Mitchell, and I. M. Banat,
444 "Adjuvant antibiotic activity of acidic sophorolipids with potential for facilitating wound
445 healing," *Antimicrob. Agents Chemother.*, vol. 61, no. 5, 2017, doi: 10.1128/AAC.02547-
446 16.
- 447 [18] S. A. Adu, M. S. Twigg, P. J. Naughton, R. Marchant, and I. M. Banat, "Characterisation of
448 cytotoxicity and immunomodulatory effects of glycolipid biosurfactants on human
449 keratinocytes," *Appl. Microbiol. Biotechnol.*, vol. 107, no. 1, pp. 137–152, 2023, doi:
450 10.1007/s00253-022-12302-5.
- 451 [19] S. A. Adu, M. S. Twigg, P. J. Naughton, R. Marchant, and I. M. Banat, "Purified Acidic
452 Sophorolipid Biosurfactants in Skincare Applications: An Assessment of Cytotoxic Effects
453 in Comparison with Synthetic Surfactants Using a 3D In Vitro Human Skin Model,"
454 *Fermentation*, vol. 9, no. 985, 2023, doi: 10.3390/fermentation9110985.
- 455 [20] Adu, S. A., Twigg, M. S., Naughton, P. J., Marchant, R., & Banat, I. M. (2022).
456 Biosurfactants as Anticancer Agents: Glycolipids Affect Skin Cells in a Differential Manner
457 Dependent on Chemical Structure. *Pharmaceutics*, 14(2), 360.
458 doi.org/10.3390/pharmaceutics14020360.
- 459 [21] R. Maget-Dana, M. Ptak (1995). Interactions of surfactin with membrane models.
460 *Biophysical Journal*, 68 (5), pp. 1937-1943. doi.org/10.1016/S0006-3495(95)80370-X.
- 461 [22] Ortiz, A., Teruel, J. A., Espuny, M. J., Marqués, A., Manresa, A., & Aranda, F. J. (2006).
462 Effects of dirhamnolipid on the structural properties of phosphatidylcholine
463 membranes. *International journal of pharmaceutics*, 325(1-2), pp. 99–107.
464 doi.org/10.1016/j.ijpharm.2006.06.028
- 465 [23] Herzog, M., Tiso, T., Blank, L. M., & Winter, R. (2020). Interaction of rhamnolipids with
466 model biomembranes of varying complexity. *Biochimica et biophysica acta.*
467 *Biomembranes*, 1862(11), 183431. doi.org/10.1016/j.bbamem.2020.183431
- 468 [24] F. J. Aranda, J. A. Teruel, and A. Ortiz, "Recent advances on the interaction of glycolipid
469 and lipopeptide biosurfactants with model and biological membranes," *Curr. Opin.*
470 *Colloid Interface Sci.*, vol. 68, pp. 101748, 2023, doi: 10.1016/j.cocis.2023.101748.
- 471 [25] G. Ben Messaoud *et al.*, "pH-Controlled Self-Assembled Fibrillar Network Hydrogels:
472 Evidence of Kinetic Control of the Mechanical Properties," *Chem. Mater.*, vol. 31, no. 13,
473 pp. 4817–4830, 2019, doi: 10.1021/acs.chemmater.9b01230.
- 474 [26] A. Poirier *et al.*, "Energy Landscape of the Sugar Conformation Controls the Sol-to-Gel
475 Transition in Self-Assembled Bola Glycolipid Hydrogels," *Chem. Mater.*, vol. 34, no. 12,
476 pp. 5546–5557, 2022, doi: 10.1021/acs.chemmater.2c00580.
- 477 [27] A. Poirier, P. Le Griel, J. Perez, D. Hermida-Merino, P. Pernot, and N. Baccile, "Metallogels
478 from a Glycolipid Biosurfactant," *ACS Sustain. Chem. Eng.*, vol. 10, pp. 16503–16515,
479 2022, doi: 10.1021/acssuschemeng.2c01860.
- 480 [28] A. Poirier *et al.*, "Ca²⁺ and Ag⁺ orient low-molecular weight amphiphile self-assembly
481 into 'nano-fishnet' fibrillar hydrogels with unusual β -sheet-like raft domains," *Soft*
482 *Matter*, vol. 19, pp. 378–393, 2022, doi: 10.1039/d2sm01218a.
- 483
484

- 485 [29] G. Ben Messaoud *et al.*, "Single-molecule lamellar hydrogels from bolaform microbial
486 glucolipids," *Soft Matter*, vol. 16, no. 10, pp. 2528–2539, 2020, doi:
487 10.1039/c9sm02158b.
- 488 [30] P. Puyol McKenna, P. J. Naughton, J. S. G. Dooley, N. G. Ternan, P. Lemoine, and I. M.
489 Banat, "Microbial Biosurfactants: Antimicrobial Activity and Potential Biomedical and
490 Therapeutic Exploits," *Pharmaceuticals*, vol. 17, no. 1, pp. 1–13, 2024, doi:
491 10.3390/ph17010138.
- 492 [31] N. Baccile *et al.*, "Self-Assembly Mechanism of pH-Responsive Glycolipids: Micelles,
493 Fibers, Vesicles, and Bilayers," *Langmuir*, vol. 32, no. 42, pp. 10881–10894, 2016, doi:
494 10.1021/acs.langmuir.6b02337.
- 495 [32] S. L. K. W. Roelants *et al.*, "Towards the industrialization of new biosurfactants:
496 Biotechnological opportunities for the lactone esterase gene from *Starmella bambicola*,"
497 *Biotechnol. Bioeng.*, vol. 113, pp. 550–559, 2016, doi: 10.1002/bit.25815.
- 498 [33] L. Van Renterghem *et al.*, "From lab to market: An integrated bioprocess design approach
499 for new-to-nature biosurfactants produced by *Starmerella bambicola*," *Biotechnol.*
500 *Bioeng.*, vol. 115, pp. 1195–1206, 2018, doi: 10.1002/bit.26539.
- 501 [34] N. Baccile, V. Chaleix, and I. Hoffmann, "Measuring the bending rigidity of microbial
502 glucolipid (biosurfactant) bioamphiphile self-assembled structures by neutron spin-echo
503 (NSE): Interdigitated vesicles, lamellae and fibers," *Biochim. Biophys. Acta - Biomembr.*,
504 vol. 1866, p. 184243, 2024, doi: 10.1016/j.bbamem.2023.184243.
- 505 [35] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR,"
506 *Nucleic Acids Res.*, vol. 29, no. 9, pp. 2002–2007, 2001, doi: 10.1111/j.1365-
507 2966.2012.21196.x.
- 508 [36] F. Z. Chen, P. C. Tan, Z. Yang, Q. Li, and S. B. Zhou, "Identifying characteristics of dermal
509 fibroblasts in skin homeostasis and disease," *Clin. Exp. Dermatol.*, vol. 48, no. 12, pp.
510 1317–1327, 2023, doi: 10.1093/ced/llad257.
- 511 [37] R. L. Thangapazham, T. N. Darling, and J. Meyerle, "Alteration of skin properties with
512 autologous dermal fibroblasts," *Int. J. Mol. Sci.*, vol. 15, no. 5, pp. 8407–8427, 2014, doi:
513 10.3390/ijms15058407.
- 514 [38] R. Atit, V. Thulabandu, and D. Chen, "Dermal fibroblast in cutaneous development and
515 healing," *Wiley Interdiscip. Rev. Dev. Biol.*, vol. 7, no. 2, 2018, doi:
516 10.4049/jimmunol.1801473.The.
- 517 [39] A. Zorina, V. Zorin, A. Isaev, D. Kudlay, M. Vasileva, and P. Kopnin, "Dermal Fibroblasts as
518 the Main Target for Skin Anti-Age Correction Using a Combination of Regenerative
519 Medicine Methods," *Curr. Issues Mol. Biol.*, vol. 45, no. 5, pp. 3829–3847, 2023, doi:
520 10.3390/cimb45050247.
- 521 [40] C. Seyrig, P. Le Griel, N. Cowieson, J. Perez, and N. Baccile, "Synthesis of multilamellar
522 walls vesicles polyelectrolyte-surfactant complexes from pH-stimulated phase transition
523 using microbial biosurfactants," *J. Colloid Interface Sci.*, vol. 580, pp. 493–502, 2020, doi:
524 10.1016/j.jcis.2020.07.021.
- 525 [41] J. R. Hughes, A. S. Miller, C. E. Wallace, G. N. Vemuri, and P. M. Iovine, "Biomedically
526 Relevant Applications of Bolaamphiphiles and Bolaamphiphile-Containing Materials,"
527 *Front. Chem.*, vol. 8, pp. 1–18, 2021, doi: 10.3389/fchem.2020.604151.
- 528 [42] P. K. Singh, S. S. R. Bohr, and N. S. Hatzakis, "Direct observation of sophorolipid micelle
529 docking in model membranes and cells by single particle studies reveals optimal fusion
530 conditions," *Biomolecules*, vol. 10, no. 9, pp. 1–16, 2020, doi: 10.3390/biom10091291.
- 531
532

- 533 [43] M. Pala, M. G. Castelein, C. Dewaele, S. L. K. W. Roelants, W. K. Soetaert, and C. V.
534 Stevens, "Tuning the antimicrobial activity of microbial glycolipid biosurfactants through
535 chemical modification," *Front. Bioeng. Biotechnol.*, vol. 12, pp. 1–13, 2024, doi:
536 10.3389/fbioe.2024.1347185.
- 537 [44] T. Cui, Y. Tang, M. Zhao, Y. Hu, M. Jin, and X. Long, "Preparing Biosurfactant Glucolipids
538 from Crude Sophorolipids via Chemical Modifications and Their Potential Application in
539 the Food Industry," *J. Agric. Food Chem.*, vol. 71, no. 6, pp. 2964–2974, 2023, doi:
540 10.1021/acs.jafc.2c06066.
- 541 [45] Aderibigbe, B. A., & Buyana, B. (2018). Alginate in Wound
542 Dressings. *Pharmaceutics*, 10(2), 42. doi.org/10.3390/pharmaceutics10020042.
- 543 [46] E. Bell, B. Ivarsson, C. Merrill (1979) Production of a tissue-like structure by contraction of
544 collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc.*
545 *Natl. Acad. Sci. U.S.A.* 76 (3), pp. 1274-1278. doi.org/10.1073/pnas.76.3.1274.
- 546 [47] Helary, C., Ovtracht, L., Coulomb, B., Godeau, G., & Giraud-Guille, M. M. (2006). Dense
547 fibrillar collagen matrices: a model to study myofibroblast behaviour during wound
548 healing. *Biomaterials*, 27(25), pp. 4443–4452.
549 doi.org/10.1016/j.biomaterials.2006.04.005.
- 550 [48] Seyrig, C., Poirier, A., Perez, J., Bizien, T., & Baccile, N. (2023). Interpenetrated
551 Biosurfactant-Biopolymer Orthogonal Hydrogels: The Biosurfactant's Phase Controls the
552 Hydrogel's Mechanics. *Biomacromolecules*, 24(1), pp. 33–42.
553 doi.org/10.1021/acs.biomac.2c00319.
- 554 [49] Lassenberger, A., Martel, A., Porcar, L., & Baccile, N. (2021). Interpenetrated
555 biosurfactant-silk fibroin networks - a SANS study. *Soft matter*, 17(8), pp. 2302–2314.
556 doi.org/10.1039/d0sm01869d.
- 557 [50] Dubey P., Nawale L., Sarkar D., Nisal A., Prabhune A.(2015) Sophorolipid assisted tunable
558 and rapid gelation of silk fibroin to form porous biomedical scaffolds. *RSC Advances* 5
559 (43) pp. 33955-33962. doi.org/10.1039/C5RA04317D
- 560 [51] Nawale, L., Dubey, P., Chaudhari, B., Sarkar, D., & Prabhune, A. (2017). Anti-proliferative
561 effect of novel primary cetyl alcohol derived sophorolipids against human cervical cancer
562 cells HeLa. *PloS one*, 12(4), e0174241. doi.org/10.1371/journal.pone.0174241
- 563 [52] E. Rognoni and F. M. Watt, "Skin Cell Heterogeneity in Development, Wound Healing,
564 and Cancer," *Trends Cell Biol.*, vol. 28, no. 9, pp. 709–722, 2018, doi:
565 10.1016/j.tcb.2018.05.002
- 566 [53] R. N. Gomes, F. Manuel, and D. S. Nascimento, "The bright side of fibroblasts: molecular
567 signature and regenerative cues in major organs," *npj Regen. Med.*, vol. 6, no. 1, pp. 1–
568 12, 2021, doi: 10.1038/s41536-021-00153-z.
- 569 [54] M. V. Plikus *et al.*, "Fibroblasts: Origins, definitions, and functions in health and disease,"
570 *Cell*, vol. 184, no. 15, pp. 3852–3872, 2021, doi: 10.1016/j.cell.2021.06.024
- 571 [55] J. Wang, Z. Duan, X. Chen, and M. Li, "The immune function of dermal fibroblasts in skin
572 defence against pathogens," *Exp. Dermatol.*, vol. 32, no. 9, pp. 1326–1333, 2023, doi:
573 10.1111/exd.14858
- 574 [56] M. Ghetti *et al.*, "Subpopulations of dermal skin fibroblasts secrete distinct extracellular
575 matrix: implications for using skin substitutes in the clinic," *Br. J. Dermatol.*, vol. 179, no.
576 2, pp. 381–393, 2018, doi: 10.1111/bjd.16255
- 577 [57] S. Knoedler *et al.*, "Fibroblasts – the cellular choreographers of wound healing," *Front.*
578 *Immunol.*, vol. 14, pp. 1–16, 2023, doi: 10.3389/fimmu.2023.1233800

