

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

**Ceresa C<sup>1†</sup>, Fracchia L<sup>1†</sup>, Banat IM<sup>2</sup>, Williams M<sup>3</sup> and Díaz De Rienzo, MA<sup>3</sup>**

1. Department of Pharmaceutical Sciences. Università del Piemonte Orientale "A. Avogadro", Novara, 28100, Italy.

2. School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA Northern Ireland, UK.

3. School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, L3 3AF Liverpool, UK.

\* Corresponding author:

Dr M. A. Diaz De Rienzo

Lecturer in Biotechnology

Pharmacy and Biomolecular Sciences

James Parsons Building 10.05C, Byrom Street, Liverpool, L3 3AF

t: 01512312202

e: [m.a.diaz@ljmu.ac.uk](mailto:m.a.diaz@ljmu.ac.uk)

†These authors equally contributed to this work.

## ABSTRACT

Biosurfactants have recently been shown to have properties as potentially new generation of antiadhesive and antibiofilm agents. Sophorolipids in particular are a relevant biosurfactant molecules known as therapeutic agents. The aim of this study was 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). The effect of SLA, SL18 and SLV were tested against *Staphylococcus aureus* ATCC 6538 and *Candida albicans* IHEM 2894. All three congener mixtures showed a disruption effect ( $> 0.1\%$  w/v) of 70% for *S. aureus* biofilms and 80% on *C. albicans* biofilms. On pre-coated silicone disks, biofilm formation of *S. aureus* was reduced by 75% using SLA 0.8% w/v. After 1.5h the inhibition of *C. albicans* attachment was between 45-56% whilst after 24h incubation the percentage of inhibition for the cell attachment increased to a range between 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 reduced significantly 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 bacterial and yeast infections.

## Keywords

Sophorolipids, *Candida albicans*, *Staphylococcus aureus*, medical-grade silicone

## INTRODUCTION

Surfactants are amphiphilic molecules that are contained in a significant number of products in use daily and therefore are part of all aspects of our daily lives. Their properties make them very useful to many industrial and domestic applications, with a global production exceeding 13 million tonnes per year (Marchant and Banat, 2012). Approximately half of this amount is used in domestic and laundry detergents and as a consequence of their use they ultimately end up in the environment (Van Bogaert et al., 2010). Surfactants of biological origin, biosurfactants, unlike the chemical counterparts, are often synthesized from renewable resources or waste through fermentation processes. Such methods produce compounds with a higher biodegradability and lower eco-toxicity (Van Bogaert et al., 2010). Nowadays the industrial demand of new surfactants that are both effective and eco-friendly is continuously growing. Sophorolipids are obtained from non-pathogenic yeast strains and also stand out for their high production yield (Van Bogaert et al., 2010).

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 microorganisms adhesion step of 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 showing 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 have been shown to affect the

adhesion characteristics of single and mixed cultures (Zezzi do Valle Gomes and Nitsche, 2012). This work aims to target the antimicrobial effect of sophorolipids on microbial strains of clinical relevance: *Candida albicans* and *Staphylococcus aureus*. *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* is a pathogen microorganism responsible for an important number of clinical infections, including bacteraemia, and device-related infections among others (Tong et al., 2015). In this study we aim to investigate the effects of sophorolipids on the disruption of biofilm formation, and their antimicrobial properties against *Candida albicans* IHEM 2894 and *Staphylococcus aureus* ATCC 6538 using medical grade silicone material.

## **MATERIALS AND METHODS**

### **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 GYU: glucose/yeast extract/urea (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 YNBD: Yeast Nitrogen Base broth + 50 mM Dextrose and stored at -80°C until further use. *Staphylococcus aureus* ATCC 6538, was cultivated in Tryptic Soy Broth + 10 g/L glucose and stored at -80°C until further use.

### **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/mins in methanol. A desolvation temperature of 200°C was applied together with a Desolvation Gas Flow (L/hr) of 694 and a capillary voltage of 3000V.

### **Medical-grade silicone elastomeric disks preparation.**

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

### **Antimicrobial susceptibility of *Candida albicans* and *Staphylococcus aureus* biofilm towards sophorolipids.**

*C. albicans* IHEM 2894 biofilm were formed according to Chandra et al., 2008. Fungal cells were suspended in Phosphate Buffered Saline +10% Fetal Bovine Serum and adjusted up to  $1.10^7$  CFU/ml. The discs were inoculated with 1ml of the suspension, after the adhesion phase (1.5h), the disks were moved into a new 24-well plate in the presence of 1ml of YNBD +10% FBS and incubated for 24h at 37°C at 90rpm to promote the biofilm growth phase.

*S. aureus* ATCC 6538 was grown in TSB + 10g/L glucose, and the suspension was adjusted up to a concentration of  $1.10^7$  CFU/ml. Silicone disks were submerged with 1 ml of bacterial suspension and incubated for 24h at 37°C in static conditions as described before.

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 24h at 37°C. The antifungal activity of SLA, SLV and SL18 was evaluated according to the method described by Ceresa et al. 2016. Disks incubated for 48h *C. albicans* IHEM 2894 biofilm and 24h for *S. aureus* ATCC 6538 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% glucose

and 1 $\mu$ M 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, 200  $\mu$ l 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).

### **Disruption properties of sophorolipids towards *Candida albicans* and *Staphylococcus aureus*.**

#### *Co-Incubation*

Silicone disks were submerged in 500  $\mu$ l 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, disks were placed in a new plate containing 1 ml of YNBD + 10% FBS + 0%, 0.025%, 0.05%, 0.1% SLs and incubated for 24h at 37°C and 90rpm.

For *S. aureus* ATCC 6538, silicone disks 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 24h.

The biofilm biomass was quantified by the crystal violet (0.2%) essay. Biofilms were washed three times with PBS, air-dried and coloured for 10 min and the absorbance at 570nm was measured. Assays were carried out in triplicate and the experiments were repeated three times (n=9).

#### *Pre-coating*

Elastomeric disks 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 24h at 37°C and 180rpm.

In the case of *C. albicans*, disks 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 disks were transferred into a new plate as described before in the co-incubation section.

In the case of *S. aureus*, disks were incubated with 1 ml of the bacterial suspension at the concentration of  $1.10^7$  CFU/mL at 37°C for 24h.

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

### **SEM Analysis**

The effect of SLA, SL18 and SLV on cells of *Candida albicans* IHEM 2894 and *Staphylococcus aureus* ATCC 6538 were evaluated through SEM according to the method described by Ceresa et al., 2016. Each disk was washed three times in PBS, fixed in a 2.5% glutaraldehyde solution for 24h 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.

### **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 and *S. aureus* ATCC 6538 biofilm formation and pre-formed biofilm in comparison with positive growth controls.



## RESULTS AND DISCUSSION

### **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 120h. 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 as having 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. 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.

**The effect of acidic SLA, lactonic SL18 and mixed SLV sophorolipids on pre-formed biofilms by *C. albicans* IHEM 2894 and *S. aureus* ATCC 6538 on medical-grade silicone elastomeric disks.**

The ability of SLA, SL18 and SLV to disrupt biofilms formed by either *C. albicans* or *S. aureus* was tested through the MTT assay. Optical densities at 570nm of each microorganism vs individual biosurfactant concentrations are shown in Fig. 2. Both of the 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 on the *S. aureus* biofilms was significant. An average of 70% 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) (Fig. 2). However, analysis of the SEM images revealed a less significant effect, indicating that the biofilm structure was preserved post-treatment (Fig. 3).

The Gram-positive microorganism *S. aureus* is able to 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. Such bactericidal effect of sophorolipids on mixed cultures of *B. subtilis* and *Staphylococcus aureus* have been reported before (Diaz De Rienzo et al., 2015), which gives an added value to these molecules for potential biomedical applications.

*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. 4). This effect was confirmed by SEM images, which showed clear biofilm disruption and lower number of cells attached (Fig. 5). 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.

### **Antimicrobial properties of SLA, SL18 and SLV on *C. albicans* IHEM 2894 and *S. aureus* ATCC 6538**

The antimicrobial effect of SLA, SL18 and SLV (at different concentrations) on both microbial strains were evaluated under co-incubation experimental conditions. All the treatments resulted in a significant reduction of the total adherent cells and biofilm biomass compared to the controls. SLA (at 0.05% and 0.1% w/v) was the treatment that showed the highest impact in preventing the attachment of both *S. aureus* and *C. albicans* cells, although lactonic SLs have been reported to have better surface tension lowering and antimicrobial activity as compared to the acidic form (de Oliveira et al., 2015). Under the conditions of the present study, the acidic form displayed superior antimicrobial activity. The findings presented here are thought to be mainly due to the hydrophilic properties of the SLs in solution enabling the formation of smaller globular micelles, which therefore interact more closely with the microbial cells.

The antimicrobial effect of sophorolipids on *S. aureus* cells have been reported before (Diaz De Rienzo et al., 2015) where sophorolipids (a congeners mix) at 5% v/v induced disruption on mature maximal biofilms of *B. subtilis* BBK006 as well as a mixed culture containing *B. subtilis* BBK006 and *S. aureus* ATCC 9144. In both cases, the cells exhibited an outpouring of cytoplasmic contents due to the presence of the intracellular enzyme malate dehydrogenase, indicating the interaction of sophorolipids with the cellular membrane increasing permeability (Dengle-Pulate et al., 2014). In this study, the concentration used was

50 times lower (0.05% w/v) and the inhibition on the biofilm biomass was up to 90% with no visible cytoplasmic content.

To our knowledge, there is no report on the antimicrobial effect of sophorolipids on the 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: 1.5h and 24h. 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).

#### **Anti-adhesive properties of SL18 on biofilms formed by *Candida albicans* IHEM 2894 and *Staphylococcus aureus* ATCC 6538**

The anti-adhesive properties of SL18 were tested on cells of both *S. aureus* (after 24h) and *C. albicans* (at 1.5h and after 24h). Concentrations from 0.2-0.8% w/v were tested, and total biofilm biomass was quantified (Fig. 6). Pre-coating experiments revealed the biofilm formation and adhesion properties of all microorganisms were progressively reduced as a function of increased SL concentrations, with SL18 showing the greatest inhibitory effect towards cell attachment to the silicone disks. Biosurfactants are able to 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 *Bacillus subtilis*, *Micrococcus luteus*, 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), and *Neisseria mucosa* (Elshikh et al., 2017) when deposited onto polystyrene surfaces. Antimicrobial activity of lactonic sophorolipids (98% lactonic SL mixture composed of C18:1 and C18:0) was previously reported (Ashby et al., 2011) for action against *Propionibacterium acnes*, and demonstrate inhibitory action at 2.4 mg/ml on films of pectin- and alginate-based SL composites.

In this study *S. aureus* ATCC 6538 cells were incubated for 24h (Fig. 6A), and a 75% inhibition on the cell attachment was visible when SL18 (0.8% w/v) was used, in comparison to 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 24h incubation (Fig. 6B and 6C). At 1.5h the *C. albicans* cells were in the initial phase of adhesion and the yeast cell

counts were very low compared with the 24h incubation (as can be seen through the SEM images). After 1.5h the inhibition was in the range of 45-56% whilst after 24h 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 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 24h of incubation. This is a clear indication that the inhibition at different stages depends on the disk treatment, referring to the nature of the antimicrobial agent.

Our results indicate that sophorolipids (in any 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 of extend the use of sophorolipids as antimicrobial molecules with commercial impact in different biotechnology fields.

## **ACKNOWLEDGMENTS**

The authors acknowledge the assistance of Dr Lakshmi Tripathi (University of Ulster), with the ESI-MS experiments; MSs Marta Lajarin-Cuesta (Liverpool John Moores University) with the sophorolipids production and Erica Trambone with the biofilms experiments. We also acknowledge the funding support from the Faculty of Science, Liverpool John Moores University ECR Fellowship 2017-2018 and the support by the Compagnia di San Paolo (Excellent Young PI-2014 Call).



## REFERENCES

Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. 2011. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. Virulence. 2(5), 445-459.

Ashby RD, Zerkowski JA, Solaiman DK, Liu LS. 2011. Biopolymer scaffolds for use in delivering antimicrobial sophorolipids to the acne-causing bacterium *Propionibacterium acnes*. N biotechnol. 28(1), 24-30.

Berridge MV, Herst PM, Tan AS. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev. 11, 127-152.

Cameotra SS, Makkar RS. 2004. Recent applications of bio- surfactants as biological and immunological molecules. Curr Opin Microbiol. 7, 262–266.

Ceresa C, Rinaldi M, Chiono V, Carmagnola I, Allegrone G, Fracchia L. 2016. Lipopeptides from *Bacillus subtilis* AC7 inhibit adhesion and biofilm formation of *Candida albicans* on silicone. Anton Van Leeuw. 109, 1375–1388.

Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol. 183, 5385–5394.

Chandra J, Mukherjee PK, Ghannoum, MA. 2008. In vitro growth and analysis of *Candida* biofilms. Nat Protoc. 3, 1909–1924.

Costa JAV, Treichel H, Santos LO, Martins VG. 2018. Chapter 16 - Solid-State Fermentation for the Production of Biosurfactants and Their Applications. In: Pandey A, Larroche C, Soccol CR, editors. Current Developments in Biotechnology and Bioengineering: Elsevier, pp. 357-72.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. Annu Rev Microbiol. 49, 711–745.

Daniel HJ, Reuss M, Syltatk C. 1998. Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage fed batch process using *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509. Biotechnol Lett. 20(12), 1153-1156.

Davila A, Marchal R, Vandecasteele J. 1992. Kinetics and balance of a fermentation free from product inhibition: sophorose lipid production by *Candida bombicola*. Appl Microbiol Biotechnol. 38(1), 6-11.

de Oliveira MR, Magri A, Baldo C, Camilios-Neto D, Minucelli T, Colabone MA. 2015. Sophorolipids A Promising Biosurfactant and it's Applications. Int J Adv Biotechnol. 6(2), 161-174.

Delbeke E, Movsisyan M, Van Geem K, Stevens C. 2016. Chemical and enzymatic modification of sophorolipids. Green Chem. 18(1), 76-104.

Dengle-Pulate V, Chandorkar P, Bhagwat S, Prabhune AA. 2014. Antimicrobial and SEM studies of sophorolipids synthesized using lauryl alcohol. J Surfact Deterg.17, 543-552.

Diaz De Rienzo, MA, Banat IM, Dolman B, Winterburn J, Martin PJ. 2015. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. N Biotechnol. 32(6), 720-726.

Dominic RM, Shenoy S, Baliga S. 2007. *Candida biofilms* in medical devices: evolving trends. Kathmandu Univ Med J. 5, 431–436.

Elshikh M, Moya-Ramírez I, Moens H, Roelants S, Soetaert W, Marchant R, Banat IM. 2017. Rhamnolipids and lactonic sophorolipids: natural antimicrobial surfactants for oral hygiene. J Appl Microbiol. 23(5),1111-1123.

Fracchia L, Banat JJ, Cavallo M, Ceresa C, Banat IM. 2015. Potential therapeutic applications of microbial surface-active compounds. AIMS Bioeng. 2, 144–162.

Ganguly S, Mitchell A. 2011. Mucosal biofilms of *Candida albicans*. Curr Opin Microbiol. 14(4), 380-385.

Kasturi J, Prabhune A. 2013. A Biosurfactant-Sophorolipid Acts in Synergy with Antibiotics to Enhance Their Efficiency. Biomed Res Int. 1-8.

Kitamoto DH, Isoda T. 2002. Functions and potential applications of glycolipids surfactants -from energy- saving materials to gene delivery carriers. J Biosci Bioeng. 94, 187-201.

Krasowska A. 2010. Biomedical activity of biosurfactants. Postepy Hig Med Dosw. 64, 310–313.

Marchant R, Banat IM. 2012. Biosurfactants: a sustainable replacement for chemical surfactants. Biotechnol Lett. 34, 1597-1605.

Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 20, 133-163.

Ramos ER, Reitzel R, Jiang Y, Hachem RY, Chaftari AM, Chemaly RF, Hackett B, Pravinkumar SE, Nates J, Tarrand JJ, Raad II. 2011. Clinical effectiveness and risk of emerging resistance associated with prolonged use of antibiotic-impregnated catheters: more than 0.5 million catheter days and 7 years of clinical experience. Crit Care Med 39, 245–251.

Rautela R, Singh AK, Shukla A, Cameotra SS. 2014. Lipopeptides from *Bacillus* strain AR2 inhibits biofilm formation by *Candida albicans*. *Antonie Van Leeuwenhoek*. 105:809–821.

Rodrigues L, Banat IM, Teixeira J, Oliveira R. 2006a. Biosurfactants: potential applications in medicine. *J Antimicrob Chemother*. 57, 609–618.

Saerens KM, Van Bogaert IN, Soetaert W. 2015. Characterization of sophorolipid biosynthetic enzymes from *Starmerella bombicola*. *FEMS Yeast Res*. 15(7), fov075.

Samadi N, Abadian N, Ahmadkhaniha R, Amini F, Dalili D, Rastkari N, Safaripour E, Mohseni FA. 2012. Structural characterization and surface activities of biogenic rhamnolipid surfactants from *Pseudomonas aeruginosa* isolate MN1 and synergistic effects against methicillin-resistant *Staphylococcus aureus*. *Folia Microbiol (Praha)*. 57(6), 501-508.

Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim, A, Gross R. 2005. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. *Antimicrob Agents Chemother*. 49(10), 4093-4100.

Shah V, Badia D, Ratsep P. 2007. Sophorolipids having enhanced antibacterial activity. *Antimicrob Agents Chemother*. 51(1):397–400.

Shao L, Song X, Ma X, Li H, Qu Y. 2012. Bioactivities of Sophorolipid with Different Structures Against Human Esophageal Cancer Cells. *J Surg Res*. 173(2), 286-291.

Singh R, Ray P, Das A, Sharma M. 2010. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Antimicrob Chemother. 65(9), 1955-1958.

Smyth TJP, Perfumo A, Marchant R, Banat IM. 2009. Isolation and analysis of low molecular weight microbial glycolipids. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin. pp. 3705–3724.

Tong S, Davis J, Eichenberger E, Holland T, Fowler V. 2015. *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. Clin Microbiol Rev. 28(3): 603–661.

Van Bogaert INA, Saerens K, De Muynck C, Develter D, Soetaert W, Vandamme EJ. 2007. Microbial production and application of sophorolipids. Appl Microbiol Biotechnol. 76(1), 23-34.

Van Bogaert IN, Roelants S, Develter D, Soetaert W. 2010. Sophorolipid production by *Candida bombicola* on oils with a special fatty acid composition and their consequences on cell viability. Biotechnol Lett. 32(10), 1509-1514.

Van Bogaert I, Zhang J, Soetaert W. 2011. Microbial synthesis of sophorolipids. Process Biochem. 46(4), 821-833.

Wenzel RP. 1995. Nosocomial candidemia: risk factors and attributable mortality. Clin Infect Dis. 20, 1531–1534.

Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis. 39, 309–317.

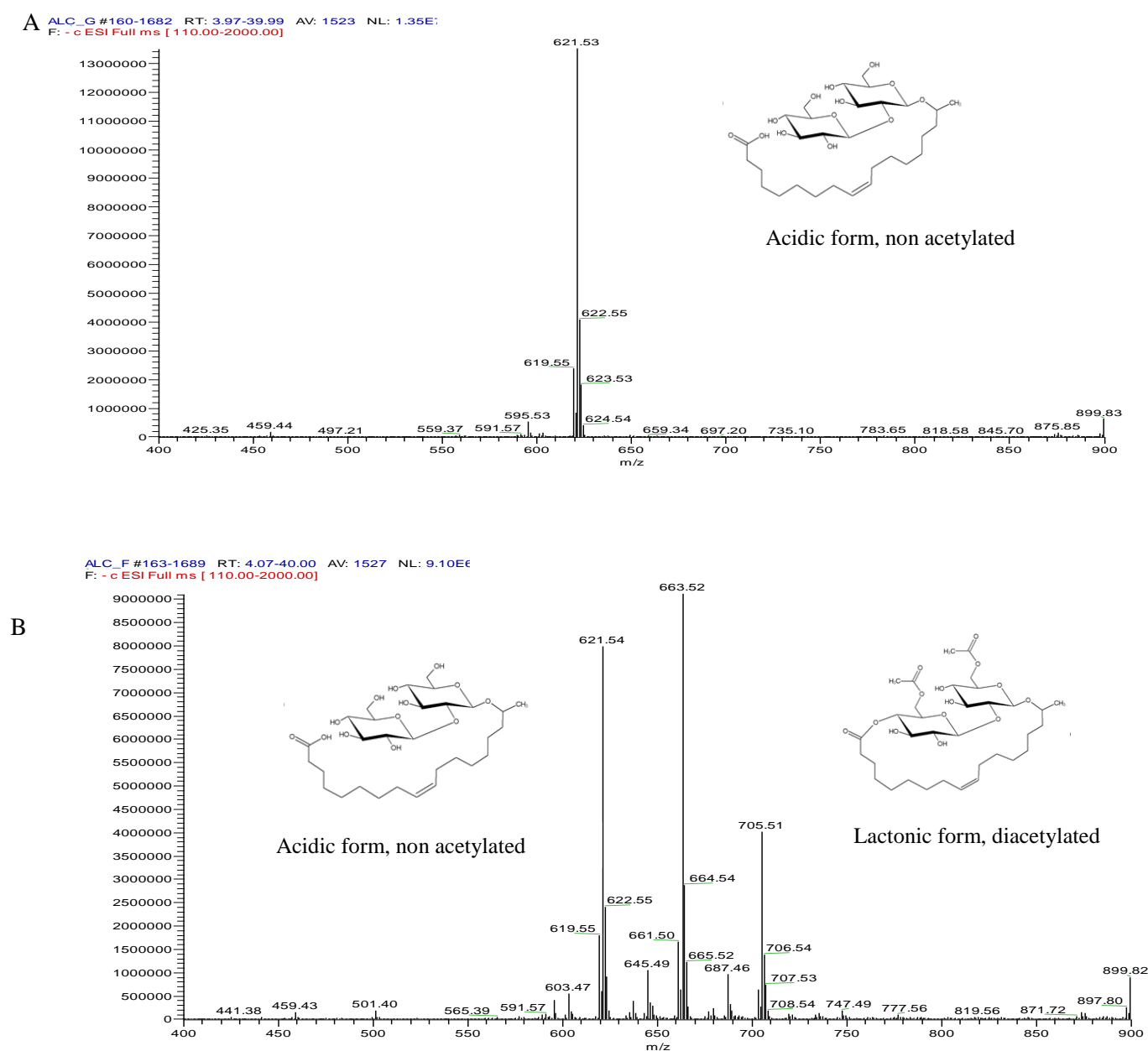
Xu KD, McFeters GA, Stewart PS. 2000. Biofilm resistance to antimicrobial agents. Microbiology. 146(3), 547-549.

Zezi do Valle Gomes M, Nitschke M. 2012. Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. Food Control. 25, 441–447.

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

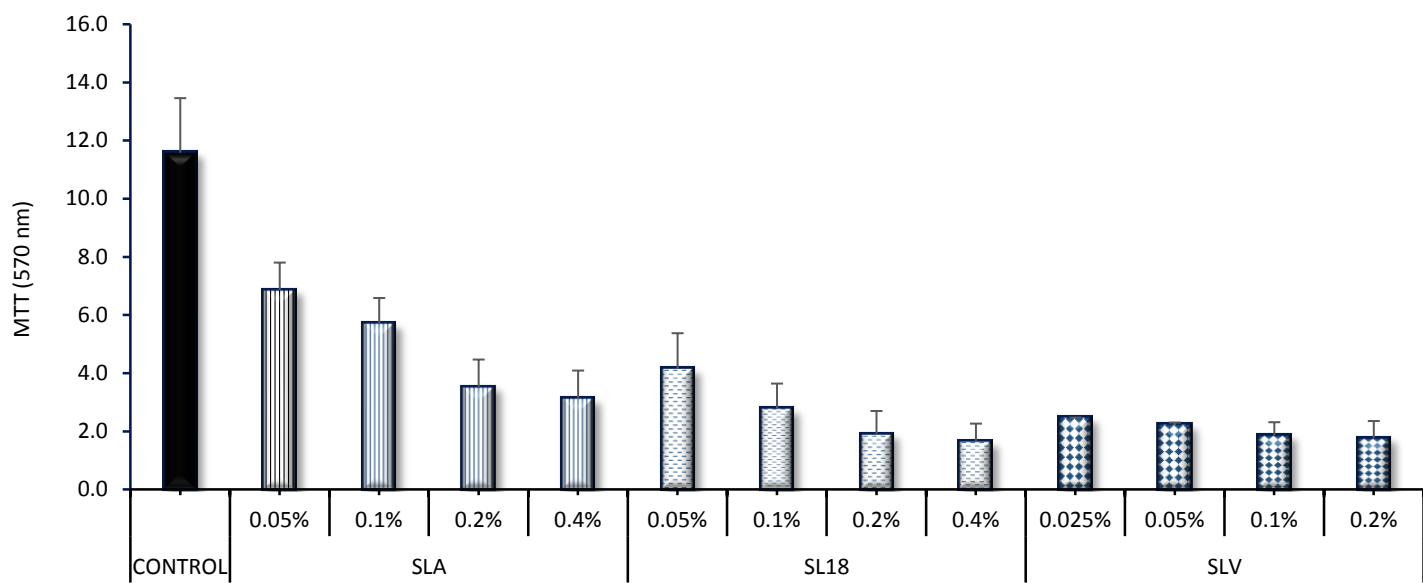
SL structural forms	<i>m/z</i> [M- H <sup>+</sup> ]
Nonacetylated SL of C18:0, acidic form	623
Diacetylated SL of C16:0, lactonic form	661
Monoacetylated SL of C18:1, acidic form	663
Diacetylated SL of C18:2, lactonic form	685
Diacetylated SL of C18:1, acidic form	705





**Figure 1.** A. ESI-MS analysis of SLA. Spectrum of partially purified extracts from fermented cells of *Candida bombicola* ATCC 22214. Oleic acid 2% was induced after 48h and 120h. B. ESI-MS analysis of SLV. Spectrum of partially purified extracts from fermented cells of *Candida bombicola* ATCC 22214. Oleic acid 4% was induced once after 48h.

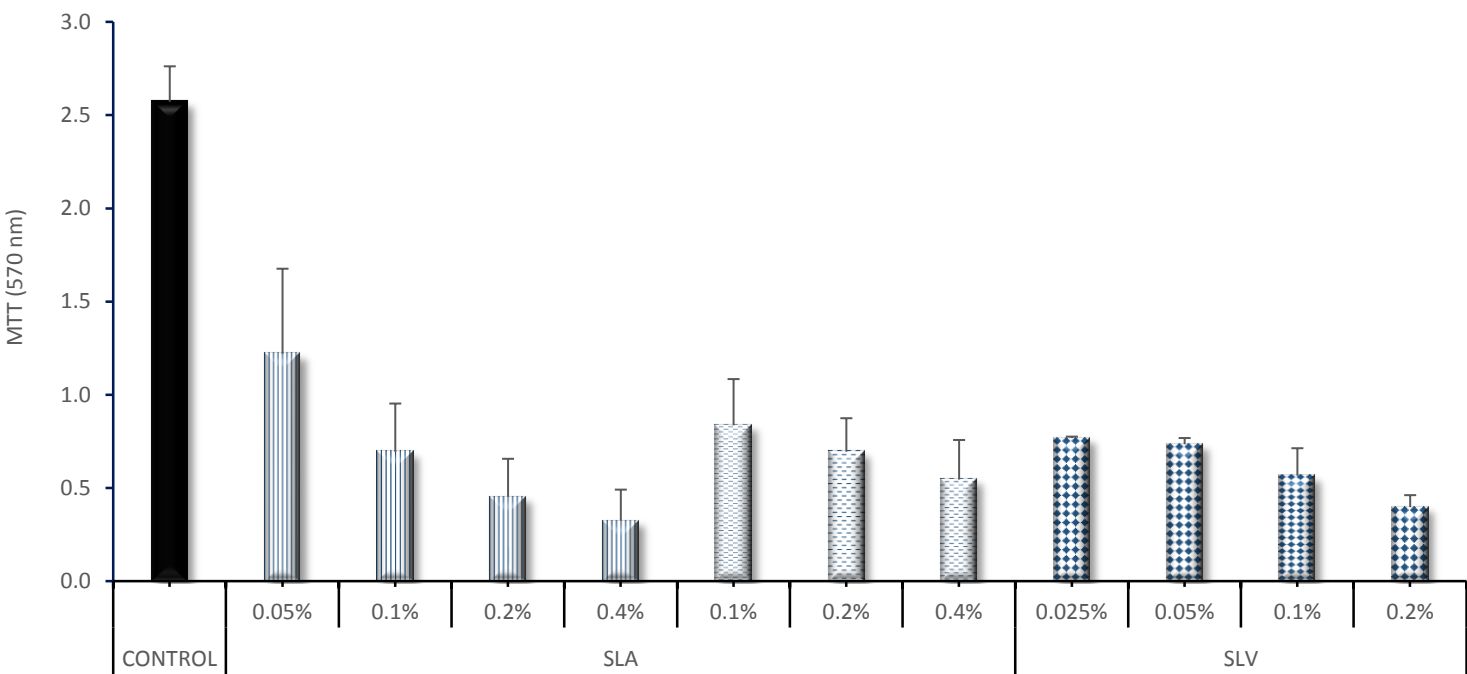
Figure 2



**Figure 2.** Sophorolipids activity against *S. aureus* 24h pre-formed biofilm on medical grade silicone disks, evaluated by the MTT assay. Three different products were used SLA, SL18 and SLV at different concentrations.

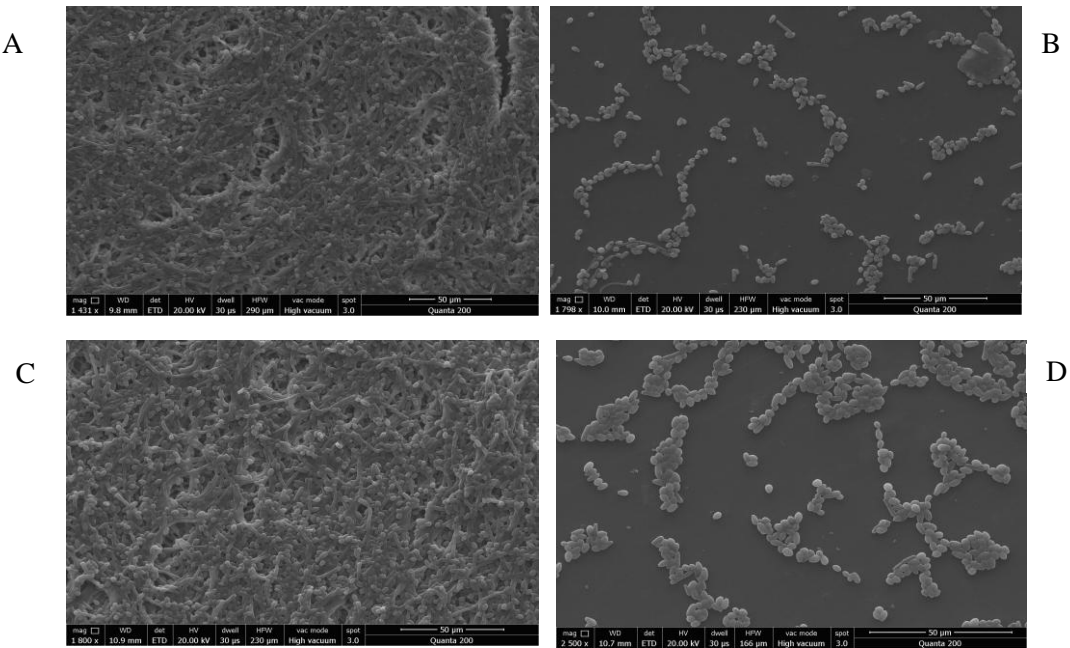


Figure 4



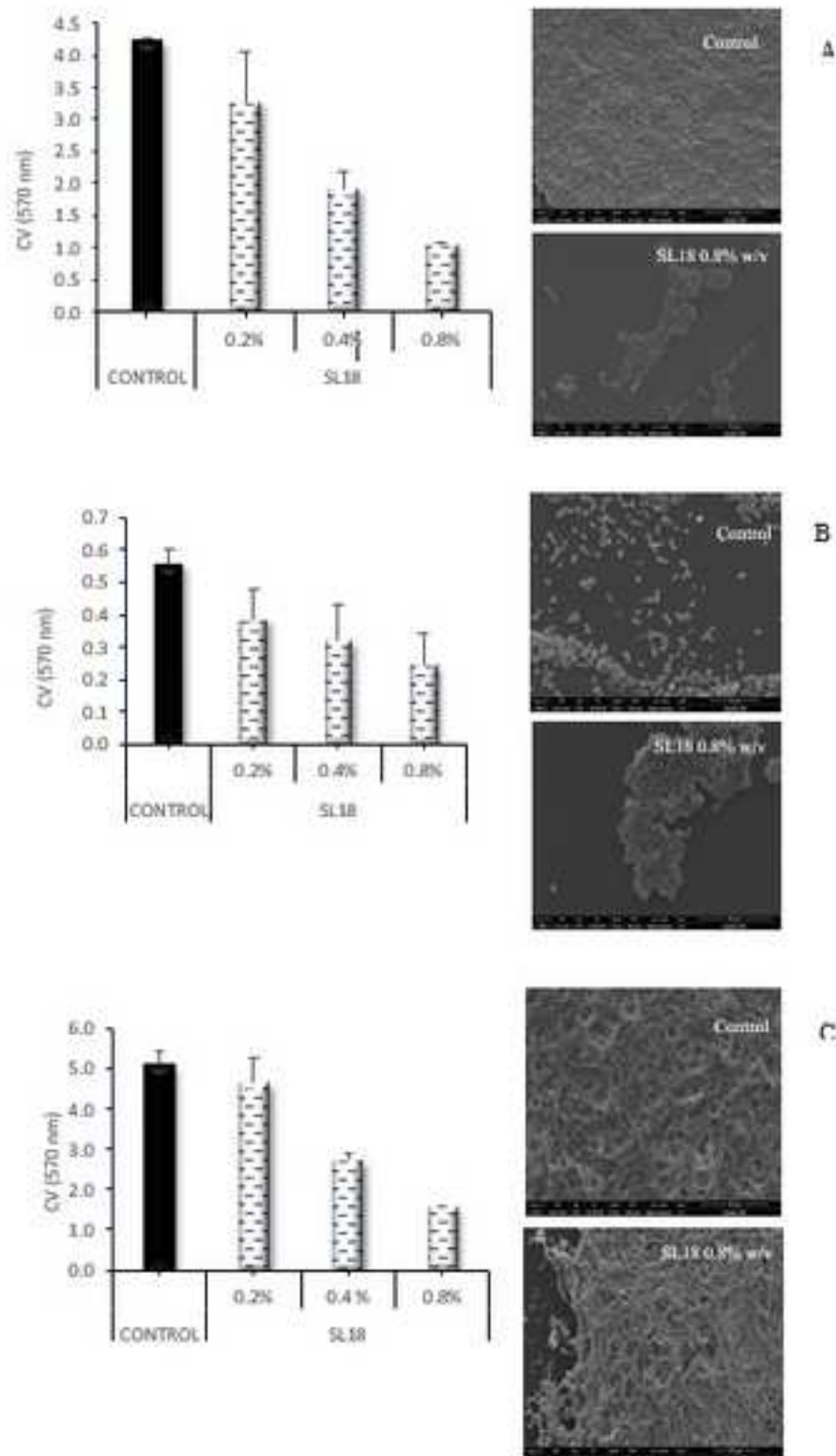
**Figure 4.** Sophorolipids activity against *C. albicans* 24h pre-formed biofilm on medical grade silicone disks, evaluated by the MTT assay. Three different products were used SLA, SL18 and SLV at different concentrations.

Figure 5



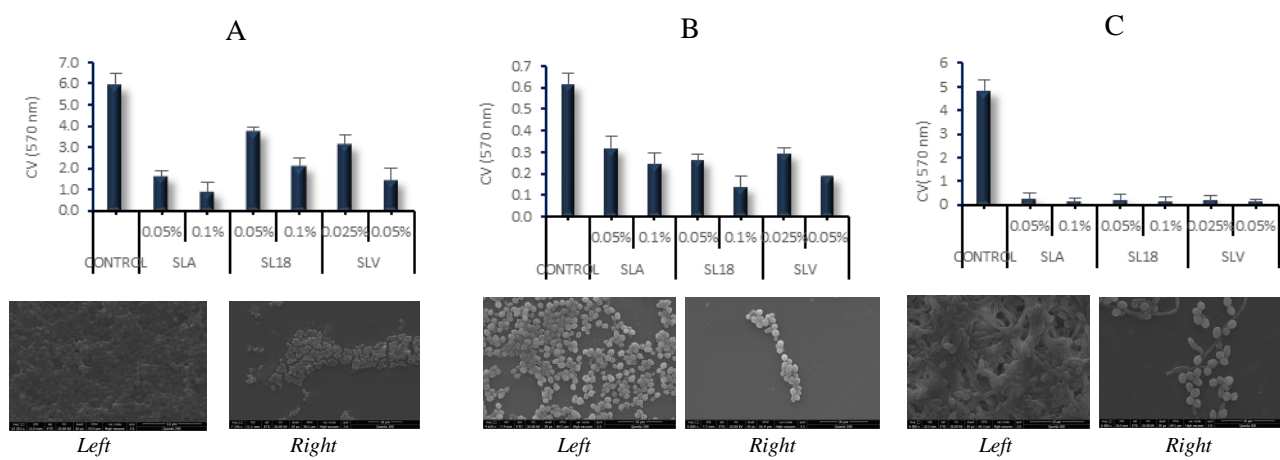
**Figure 5.** Scanning electron micrographs of *C. albicans* 24h pre-formed biofilm after 24h (A), under SLA 0.1% treatment (B), under SL18 0.1% treatment (C) and SLV 0.1% treatment (D).

**Figure 6**  
[Click here to download high resolution image](#)



**Figure 6.** Activity of SL18 adhered to medical grade silicone discs on biofilm formation of (A) *S. aureus* 24h and (B) *C. albicans* 1.5h and (C) *C. albicans* 24h evaluated by crystal violet assay. Scanning electron micrographs showed the control cells and treated with SL18 0.8% (w/v).

Figure 7



**Figure 7.** Activity of sophorolipids on co-incubation experiments. Biofilms formed by (A) *S. aureus* 24h and (B) *C. albicans* 1.5h and (C) *C. albicans* 24 evaluated by crystal violet assay. Scanning electron micrographs showed the control cells (left) and treated with SLA 0.1% w/v (right).