

1 **The effect of sophorolipids against microbial biofilms on medical-grade silicone.**

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26 **ABSTRACT**

27 Recent medical strategies rely on the search for effective antimicrobials as surface coatings to
28 prevent and treat infections in humans and animals. Biosurfactants have recently been shown
29 to have properties as antiadhesive and antibiofilm agents. Sophorolipids in particular are
30 biosurfactant molecules known to act as therapeutic agents. This study aimed to evaluate
31 antimicrobial properties of sophorolipids in medical-grade silicone discs using strains of
32 clinical relevance. Sophorolipids were produced under fed batch conditions, ESI-MS analyses
33 were carried out to confirm the congeners present in each formulation. Three different
34 products were obtained SLA (acidic congeners), SL18 (lactonic congeners) and SLV
35 (mixture of acidic and lactonic congeners) and were tested against *Staphylococcus aureus*
36 ATCC 6538, *Pseudomonas aeruginosa* ATCC 10145 and *Candida albicans* IHEM 2894. All
37 three congener mixtures showed a biofilms disruption effect (> 0.1% w/v) of 70%, 75% and
38 80% for *S. aureus*, *P. aeruginosa* and *C. albicans*, respectively. On pre-coated silicone discs,
39 biofilm formation of *S. aureus* was reduced by 75% using SLA 0.8% w/v. After 1.5 h the
40 inhibition of *C. albicans* attachment was between 45-56% whilst after 24 h incubation the
41 percentage of inhibition for the cell attachment increased to 68-70% when using SLA 0.8%
42 w/v. Finally, in co-incubation experiments SLA 0.05% w/v significantly reduced the ability
43 of *S. aureus* and *C. albicans* to form biofilms and to adhere to surfaces by 90-95% at
44 concentrations between 0.025-0.1% w/v. In conclusion sophorolipids significantly reduced
45 the cell attachment of both tested strains which suggests that these molecules could have a
46 potential role as coating agents on medical grade silicone devices for the preventions of Gram
47 positive bacteria and yeast infections.

48 **Keywords**

49 Sophorolipids, biofilms, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas*
50 *aeruginosa*, medical-grade silicone.

51 **1. INTRODUCTION**

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53 It is well known that up to 80% of microbial infections that develop in humans are due to
54 biofilm development (Römling and Balsalobre, 2012). Biofilm infections are associated with
55 pathogenic or opportunistic bacteria linked to chronic condition with recurrent or long lasting
56 infections despite the host's immune response and antibiotic therapy (Hall-Stoodley and
57 Stoodley, 2009). Biofilms develop preferentially on dead tissues or on inert surfaces, such as
58 medical devices (Lambe et al., 1991), but may also form on living tissues, as in the case of
59 endocarditis (Costerton et al., 1999). Therefore, although many biofilm infections develop
60 slowly and initially produce few symptoms, they represent serious clinical problems because
61 they promote complex responses by the immune system and act as reservoirs of acute
62 infections (Donlan and Costerton, 2002).

63 It is difficult however to evaluate the contribution of biofilms in human disease due to the
64 lack of criteria to characterize the biofilm-induced pathogenesis (Hall-Stoodley and Stoodley,
65 2009). About 60-70% of nosocomial infections are due to the implantation of medical devices
66 to improve the quality of life for patients and to provide better medical care (Darouiche,
67 2001; Bryers, 2008). The cause of these infections is often attributed to the development of
68 microbial biofilms on devices, and it has been observed that the onset of an inflammatory
69 response following implantation can lead to the formation of molecules favouring biofilm
70 adhesion (Hall-Stoodley et al., 2004).

71 Almost all surfaces can be colonized by biofilms and practically all medical devices or tissue
72 engineering constructs are susceptible to colonization and microbial infection (Castelli et al.,
73 2007). Biofilm development is often observed on urinary catheters (Stickler, 2008), central
74 venous catheters (Petrelli et al., 2006), catheters in the cerebrospinal fluid (Odds, 1988),
75 cardiac prosthetic valves (Litzler et al., 2007), pacemakers (Kojic and Darouiche, 2004),
76 endotracheal tubes (Odds, 1988), silicone vocal prostheses (Buijssen et al., 2007), contact

77 lenses (Imamura et al., 2008), hip prostheses (Dempsey et al. , 2007) and intrauterine devices
78 (Chassot et al., 2008).

79 The colonization of the medical device can lead to tissue damage, systemic infection and
80 altered device functioning, therefore, once the biofilm has formed it is almost always
81 necessary to remove the device to eliminate the infection. The elimination of the infected
82 device and the use of high dose antimicrobial agents for long periods of time are essential for
83 successful therapy against these infections. The main problem with this approach is the
84 frequent development of antibiotic resistance (Rodrigues, 2011). Therefore, it is clinically
85 very important to develop technologies to control the formation and growth of biofilms
86 (Fracchia et al., 2012). For this reason, medical devices are often coated with antimicrobial
87 and anti-adhesive agents in order to prevent the adhesion and development of biofilm with a
88 consequent reduction of infections related to them (von Eiff et al., 2005; Basak et al., 2009).

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90 Surfactants are amphiphilic molecules that are contained in a significant number of
91 products in use daily and therefore are part of all aspects of our daily lives. Their properties
92 make them very useful for many industrial and domestic applications, with a global
93 production exceeding 13 million tonnes per year (Marchant and Banat, 2012). Interest in the
94 use of biosurfactants in general is steadily increasing in healthcare associated applications to
95 reduce infections (Krasowska, 2010) particularly, involving their use in controlling biofilms
96 formation and/or their disruption. Previous studies have shown that the interaction of
97 biosurfactants with different surfaces can affect their hydrophobic properties affecting the
98 microorganism's adhesion abilities and consequent biofilm formation (Shah et al., 2007).
99 Sophorolipids showed bactericidal properties when compared to conventional antimicrobial
100 agents with bacteriostatic effects (Diaz De Rienzo et al., 2015). Previous studies indicating
101 the anti-adhesive properties of biosurfactants have used pure cultures of microorganisms,

102 however, analysis of a typical biofilm reveals predominantly mixed cultures. Additionally,
103 the nutritional composition of biofilms has been shown to affect the adhesion characteristics
104 of single and mixed cultures (Zezzi do Valle Gomes and Nitscke, 2012). This work aims
105 investigate antimicrobial effect of sophorolipids on medical grade silicon material surfaces
106 using microbial strains of clinical relevance: *Candida albicans*, *Staphylococcus aureus* and
107 *Pseudomonas aeruginosa*. *C. albicans* is the most common fungal human pathogen causing
108 diseases ranging from superficial mucocutaneous infections to life-threatening candidiasis
109 (Pfaller and Diekema, 2007; Ganguly and Mitchell, 2011). *S. aureus* and *Pseudomonas*
110 *aeruginosa* are pathogen microorganisms responsible for an important number of clinical
111 infections, including bacteraemia, and device-related infections among others (Tong et al.,
112 2015, Zhang et al., 2018).

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127 2. MATERIALS AND METHODS

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129 2.1 Microorganisms and media

130 *Candida bombicola* ATCC 22214 was the microorganism used to produce sophorolipids, it
131 was stored in nutrient broth with 20% glycerol at -80°C until further use. The culture medium
132 for the production of sophorolipids was glucose/yeast extract/urea (GYU) (Diaz De Rienzo,
133 et al 2015). Rapeseed oil, was used as a second carbon source, fed at regular intervals to
134 induce sophorolipid production. *Candida albicans* IHEM 2894 strain was cultivated in Yeast
135 Nitrogen Base broth (YNBD) + 50 mM Dextrose and stored at -80°C until further use.
136 *Staphylococcus aureus* ATCC 6538, was cultivated in Tryptic Soy Broth (TSB) + 1%
137 glucose (G) and stored at -80°C until further use. *Pseudomonas aeruginosa* ATCC 10145,
138 was cultivated in Brain Heart Infusion (BHI) + 1% G and stored at -80°C until further use.

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140 2.2 Production of sophorolipids

141 Crude SL mixtures were obtained as crude extract from fed batch cultivation of *C. bombicola*
142 ATCC 22214 (Shah et al., 2005), feeding glucose and oleic acid rather than waste frying oil
143 at 1.5%, 2% and 4% w/v to induce the production of different congeners. The dry matter
144 content was classified as SLA (acidic congeners), SL18 (lactonic congeners) and SLV
145 (mixture of both congeners). Sophorolipids were extracted and partially purified by chemical
146 extraction (Smyth et al., 2009). For mass analysis, partially purified sophorolipids were
147 dissolved in methanol and characterised by electrospray ionisation–mass spectrometry (ESI–
148 MS) using a Waters LCT mass spectrometer in negative-ion mode. Data was collected via
149 direct infusion using a syringe pusher over 0.5/min in methanol. A desolvation temperature of
150 200°C was applied together with a Desolvation Gas Flow (L/h) of 694 and a capillary voltage
151 of 3000V.

152 **2.3 Medical-grade silicone elastomeric discs preparation.**

153 Medical-grade silicone elastomeric discs (SEDs) of 10 mm in diameter, 1.5 mm in thickness
154 were used for experiments in 24-well culture tissue plates, each silicone disc was cleaned,
155 sterilized and conditioned according to Ceresa et al., 2016 with minor modifications. The
156 discs were sonicated for 5 min at 60 kHz using Elma S30H and rinsed two times with
157 distillate water. Then, discs were submerged in 20 mL of MeOH, sonicated for 5 min at 60
158 kHz, rinsed twice and steam sterilized for 15 min at 121°C.

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160 **2.4 Antimicrobial susceptibility of *C. albicans*, *S. aureus* and *P. aeruginosa* biofilm**
161 **towards sophorolipids.**

162 *C. albicans* IHEM 2894 biofilm were formed according to Chandra et al., 2008. Fungal cells
163 were suspended in Phosphate Buffered Saline (PBS) +10% Fetal Bovine Serum (FBS) and
164 adjusted up to 1×10^7 CFU/mL. The discs were inoculated with 1mL of the suspension and,
165 after cell adhesion (1.5 h), were moved into a new 24-well plate in the presence of 1mL of
166 YNBD +10% FBS and incubated for 24 h at 37°C at 90 rpm to promote the biofilm growth
167 phase.

168 *S. aureus* ATCC 6538 was grown in TSB + 1% G, and the suspension was adjusted up to a
169 concentration of 1×10^7 CFU/mL. Silicone discs were submerged with 1 mL of bacterial
170 suspension and incubated for 24 h at 37°C in static conditions as described before.

171 *P. aeruginosa* ATCC 10145 was grown in BHI + 1% G, and the suspension was adjusted up
172 to a concentration of 1×10^6 CFU/mL. Silicone discs were submerged with 1 mL of bacterial
173 suspension and incubated for 24 h at 37°C and 140 rpm.

174 Microbial pre-formed biofilms were then treated with different concentrations of SLA and
175 SL18 ranging from 0.05%-0.4%, of SLV ranging from 0.025%-0.2% and incubated for 24 h
176 at 37°C. The antimicrobial activity of SLA, SLV and SL18 was evaluated using 3-[4,5-

177 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay
178 (Trafny et al., 2013). Biofilms were washed three times with PBS for removal of non-
179 adherent cells and moved in 1 mL of 0.3% MTT solution supplemented with 0.01% G and
180 1µM menadione. After 30 min of incubation time at 37°C, formazan crystals were dissolved
181 with 1ml of DMSO/0.1M glycine buffer (pH 10.2) solution (7:1). From each biofilm, 200µL
182 were transferred to a new 96-well plate and the absorbance was measured at 570 nm. The
183 assay was carried out in triplicate and repeated three times for all of SLA, SLV and SL18
184 concentrations used for the test (n=9).

185

186 **2.5 Disruption properties of sophorolipids towards *C. albicans*, *S. aureus* and *P.*** 187 ***aeruginosa*.**

188 *2.5.1 Co-Incubation*

189 Silicone discs were submerged in 500µL of *C. albicans* IHEM 2894 inoculum (2×10^7
190 CFU/mL in PBS + 20% FBS) and an equal volume of double-concentrated SLA (0.05%),
191 SL18 (0.05%) and SLV solutions (0.025% and 0.05%) (test groups) or PBS (control group).
192 After the adhesion phase, discs were placed in a new plate containing 1 mL of YNBD + 10%
193 FBS + 0%, 0.025%, 0.05%, 0.1% SLs and incubated for 24 h at 37°C and 90 rpm.

194 For *S. aureus* ATCC 6538, silicone discs were inoculated with an equal volume of a bacterial
195 suspension (2×10^7 CFU/ml in TSB 2X + 2% G) and SLs (0.05%, 0.1%, 0.2%) or PBS and
196 incubated at 37°C for 24 h.

197 For *P. aeruginosa* ATCC 10145, silicone discs were inoculated with an equal volume of a
198 bacterial suspension (2×10^6 CFU/ml in BHI 2X + 2% G) and SLs (0.05%, 0.1%, 0.2%) or
199 PBS and incubated at 37°C for 24 h and 140rpm.

200 The biofilm biomass was quantified by the crystal violet (0.2%) assay. Biofilms were washed
201 three times with PBS, air-dried and coloured for 10 min and the absorbance at 570 nm was

202 measured. Assays were carried out in triplicate and the experiments were repeated three times
203 (n=9).

204

205 2.5.2 Pre-coating

206 Elastomeric discs were dipped in 1 mL of SLs solutions at concentrations ranging from 0.2%
207 to 0.8% (test groups) or PBS (control group) and incubated for 24 h at 37°C and 180 rpm.

208 In the case of *C. albicans*, discs were moved into 24-well plates containing 1 mL of
209 suspension, standardised to 1×10^7 CFU/mL in PBS + 10% FBS. After the adhesion phase,
210 the discs were transferred into a new plate as described before in the co-incubation section.

211 In the case of *S. aureus*, discs were incubated with 1 mL of the bacterial suspension at the
212 concentration of 1×10^7 CFU/mL at 37°C for 24 h, whilst for *P. aeruginosa*, discs were
213 incubated with 1 mL of the bacterial suspension at the concentration of 1×10^6 CFU/mL at
214 37°C and 140 rpm for 24 h.

215 The anti-adhesion and anti-biofilm activity of SLs-coated discs were evaluated respectively
216 after 1.5 h and 24 h using the previously described CV staining method. Assays were carried
217 out in triplicate and experiments were repeated two times (n=6).

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219 2.6 SEM Analysis

220 The effect of SLA, SL18 and SLV on cells of *Candida albicans* IHEM 2894, *Staphylococcus*
221 *aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 10145 were evaluated through
222 SEM according to the method described by Ceresa et al., 2015. Each disc was washed three
223 times in PBS, fixed in a 2.5% glutaraldehyde solution for 24 h at 4°C, washed twice in
224 distilled water, dehydrated and dried overnight. SEM analyses were conducted in a FEI
225 QUANTA 200 with a variable range 1-30 KV beam voltage.

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227 **2.7 Statistical Analysis**

228 Statistical analysis was carried out by means of the statistical program R (R Development
229 Core Team, <http://www.R-project.org>). ANOVA followed by Tukey's HSD post-test was
230 used to compare the effect of different SLA, SL18 and SLV concentrations against *C.*
231 *albicans* IHEM 2894, *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 6538 biofilm
232 formation and pre-formed biofilm in comparison with positive growth controls.

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252 **3. RESULTS AND DISCUSSION**

253 **3.1 Fermentation process: *Sophorolipids* production**

254 *Candida bombicola* ATCC 22214 was able to produce sophorolipids under aerobic
255 conditions, on GYU medium at 30°C using oleic acid (as a second carbon source) at different
256 concentrations after 120 h. The production of sophorolipids starts when the yeast cells enter
257 in stationary phase once they have been triggered by a high carbon/nitrogen ratio (Davila et
258 al., 1992). Typically, growth rate is dependent upon the hydrophilic substrate used; in our
259 study, glucose is the hydrophilic substrate of choice whilst oleic acid was chosen as the
260 hydrophobic substrate for sophorolipid synthesis.

261

262 Different studies have shown that the use of a second lipidic carbon source helps to increase
263 the production yield of sophorolipids. Particularly favourable sources include rapeseed oil
264 and most vegetable oils that are rich in C16-18 fatty acids; these carbon sources are more
265 favourable to renewable production practices, readily incorporated into the sophorolipid
266 molecule, and add an additional control over molecular diversity (Saerens et al., 2015;
267 Delbeke et al., 2016). During SL biosynthesis, the enzyme CYP52M1 catalyses oxygenation
268 of the fatty acids. The enzyme largely determines the length of the fatty acid chain within the
269 molecule and has a high specificity towards stearic acid (18 carbons, 0 double bonds, C18:0)
270 and oleic acid (C18:1) which are then mirrored in the fatty acid model of the sophorolipid
271 molecule. However, the sophorolipids produced by *C. bombicola* are not typically pure
272 compounds but consist of a mixture of molecules with variations in molecular weights, chain
273 length, position of hydroxylation and differences in the saturation of the fatty acid chain (Van
274 Bogaert et al., 2007). The organism has a preference to produce lactonic congeners of the
275 SLs, however they are typically produced as a mixture of different congeners with two major
276 points of variation: acetylation in the sophorose moiety, and lactonisation (Costa et al., 2018).

277 The achievement of such congeners is particularly important when considering the potential
 278 therapeutic applications, since acidic and lactonic SLs have been demonstrated to have
 279 different physicochemical and biological activities. Lactonic SLs for example show higher
 280 antimicrobial, virucide, and anti-cancer activity (Shao et al., 2012), whereas acidic SLs show
 281 higher spermicidal and proinflammatory activity (Shah et al., 2005). The predominance for
 282 the production of the acidic or lactonized form is mostly dependant on the tendency of the
 283 metabolic route, which is affected by the fermentation time and hydrophobic substrate used
 284 (Daniel et al., 1998). In this study, changing the concentration of oleic acid and varying the
 285 fermentation process in terms of time had an effect on the production of different congeners
 286 (Fig. 1).

287
 288 ESI-MS analysis of each purified product was carried out, Fig. 1A revealed the presence of a
 289 sophorolipid congener produced by *C. bombicola* ATCC 22214 when grown in 2% v/v oleic
 290 acid. A dominant peak in the ESI-MS showed a pseudomolecular ion of m/z 621-622 (Fig.
 291 1A), corresponding to a nonacetylated C18:0 SL.

292
 293 **Table 1.** Identification of sophorolipid analogs based on m/z peaks in negative mode [M- H⁺]

SL structural forms	m/z [M- H ⁺]
Nonacetylated SL of C18:0, acidic form	623
Diacetylated SL of C16:0, lactonic form	661
Monoacetylated SL of C18:1, acidic form	663
Diacetylated SL of C18:2, lactonic form	685
Diacetylated SL of C18:1, acidic form	705

294
 295 This form has previously been reported (Kasturi and Prabhune, 2013) and it is one of the
 296 acidic congeners; for this study, it has been denominated as SLA for all antimicrobial
 297 experiments carried out. The presence of the different congeners was observed when the

298 concentration of oleic acid was changed to 1.5% and 4% v/v. Different peaks were detected
299 (Fig. 1b) corresponding to different acidic and lactonic congeners (Table 1) (Fig. 1b). For all
300 the antimicrobial experiments, the product that contains a mixture of acidic and lactonic
301 congeners was called SLV. The purified product that mainly consisted of the lactonic form of
302 sophorolipids, on the other hand was designated as SL18.

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304

305 **3.2 The effect of acidic SLA, lactonic SL18 and mixed SLV sophorolipids on pre-formed** 306 **biofilms on medical-grade silicone elastomeric discs.**

307 The ability of SLA, SL18 and SLV to disrupt biofilms formed by either *C. albicans*, *S.*
308 *aureus* and *P. aeruginosa* was tested through the MTT assay. Optical densities at 570 nm of
309 each microorganism vs individual biosurfactant concentrations are shown in Fig. 2. All
310 strains tested showed the ability to form biofilms on medical-grade silicone discs under the
311 conditions described here. The combined effect of all biosurfactants used (concentrations
312 above 0.1% w/v) on the disruption of *S. aureus* and *P. aeruginosa* biofilms was significant.
313 An average of 75% lower metabolic activity was estimated through the MTT assay, using
314 NAD (P) H-dependent cellular oxidoreductase enzyme (under the conditions used in this
315 study) as a reflection of the number of viable cells present (Berridge et al., 2005). However,
316 analysis of the SEM images revealed a less significant effect, indicating that the biofilm
317 structure was preserved post-treatment (data not shown).

318

319 The Gram-positive microorganism *S. aureus* can produce a multi-layered biofilm matrix
320 representing subpopulations of bacteria embedded within a glycocalyx (Archer et al., 2011).
321 Infections caused by methicillin resistant *S. aureus* (MRSA) are a serious problem with a
322 high occurrence in hospital inpatients and healthcare professionals. Some anti-staphylococcal

323 antibiotics are available, although the treatment options for MRSA infections remain limited
324 due to the increasing occurrence of antibiotic resistant phenotypes (Samadi et al., 2012).
325 Additional environmental factors such as the selective pressures within the distinct matrix
326 layers have been shown to further encourage resistance (Xu et al., 2000; Singh et al., 2010)
327 highlighting the importance of developing effective biofilm disruptor therapies. The thickness
328 of the biofilms formed by these microorganisms are expected to be more than 400µm
329 (Costerton et al., 1995), which could be the main reason why disruption mediated by
330 sophorolipids was not evident through SEM, the 3D structure of the biofilm were still visible
331 but the cells that formed this structure were metabolically compromised as indicated by the
332 MTT assay (Fig. 2A). Such bactericidal effect of sophorolipids on mixed cultures of *B.*
333 *subtilis* and *Staphylococcus aureus* has been reported before (Diaz De Rienzo et al., 2015),
334 which gives an added value to these molecules for potential biomedical applications.

335

336 *P. aeruginosa* can form biofilms in different environments and it is the responsible for many
337 acute and chronical infections, plus it is one of the major nosocomial pathogens in patients
338 with cystic fibrosis (Chen et al., 2018). The clinical relevance and the relative ease of biofilm
339 growth has made *P. aeruginosa* a model organism on biofilm formation studies (Maurice et
340 al., 2018). There have been progress on the development of new treatments for biofilm
341 infections produced by *P. aeruginosa* which involves the use of cationic antimicrobial
342 peptides which are found naturally in a wide variety of organisms and constitute a major
343 component of the innate immune system (Beaudoin, et al., 2018), glycoclusters (Boukerb, et.
344 Al, 2014), plant extracts (Zameer, et al., 2016), biosurfactants (Diaz De Rienzo et al., 2016)
345 among others. The treatment of the *P. aeruginosa* ATCC 10145 biofilms with SLV was the
346 most effective with a disruption about 75% (Fig. 2B) on medical-grade silicone discs. SLV is
347 a mixed of lactonic and acidic isomers, and this results confirm those showed before where

348 sophorolipids from Ecover® had a bactericidal effect against cells of *P. aeruginosa* ATCC
349 15442 within a period on 30 min (Diaz De Rienzo et al., 2016).

350

351 *C. albicans* usually produces biofilms composed of multiple cell types (i.e., round, budding
352 yeast-form cells; oval pseudohyphal cells; and elongated, cylindrical hyphal cells) encased in
353 an extracellular matrix (Chandra et al., 2001; Dominic et al., 2007). These microorganisms
354 are responsible for at least 15% of the total sepsis cases acquired within a clinical setting,
355 moreover, their occurrence accounts for the fourth most common determinant of bloodstream
356 infections in clinical settings, and the predominant fungal species isolated from medical
357 device infections (Wenzel, 1995; Wisplighoff, 2004), therefore highlighting the importance
358 of the disruption on medical-grade silicone discs.

359

360 The disruptive effects of SLA, SLV (at all the concentrations tested) and SL18 (at
361 concentrations above 0.1% w/v) on *C. albicans* IHEM 2894 biofilms showed 80% inhibition
362 (evaluated as an indirect measure of the metabolic activity) (Fig. 2C). To our knowledge, this
363 study is the first reporting sophorolipids as antimicrobial disruptors of *C. albicans* biofilms.
364 The recent emergence of lipopeptide biosurfactants as a new generation of agents with anti-
365 adhesive and antimicrobial properties with enhanced biocompatibility provide potential
366 commercial applications in the pharmaceutical and biomedical fields (Cameotra and Makkar,
367 2004; Fracchia et al., 2015; Ceresa et al., 2016). This work shows the potential use of lactonic
368 sophorolipids as disruptive agents at concentrations as low as 0.05% w/v.

369

370

371 **3.3 Antimicrobial properties of SLA, SL18 and SLV on *C. albicans*, *P. aeruginosa* and *S.***
372 ***aureus***

373 The antimicrobial effect of SLA, SL18 and SLV (at different concentrations) on all the
374 microbial strains were evaluated under co-incubation experimental conditions (Fig. 3). All
375 the treatments resulted in a significant reduction of the total adherent cells and biofilm
376 biomass from *C. albicans* and *S. aureus* compared to the controls, whilst no effect was
377 detected against cells of *P. aeruginosa* (data not shown). SLA (at 0.05% and 0.1% w/v)
378 showed the highest impact in preventing the attachment of both *S. aureus* and *C. albicans*
379 cells, although lactonic SLs have been reported to have better surface tension lowering and
380 antimicrobial activity as compared to the acidic form (de Oliveira et al., 2015). Under the
381 conditions of the present study, the acidic form displayed superior antimicrobial activity. The
382 findings presented here are thought to be mainly due to the hydrophilic properties of the SLs
383 in solution enabling the formation of smaller globular micelles, which therefore interact more
384 closely with the microbial cells.

385
386 The antimicrobial effect of sophorolipids on *S. aureus* cells have been reported before (Diaz
387 De Rienzo et al., 2015) where sophorolipids (a congeners mix) at 5% v/v induced disruption
388 on mature maximal biofilms of *B. subtilis* BBK006 as well as a mixed culture containing *B.*
389 *subtilis* BBK006 and *S. aureus*. In both cases, the cells exhibited an outpouring of
390 cytoplasmic contents due to the presence of the intracellular enzyme malate dehydrogenase,
391 indicating the interaction of sophorolipids with the cellular membrane increasing
392 permeability (Dengle-Pulate et al., 2014). In this study, the concentration used was 50 times
393 lower (0.05% w/v) and the inhibition on the biofilm biomass was up to 90% with no visible
394 cytoplasmic content (Fig 3A).

395
396 To our knowledge, there is no report on the antimicrobial effect of sophorolipids on the
397 inhibition on the biofilm biomass of *C. albicans* when co-incubated with concentrations

398 between 0.025-0.1% w/v of sophorolipids concentration. In this study, the experiments were
399 carried out at two different times: 1.5 h and 24 h (fig. 3B and 3C respectively). In general, the
400 highest reduction in the cell attachment (>95%) was achieved after 24h of incubation.
401 Different studies showed the effect of different biosurfactants against *C. albicans* biofilms
402 (Ceresa et al., 2016), where the effect of a lipopeptide AC7 BS (0.5-3 mg/mL) was evaluated
403 on *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894, resulting in a significant
404 reduction of the total adherent cells and biofilm biomass (with a maximum inhibition of 68%
405 at 2mg/ml). Additionally, the influence of lipopeptides from *Bacillus amyloliquefaciens* strain
406 on polystyrene plates was shown to inhibit *C. albicans* biofilm formation between 46-100%,
407 depending on the concentration and on *Candida* strains (Rautela et al., 2014).

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409 **3.4 Anti-adhesive properties of SL18 on biofilms formed by *C. albicans*, *P. aeruginosa*** 410 **and *S. aureus***

411 The anti-adhesive properties of SL18 were tested on cells of *S. aureus*, *P. aeruginosa* (after
412 24h) and *C. albicans* (at 1.5 h and after 24 h). Concentrations from 0.2-0.8% w/v were tested,
413 and total biofilm biomass was quantified (Fig. 4). Pre-coating experiments revealed the
414 biofilm formation and adhesion properties of *S. aureus* and *C. albicans* were progressively
415 reduced as a function of increased SL18 concentrations, with SL18 0.8% showing the
416 greatest inhibitory effect towards cell attachment to the silicone discs. On the other hand,
417 under the same conditions no anti-adhesive effect was shown on cells of *P. aeruginosa*.
418 Biosurfactants can disrupt phospholipid membranes and affect the cell-to-cell surface
419 interactions by decreasing hydrophobicity and interfering with the cell deposition and
420 microbial adhesion processes (Rodrigues et al., 2006).

421 Certain structural analogues of SLs have been shown in previous studies to inhibit conidia
422 germination in the fungus *Glomerella cingulata* (Kitamoto and Isoda, 2002). SLs have also

423 demonstrated an inhibitory effect on the growth of some Gram-positive bacteria, which
424 include *B.acillus subtilis*, *Micrococcus luteus*, *Neisseria mucosa* and *Mycobacterium rubrum*
425 (Elshikh et al., 2017) and *Streptococcus oralis*, as well as Gram-negative bacteria including
426 *Escherichia coli*, *Serratia marcescens* (de Oliveira et al., 2015) when deposited onto
427 polystyrene surfaces. Antimicrobial activity of lactonic sophorolipids (98% lactonic SL
428 mixture composed of C18:1 and C18:0) was previously reported for action against
429 *Propionibacterium acnes*, and demonstrates inhibitory action at 2.4 mg/ml on films of pectin-
430 and alginate-based SL composites (Ashby et al., 2011).

431

432 In this study, *S. aureus* ATCC 6538 cells were incubated for 24h (Fig. 4A), and a 75%
433 inhibition on the cell attachment was visible when SL18 (0.8% w/v) was used, in comparison
434 with the controls where the silicone discs were not pre-treated using biosurfactants. Previous
435 studies have shown pre-treatment of catheters using minocycline and rifampin significantly
436 decreases the incidence of central line-associated bloodstream infections caused by *S. aureus*
437 in a medical intensive care unit in a manner that was independent and complimentary to
438 precautionary measures for infection control (Ramos et al., 2011). However, this is the first
439 time that pre-treatment of medical grade silicone discs with SL18 has shown a high
440 percentage of inhibition after 24h incubation.

441

442 In the assays with *C. albicans*, analysis was carried out at 1.5 and 24 h incubation (Fig. 4B
443 and 4C). At 1.5 h the *C. albicans* cells were in the initial phase of adhesion and the yeast cell
444 counts were very low compared with the 24 h incubation (as can be seen through the SEM
445 images). After 1.5 h the inhibition was in the range of 45-56% whilst after 24 h the
446 percentage of inhibition on the cell attachment increased (using 0.8% w/v) was in the range
447 of 68-70%. These results are in contrast to previous reports (Ceresa et al., 2016), where the
448 medical silicone discs treated with 2 mg/mL the lipopeptide biosurfactant AC7 BS were able

449 to significantly reduced the cell attachment (*C. albicans*) at a range of 57.7–62.0 % at 1.5 h
450 and in a range of 45.9–47.6 % after 24 h of incubation. This is a clear indication that the
451 inhibition at different stages depends on the disc treatment, referring to the nature of the
452 antimicrobial agent.

453

454 **4. CONCLUSIONS**

455 Our results indicate that sophorolipids (acidic, lactonic or mixed congener form) are able to
456 reduce the biofilm biomass that is able to form 3D mature films on medical grade silicone
457 discs under the conditions tested in this study. These results also display strong anti-adhesive
458 properties with up to 75% inhibition in the pre-treated group. However, further investigations
459 are needed to explore the effects of lower concentrations, as well as studies of cytotoxicity to
460 be able to extend the use of sophorolipids as antimicrobial molecules with commercial impact
461 in different biotechnology fields.

462

463

464 **ACKNOWLEDGMENTS**

465 The authors acknowledge the assistance of Dr Lakshmi Tripathi (University of Ulster), with
466 the ESI-MS experiments; MSc Marta Lajarin-Cuesta (Liverpool John Moores University)
467 with the sophorolipids production and Erica Tambone with the biofilm's experiments. We
468 also acknowledge the funding support from the Faculty of Science, Liverpool John Moores
469 University ECR Fellowship 2017-2018, the support of the Compagnia di San Paolo
470 (Excellent Young PI-2014 Call), and the support of the Università degli Studi del Piemonte
471 Orientale through their Research Fellowship (Bando Fondazione CRT, Id. 393).

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738 **FIGURE CAPTIONS**

739

740 **Figure 1.** A. ESI-MS analysis of SLA. Spectrum of partially purified extracts from fermented cells of *C.*
741 *bombicola*. Oleic acid 2% was induced after 48 h and 120 h. B. ESI-MS analysis of SLV. Spectrum of partially
742 purified extracts from fermented cells of *C. bombicola* . Oleic acid 4% was induced once after 48 h.

743

744 **Figure 2.** Sophorolipids activity against *S. aureus* 24 h pre-formed biofilm on medical grade silicone discs (A),
745 *P. aeruginosa* 24 h pre-formed biofilm (B) and *C. albicans* 24 h pre-formed biofilm (C) evaluated by the MTT
746 assay. Three different products were used: SLA, SL18 and SLV at different concentrations.

747

748 **Figure 3.** Activity of sophorolipids on co-incubation experiments. Biofilms formed by (A) *S. aureus* 24 h and
749 (B) *C. albicans* 1.5 h and (C) *C. albicans* 24 h evaluated by crystal violet assay. Scanning electron micrographs
750 showed the control cells (left) and treated with SLA 0.1% w/v (right). The magnification bar for image A is 10
751 μm , and for images B and C is 20 μm .

752

753 **Figure 4.** Activity of SL18 adhered to medical grade silicone discs on biofilm formation of (A) *S. aureus* 24 h
754 and (B) *C. albicans* 1.5 h and (C) *C. albicans* 24 h evaluated by crystal violet assay. Scanning electron
755 micrographs showed the control cells and treated with SL18 0.8% (w/v). The magnification bar for image A is
756 10 μm , and for images B and C is 50 μm .