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

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ABSTRACT

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

Keywords

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

1. INTRODUCTION

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

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

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

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

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

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

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

2. MATERIALS AND METHODS

2.1 Microorganisms and media

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

2.2 Production of sophorolipids

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

2.3 Medical-grade silicone elastomeric discs preparation.

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

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

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

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

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

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

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

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

2.5.1 Co-Incubation

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

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

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

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

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

2.5.2 Pre-coating

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

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

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

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

2.6 SEM Analysis

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

2.7 Statistical Analysis

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

3. RESULTS AND DISCUSSION

3.1 Fermentation process: *Sophorolipids production*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. CONCLUSIONS

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

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FIGURE CAPTIONS

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

744 **Figure 2**. Sophorolipids activity against *S. aureus* 24 h pre-fromed biofilm on medical grade silicone discs (A),
745 *P. aeruginosa* 24 h pre-fromed biofilm (B) and *C. albicans* 24 h pre-fromed 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 tretaed 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 tretaed 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.