

Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa



Antimicrobial Glycolipids: Evaluation of their Antimicrobial Activity, Antibiofilm Activity and Mechanisms of Action

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Master in **Biochemistry**

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Abstract

Biofilm formation by pathogenic agents on medical devices is a current issue and the available treatment seems ineffective in fighting these infections, therefore new effective molecules are in high demand. Biosurfactants are new promising compounds, that due to their structure can interact with cellular membrane of microorganisms, interfering with the microbial adhesion in the surface of medical devices to prevent these infections.

The aim of this thesis was to evaluate the antimicrobial and antibiofilm activities and antimicrobial mechanism of biosynthesized mixtures of two glycolipids (i.e. rhamnolipids and sophorolipids). These two were immobilized in the surface of silicone by two methods, either by adsorption or covalent bond by plasma activation of the surface, to prevent microbial adhesion.

Initially, antimicrobial activity was evaluated against different planktonic bacteria and *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, clinic *MRSA* and *S. epidermidis* ATCC 28319 were the most vulnerable to the studied glycolipids mixtures.

The mechanism of action of both glycolipids including membrane integrity (by propidium iodide uptake) and cellular viability (by resazurin reduction), cells' surface hydrophobicity and cells' surface charge modifications were studied. Rhamnolipids were the most active on disturbing membrane integrity, while sophorolipids were the most active on disturbing cellular viability. Both glycolipids increased cell surface hydrophobicity more effectively against *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *MRSA* and *P. aeruginosa* ATCC 15442, nevertheless rhamnolipids were more active. Furthermore, both compounds caused a decrease in cell surface charge, however only in a significant way against *S. aureus* ATCC 25923.

Rhamnolipids adsorbed on the surface of silicone caused a decrease of the hydrophobicity of the material. Furthermore, using crystal violet assay and counting of colony forming units' methods, it was verified that both glycolipids previously adsorbed on silicone caused a reduction in biofilm formation of sessile bacteria, most effectively against *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and *MRSA*. However, sophorolipids showed higher activity.

Lastly, plasma treatment on silicone specimens functionalized with rhamnolipids provided good results, showing that this can be a good strategy to create a permanent functionalization to these surfaces.

In conclusion, both glycolipids seem an optimistic approach in preventing biofilm formation on the surface of medical grade silicone, decreasing the risk for the development of these infections.

Keywords: medical device-associated infections, biofilm, rhamnolipids, sophorolipids, silicone, antimicrobial-functionalization.

Resumo

A formação de biofilme por agentes patogénicos em dispositivos médicos é um problema atual e o tratamento disponível é ineficaz no combate destas infeções, portanto, novas moléculas eficazes são necessárias. Biotensoativos são compostos novos promissores que devido à sua estrutura conseguem interagir com a membrana celular dos microorganismos interferindo com a adesão microbiana à superfície dos dispositivos médicos prevenindo o desenvolvimento destas infeções.

O objetivo desta tese foi avaliar a atividade antimicrobiana e antibiofilme, bem como o mecanismo de ação de misturas de dois glicolípidos biossintetizadas (i.e. ramnolípidos e soforolípidos). Estes dois compostos foram imobilizados à superfície do silicone por dois métodos, quer por adsorção quer por ligação covalente através da ativação da superfície com plasma, prevenindo a adesão microbiana.

Inicialmente, a atividade antimicrobiana foi avaliada para diferentes bactérias planctónicas e *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *MRSA* clínico e *S. epidermidis* ATCC 28319 foram identificadas as mais vulneráveis às misturas de glicolípidos.

O mecanismo de ação de ambos, incluiu a avaliação de modificações na integridade da membrana (pela captação de iodeto de propídeo) e viabilidade celular (pela redução de resazurina), hidrofobicidade e carga da superfície celular. Os ramnolípidos foram mais ativos na perturbação da integridade da membrana, enquanto que os soforolípidos foram mais ativos na viabilidade celular. Concluiu-se que ambos aumentaram a hidrofobicidade celular de forma mais eficaz de *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *MRSA* e *P. aeruginosa* ATCC 15442, porém os ramnolípidos foram mais ativos. Ainda, ambos causaram a diminuição da carga celular, mas de forma significativa apenas para *S. aureus* ATCC 25923.

Os ramnolípidos foram usados para revestir a superfície do silicone o que causou uma diminuição da hidrofobicidade do material. Usando dois métodos, violeta de cristal e contagem de unidades formadoras de colónias, verificou-se que ambos os glicolípidos adsorvidos previamente à superfície do silicone causaram uma redução na formação de biofilme de bactérias sésseis de forma mais eficaz para *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 e *MRSA*. No entanto, os soforolípidos apresentaram maior atividade.

Por último, o tratamento com plasma em espécimes de silicone funcionalizados com ramnolípidos deu bons resultados, o que demonstra que esta pode ser uma boa estratégia para criar uma funcionalização permanente destas superfícies.

Concluindo, ambos os glicolípidos demonstraram ser uma abordagem otimista na prevenção da formação de biofilme na superfície de silicone, diminuindo o risco de desenvolvimento destas infeções.

Palavras-chave: infecções associadas aos dispositivos médicos, biofilme, ramnolípidos, soforolípidos, silicone, funcionalização-antimicrobiana.

Index

Acknowledgments	III
Abstract	V
Resumo	VII
Index of figures	XIII
Index of tables	XV
Abbreviations	XVII
1. Introduction	3
1.1. Healthcare-associated infections.....	3
1.2. Why are Healthcare-associated infections an emergent problem?	4
1.2.1. Medical device-associated infections.....	4
1.2.2. The role of Biofilms on medical devices-associated infections	5
1.2.3. Antibiotic Resistance	9
1.3. Strategies to fight medical devices-associated infections	10
1.4. Using Biosurfactants as antimicrobial agents	12
1.4.1. Biosurfactants	12
1.4.2. RLs	13
1.4.3. SLs	14
1.4.4. RLs and SLs Antimicrobial Mechanisms of action	20
2. Thesis Aim and outline	25
3. Materials and Methods	29
3.1. Chemicals	29
3.2. Culture Media	29
3.3. Culture Media Preparation	29
3.4. SLs biosynthesis and extraction	29
3.5. Glycolipids Isolation and Identification	30
3.5.1 Preliminary Glycolipids Mixture Identification	30
3.5.2. Characterization and Identification of Glycolipids Mixture	30
3.6. Antimicrobial Activity of planktonic bacteria	31
3.6.1. Strains and Culture Conditions	31

3.6.2. Minimum Inhibitory Concentration Assay	31
3.6.3. Minimum Bactericidal Concentration Assay	32
3.6.4. Minimum Biofilm Inhibitory Concentration Assay	32
3.7. Antimicrobial mechanism of action of SLs and RLs on planktonic bacteria	32
3.7.1. Membrane Integrity and Cellular Viability	32
3.7.2. Bacterial Surface Hydrophobicity	33
3.7.3. Bacterial Surface Charge	34
3.8. Antibiofilm Activity on PDMS towards sessile bacteria.....	35
3.8.1. Adsorption functionalization with RLs or SLs	35
3.8.2 Contact angle measurements	35
3.8.3. Inoculum Preparation	35
3.8.4. Biofilm formation on glycolipids functionalized PDMS	36
3.8.5. Biofilm cellular viability on glycolipids functionalized PDMS	36
3.9. Plasma Functionalization	37
3.9.1. Functionalization of PDMS with RLs after treatment with plasma	37
3.9.2. Contact angle measurements	37
3.9.3. Fourier transform infrared- attenuated total reflectance	37
3.9.4. Antibiofilm Activity towards sessile bacteria	37
3.10. Statistical Analysis	38
4. Results and Discussion	41
4.1. RLs characterization	41
4.2. SLs biosynthesis and characterization	42
4.3. Antimicrobial activity towards planktonic bacteria	43
4.4. Antimicrobial mechanism of action of SLs and RLs	44
4.4.1. Method Optimization	44
4.4.1.1. Optimization of parameters	45
4.4.1.2. Optimization of measuring times	45
4.4.2. Propidium uptake and Rezasurin reduction	46
4.4.3. Bacterial Surface Hydrophobicity	48
4.4.4. Bacterial Surface Charge	50
4.5. Antimicrobial activity of RLs towards sessile bacteria	51
4.5.1. Surface characterization of PDMS surface by contact angle measurements	51
4.5.2. Biofilm quantification	52
4.5.3. Biofilm cellular viability	54
4.7. Antimicrobial activity of SLs towards sessile bacteria	56

4.7.1. Biofilm quantification	56
4.7.2. Biofilm cellular viability	57
4.8. Plasma treatment and RLs functionalization on PDMS Specimens	59
4.8.1. Characterization of PDMS by Contact Angle Measurements	59
4.8.2. Fourier transform infrared spectroscopy- Attenuated Total Reflectance	61
4.8.3. Cellular Viability towards sessile bacteria	63
5. Conclusions and Future Perspectives	67
6. Bibliography	73

Figure Index

Figure 1.1. Medical-device colonization by microorganisms'	6
Figure 1.2. Quorum Sensing cell-density dependent process	8
Figure 1.3. Chemical structure of mono-SLs (A) and di-RLs (B)	14
Figure 1.4. Chemical structure of acidic (A) and lactonic (B) SLs.....	15
Figure 4.5(A). TLC separation of RLs mixture produced by <i>Pseudomonas aeruginosa</i>	41
Figure 4.5(B). RLs present in the mixture produced by <i>Pseudomonas aeruginosa</i>	41
Figure 4.6(A). TLC separation of SLs mixture produced by <i>Starmerella bombicola</i> supplemented with oleic acid	42
Figure 4.6(B). SLs present in the mixture produced by <i>Starmerella bombicola</i> supplemented with oleic acid	42
Figure 4.7. Contact angle measurements PDMS specimens functionalized with RLs	51
Figure 4.8. Biofilm formation assessed through crystal violet staining on PDMS specimens functionalized with RLs	53
Figure 4.9. Biofilm formation assessed through CFU counting on PDMS functionalized with RLs	54
Figure 4.10. Biofilm formation assessed through crystal violet staining on PDMS functionalized with SLs.....	56
Figure 4.11. Biofilm formation assessed through CFU counting on PDMS specimens functionalized with SLs	57
Figure 4.12. Contact angle measurements performed for 5 (A) and 10 min (B) of plasma treatment on PDMS functionalized with RLs	59

Figure 4.13. FTIR-ATR spectra on PDMS specimens treated with plasma and functionalized with RLs	61
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Figure 4.14. CFU counting of biofilm on PDMS specimens treated with plasma for 5 (A) and 10 min (B) and functionalized with RLs	63
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Table Index

Table 1.1. Most common responsible microorganisms for the main Healthcare-associated infections	4
Table 1.2. Classification of the class of biosurfactants glycolipids, subclasses and producer microorganisms	13
Table 1.3. Antimicrobial activity displayed by RLs	16
Table 1.4. Antimicrobial activity displayed by SLs	18
Table 4.5. Minimum Inhibitory Concentrations, Minimum Bactericidal Concentrations and Minimum Biofilm Inhibitory Concentrations of RLs and SLs.....	43
Table 4.6. PI uptake ratios and Rz reduction percentages obtained for <i>S. aureus</i> ATCC 25923	45
Table 4.7. PI uptake ratios obtained at 30 min, 1 h and 3 h for <i>S. aureus</i> ATCC 25923 and <i>E. coli</i> ATCC 25922	45
Table 4.8. PI uptake ratio and resazurin reduction percentages with RLs and SLs	46
Table 4.9. Bacterial surface hydrophobicity of six strains with RLs and SLs	48
Table 4.10. Bacterial surface charge of six strains with RLs and SLs	50

Abbreviations

ACS	American Chemical Society
AHL	Acetyl homoserine lactone
AI-2	Autoinducer 2
AIP	Autoinducing peptide
AMPs	Antimicrobial peptides
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BSIs	Bloodstream infections
BS	Biosurfactants
CBS	Centraalbureau voor Schimmelcultures
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CSC	Cell Surface Charge
CSH	Cell Surface Hydrophobicity
ECDC	Diseases Prevention and Control European Center
EMA	European Medicines Agency
EPS	Extracellular polymeric substances
FTIR-ATR	Fourier Transform Infrared- Attenuated total reflectance
GPY	Glucose-peptone-yeast
CPYA	Glucose-peptone-yeast agar
HAIs	Healthcare-associated Infections
HPLC-MS	High Performance Liquid chromatography- Mass Spectrometry
ISO	International Organization for Standardization
LPS	Lipopolysaccharide
LRTIs	Lower respiratory tract infections
MBC	Minimum Bactericidal Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MHB	Müller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin- resistant <i>Staphylococcus aureus</i>
MTP	Microtiter plate
OD	Optical Density
PBS	Phosphate Buffered Solution

PDMS	Polydimethylsiloxane
PI	Propidium Iodide
Ph. Eur.	European Pharmacopoeia
QS	Quorum Sensing
QSI	Quorum Sensing Inhibitors
RLs	Rhamnolipids
Ref.	References
Rz	Resazurin
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SLs	Sophorolipids
SSIs	Surgical site infections
SSLA	SLs with lauryl alcohol
TLC	Thin Layer Chromatography
TSA	Tryptic Soy Agar
UTIs	Urinary tract infections
VAP	Ventilator-associated pneumonia
WHO	World Health Organization

✓ Chapter 1: Introduction

1. Introduction

1.1 Healthcare-associated infections

Infections occurring in the Healthcare System are the most common and pose a threat to human health being considered a problematic clinical issue.^{1,2,3} Nosocomial or Healthcare-associated infections (HAIs) are infections acquired by patients in an hospital environment, whilst receiving healthcare for unrelated conditions.^{2,4} The first contact with HAIs was in 1957 and in 1958 a nationwide epidemic of *staphylococcal* infections emerged in hospitals, putting at risk many lives.⁴

Usually, a patient that is hospitalized will be affected by several common adverse effects, such as surgical complications, adverse drug events and HAIs.⁵ However, HAIs are considered the most common complications presented in hospitalized patients and are responsible for an increase in morbidity and mortality of patients, longer hospital stays and increased medical costs.^{4,5}

It has also been reported that HAIs are identified as the top 10 leading causes of death in the USA and the most common complications in hospitals by the Agency for Healthcare Research and Quality. It was identified by the US Centers for Disease Control and Prevention that annually nearly 1.7 million hospitalized patients acquire HAIs after receiving treatment.⁵ In 2018, Centers for Disease and Control Prevention reported that 1 in 15 patients admitted to a European hospital developed at least one HAI, on any given day, which resulted in 98 000 patients infected per year.⁶ In the Hospital prevalence Survey approximately 1 in 31 patients admitted to a hospital has at least one healthcare-associated infection in the USA. Nevertheless, there were estimated 687,000 HAIs in acute hospitals in 2015, and about 72 000 of these patients died during their hospitalizations.⁷

An average of 80 percent of HAIs are divided in four types: bloodstream infections (BSIs), urinary tract infections (UTIs), surgical site infections (SSIs), and lower respiratory tract infections (LRTIs) or ventilator-associated pneumonia (VAP).^{4,5} Despite, HAIs being divided in those four types, these infections can also be characterized according to other topics, such as their costs, mortality rates, frequencies, among others.⁴ Nevertheless, the most frequent are ventilator-associated pneumonia (e.g. ventilator related), urinary tract infections (e.g. urinary catheter related) and bloodstream infections (e.g. intravascular device-related).

In an Annual Epidemiological Report of 2017 by European Centre for Disease Prevention and Control (ECDC)⁸ on European HAIs acquired in intensive care units with was found that 11 787 (8.3%) of the patients being monitored for more than two days developed at least one HAI. Of these patients 6% developed pneumonia, 4% bloodstream infection and 2% urinary tract infections. It was important to note that 90% of pneumonia episodes were ventilator-related,

urinary and bloodstream infections episodes were catheter-related with a prevalence of 98% and 37%, respectively.

Normally, 80 to 87 % of HAIs are caused by 12 to 17 microorganisms, such as: *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus species* (e.g. *E. faecium*, *E. faecalis*), *Candida species* (e.g. *C. albicans*, *C. glabrata*), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and many others.^{5,9} The most common responsible microorganisms for the described Healthcare-associated infections are present as described in **Table 1.1**.¹⁰

Furthermore, almost 70 % of these infections are caused by microorganisms with resistance to one or more antibiotics and one fourth of the patients presenting these infections are being treated in intensive care units.⁴

Table 1.1. Most common responsible microorganisms for the main Healthcare-associated infections (bloodstream infections (BSIs), urinary tract infections (UTIs), surgical site infections (SSIs), and lower respiratory tract infections (LRTIs)).^{9,10}

Healthcare-associated Infections	Medical device	Microorganisms
Urinary Tract Infections (UTIs)	Urinary Catheter	<i>Coagulase-negative staphylococci (CNS)</i> , <i>C. albicans</i> , <i>A. baumannii</i> , <i>P. mirabilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. epidermidis</i> , <i>K. pneumoniae</i> / <i>K. oxytoca</i> , <i>Enterococcus species</i> .
Bloodstream Infections (BSIs)	Central line or Central Venous Catheter	<i>Candida species (C. albicans)</i> , <i>coagulase-negative</i> <i>Staphylococci</i> , <i>S. aureus</i> , <i>S. epidermidis</i> , <i>Enterococcus species</i> , <i>E. coli</i> , <i>P. aeruginosa</i> . <i>Candida</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> ,
Lower respiratory tract Infections (LRTIs)	Ventilator	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>K. pneumoniae</i> / <i>K. oxytoca</i> , <i>P. aeruginosa</i> , <i>Streptococcus species</i> , <i>Stenotrophomonas maltophilia</i> .
Surgical Site Infections (SSIs)	Implant, Prosthesis or Wound	<i>Candida albicans</i> , <i>E. coli</i> , <i>Staphylococcus spp.</i> , <i>MRSA</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. epidermidis</i> , <i>Enterococcus species</i> , <i>E. coli</i> , <i>K. pneumoniae</i> / <i>K. oxytoca</i> , <i>Streptococcus species</i> .

1.2 Why are Healthcare-associated infections an emergent problem?

HAIs are an emergent global issue that is mainly caused by the increase use of medical devices in the medical field, the adherence and colonisation of different bacteria and formation of biofilm in the surface of implanted medical devices and due to the emergence of antibiotic resistance that limits the available treatments.

1.2.1. Medical device-associated infections

Medical device-associated infections are responsible for a large percentage of HAIs, ranging from 60 to 70%, and usually related to the treatment of critically ill patients.^{11,12} Meaning that most patients whilst receiving healthcare, for unrelated reasons, can develop infections associated to the use of medical devices. Therefore, the emergence of these infections was related

to the increase use of these devices, that due to their effectiveness have improved patient's health and quality of life immensely.¹²

Firstly, the implantation of the medical device is normally accompanied by contamination with bacteria, which is almost inevitable despite the efforts to keep sterile by the medical professionals.¹³ The initial colonisation starts with a small number of microorganisms that are present on the skin of the patient itself or healthcare professionals, or even contaminated food or water.⁹ The interface between the medical device and the human tissue is an ideal environment for the initial attachment and colonisation of microorganisms resulting in the growth and formation of biofilm.^{9,12} Furthermore, surface composition and physicochemical properties of medical devices, such as hydrophobicity, also facilitate the adhesion of microorganisms and formation of biofilm promoting the emergence of these infections.^{9,14} Therefore, any foreign object inserted in the human body can potentialize the formation of biofilms, such as intrauterine contraceptive devices, implants, prosthetics, catheters, dental material, contact lenses and much more.^{15,16}

Additionally, medical grade silicone, i.e. polydimethylsiloxane (PDMS), is recognized as the most used material in medical devices, such as catheters.⁹ The common use of this material is due to the high biocompatibility, softness and flexibility that it provides when introduced, causing less damage.¹⁷

These infections are becoming more and more prevalent throughout time and have developed to a worrying problem, concerning not only public health, but also patient's welfare. An effective strategy would be the removal of the medical device and replacement for another, however due to various reasons this is not always possible to accomplish.¹² Therefore, this global crisis of HAIs highlighted the need to research and find a strategy to prevent and control these infections and ensure hospital safety for the patients.^{3,4,5}

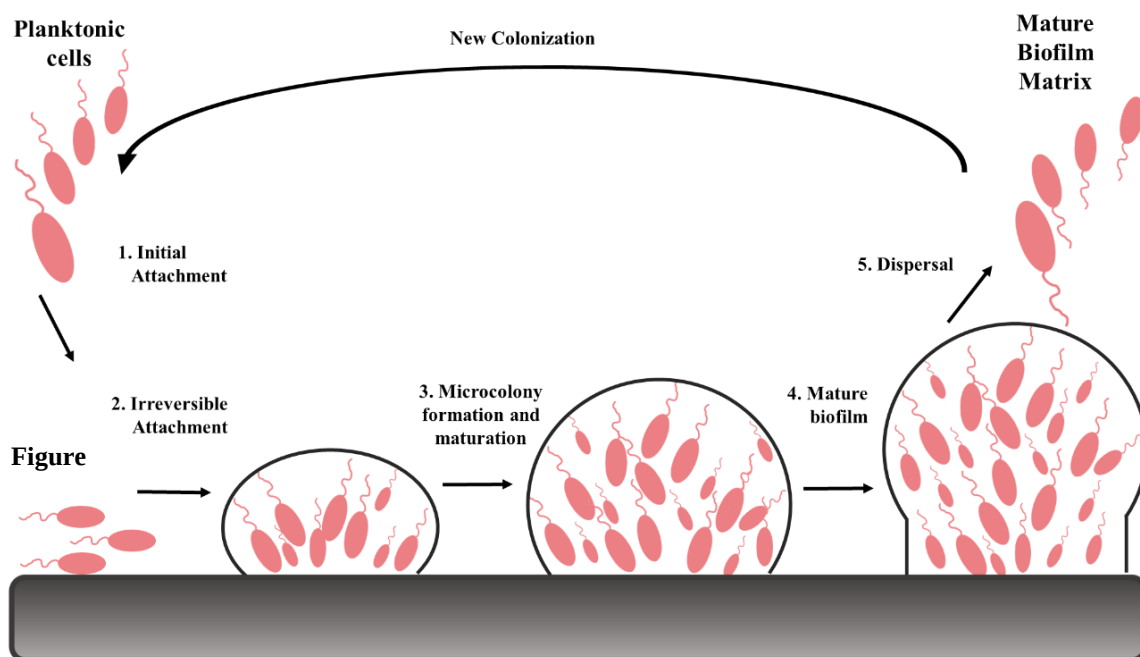
1.2.2. The role of Biofilms on medical devices-associated infections

Microorganisms have existed for millions of years and throughout history have been easily adjusting to their environment by the emergence of new versatile mechanisms for the colonization of surfaces.^{18,19} Research showed that is more likely for bacteria to attach and colonise surfaces than to remain in a planktonic state, because of the advantages that the attachment presents.^{18,20} Consequently, a microbial strategy that microorganisms created for survival was the attachment to a surface and production of biofilm and it is a characteristic found possible in 99% of microorganisms.^{16,21}

Biofilms are significant in the medical field for their participation in the emergence of several bacterial infections due to the microbial adhesion to medical devices, as previously mentioned.^{15,22} Thus, to purpose a newer strategy for the treatment of medical device-associated infections caused by biofilm colonization it is important to first understand the physiology and pathogenesis of biofilms.^{15,23}

Biofilms are described differently in many reports, being that the term itself it is constantly changing and being updated accordingly to new research.^{15,16} Biofilms are characterized as a microbial community composed by different bacterial cells adhered to abiotic and biotic surfaces encased in a self-produced matrix of extracellular polymeric substances.^{16,22}

The formation of biofilm on a surface is a dynamic and cyclical process that includes different stages, such as initiation described as reversible attachment, irreversible attachment, microcolony formation, maturation along with extracellular polymeric substances production, and finally dispersal of individual cells as illustrated in **Figure 1.1**.^{9,16} This process is accompanied by diffusion, transportation, and some chemical reactions.¹⁶



1.1. Medical-device colonization by microorganisms' illustration. The 5 stages of biofilm formation are described 1. reversable initial attachment, 2. irreversible attachment, 3. Microcolony formation and maturation, 4. mature biofilm (with mushroom-like structure) and 5. dispersion of microorganisms.

The first stage is reversible adherence of planktonic bacterial cells to a surface, which normally is assisted by physical forces, such as electrostatic interactions (van der Waals and steric) and by bacterial appendages (pili, fimbriae and flagella).^{9,16} Reports hypothesize that the reversible attachment to a surface is a state of equilibrium between the cells attached and the free-floating cells.⁹ At this initial stage planktonic bacteria are still susceptible to antibiotic treatment,

because planktonic bacteria attached to surfaces reversibly are covered only by a layer of proteins.²⁴

When, this equilibrium is not maintained the next stage is introduced by irreversible attachment.⁹ The attachment to a surface results in the depositing of organic material, that once was suspended, allowing an increase in the local concentration of nutrients.²⁰ This increase in nutrients will promote the growth of bacteria. When bacteria is adhered irreversibly to a surface development of biofilm formation and maturation stage follows after.¹⁶

In this stage, the aggregation of cells will grow, both in density and size, followed by production of extracellular polymeric substances (EPS), resulting in maturation of biofilm.^{15,24} These extracellular substances are excreted, as polysaccharides, lipopolysaccharides, DNA, lipids and proteins that consequently produce a matrix. Depending on the availability of carbon sources or nutrients in the environment the quantity and composition of EPS will differ.²⁵

The cells will become involved by a extracellular polymeric matrix that is self-produced that comprises 50-90 % of the biomass of the biofilm.^{19,22,26} The matrix will hold the aggregation of cells of the biofilm, maintaining the function and structure.^{15,16,19} Consequently, it will protect bacteria from the physical environment, retain water to help with dehydration, enhance the physical integrity of the structure and serve as binding sites for additional bacteria to adhere resulting in the increase of the probability of survival.²⁵ In this stage, the aggregation of cells to produce biofilm can encompass many three-dimensional structures, according to the environment and conditions present.¹⁵ Spherical in static systems or mushroom-like structures in flow cell systems, depending on the availability of oxygen and nutrients.^{15,24} The stage of the mature biofilm demonstrates the maximum resistance to antibiotics.²⁴

The final stage is the biofilm dispersal, which is characterized by dispersal of the cells that once formed the biofilm.⁹ In this stage cells can be relatively static and growing slowly, due to changes in temperature, oxygen levels or nutrients.⁹ Chemical signals can also trigger the dispersal by the presence of other microorganisms in the biofilm.⁹ The cells dispersed from the biofilm can then attach to new surfaces and initiate biofilm growth once more, leading to systemic infections.^{9,27}

The growth and formation of the biofilm itself is accompanied by quorum sensing (QS), which is characterized as the mechanism that allows communication between the cells involved by the EPS matrix.²⁴ QS is a cellular process that allows bacteria to sense other bacteria, when present in a critical number, normally concentration, in a limited space in the environment as illustrated in **Figure 1.2**.^{24,28} So, QS is a cell-density depending process.^{24,25,28}

Besides, allowing bacteria to sense other bacteria, the bacteria are able to respond accordingly as a multicellular organism by synchronizing specific behaviours.^{25,28} QS in the initiation of biofilm formation results in an increase of the production of EPS. In the other stages, such as biofilm growth and maturation, there is a decrease in EPS production.²⁵

QS is implemented by modifying, producing and recognizing signal molecules that modify gene expression and regulate the production of EPS.²⁵ The secretion of these extracellular signalling molecules act as autoinducers.^{25,28}

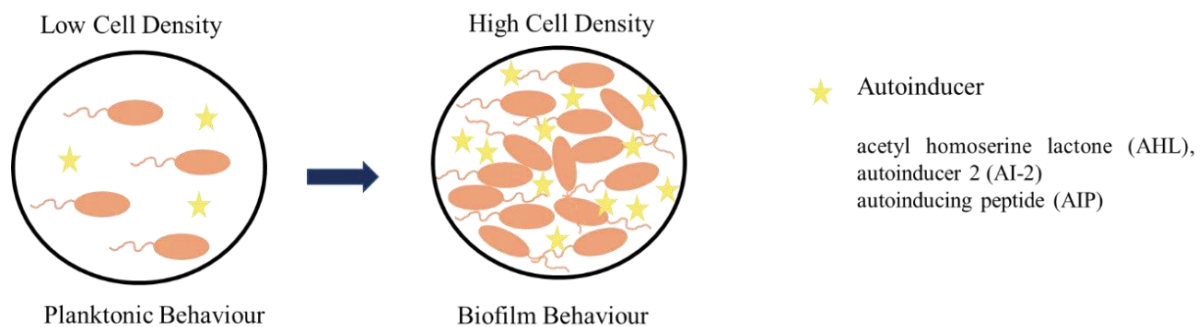


Figure 1.2. Illustration of Quorum Sensing cell-density dependent process and autoinducers that can be present.

In a low cell density environment, the present microorganisms have planktonic behaviour moving freely and producing autoinducers, which diffuse from the cells. However, when high cell density is present microorganisms will aggregate and produce the encased EPS matrix causing a higher amount of autoinducers. These can then bind to receptors within the cells and regulate the transcription of certain genes causing a positive-feedback-loop.

Molecules released that coordinate this process are for instance the acetyl homoserine lactone (AHL) in Gram negative bacteria, the autoinducer 2 (AI-2) in both Gram positive and Gram negative and the autoinducing peptide (AIP) in Gram positive bacteria.²⁸

However, the formation of biofilm is susceptible to failure being influenced by many processes, such as adhesion, mass transport, QS, detachment, cell death and active dispersal.¹⁶

The structure itself and physiological characteristics of biofilm provides protection against antimicrobial agents.¹⁶ The EPS matrix that is formed around the bacterial cells works as a barrier and promotes the protection of attached cells to medical devices.⁹ Furthermore, the matrix will not inhibit diffusion, but hinder antibiotic penetration and the current treatment with antibiotics becomes less effective.^{16,24,29} Therefore, the biofilm presence in these medical devices is one of the reasons device-associated infections are so difficult to treat.

Additionally, biofilm presence in medical devices may cause persistent inflammation and also tissue damage leading to chronic infections, despite antibiotic treatment and the innate and adaptive immune system of the host.^{9,16,24}

The presence and formation of biofilm on medical devices continuous to be a massive health hazard due to the available treatment consisting of antibiotics therapy that fails, in many clinical situations.^{2,15} To fight medical device-associated infections is required to find new molecules that can penetrate biofilms or can prevent growth and formation of biofilms that cause these persistent infections.

1.2.3. Antibiotic Resistance

The discovery of antibiotics has been recognized as an important accomplishment that revolutionized therapeutic medicine and saved human lives.^{30,31,32} This new discovery rapidly shed hope to reduce the impact that infectious diseases have been having in human health for so many years.^{30,33,34} These molecules improved the treatment of infected patients, targeting the microorganisms responsible for these infections, that until then were fatal, without risking the health of the host itself.^{32,35} Consequently, reducing incidences of infection leading to less mortality rates, better quality of life, prolonging life expectancy and saving lives.³¹

However, low priced antibiotics, easily and readily available, sometimes without medical prescription, caused a major overuse and misuse of these new drugs, which resulted in antibiotic resistance.^{30,32,34,36} The pressure felt by microorganisms to survive to these new compounds lead to the gain of this adaptative trait, diminishing the effectiveness of antibiotics.^{30,32,35}

Currently, antibacterial resistance has increased significantly worldwide and is recognized as a global issue.^{35,34,37} Many organizations have declared antibiotic resistance to be a “global public health concern”, such as Diseases Prevention and Control European Center (ECDC), Infectious Diseases Society of America, Centers for Disease Control and Prevention (CDC), the World Health Organization (WHO) and the World Economic Forum.^{30,38} A global action plan with the purpose of fighting the antibiotic resistance issue was requested from The World Health Assembly to WHO.^{30,39} Antibiotic resistance is an emergent issue causing not only morbidity but also mortality worldwide being characterized as a severe global health crisis, causing an economic burden on developed and developing economics.^{30,34,36}

Nowadays, the use of antibiotics is still one of the main strategies of modern medicine to treat infections caused by microorganisms.³⁰ However, the emergence of antibiotic resistance caused the prolonging of the prevalence of these infections and lowered the possibility for a successful treatment. Additionally, if single bacterial cells are already resistant to antibiotics, when under the form of a biofilm, finding an effective treatment can be an additional challenge.⁴⁰

The best solution for this rising concern would be to return to a pre-antibiotic era, but that doesn't seem possible.³⁰ Currently, the investment in the discovery and development of new antibiotics has been stalled for being recognized as a poor economical investment for the pharmaceutical industry.^{31,36} Furthermore, since administration of antibiotics is not an effective long-term treatment for HAIs, due to antibiotic resistance and multi-drug resistance the discovery of new active molecules effective and biocompatible is in high demand.

1.3. Strategies to fight medical devices-associated infections

Currently, the only strategy to treat infections developed by the formation of biofilm in medical devices is the use of antibiotics, apart from surgical intervention when needed or applicable to remove the foreign object.¹⁵ Antibiotics are approved by the EMA (European Medicines Agency) and are still the best strategy to treat these infections. Normally, is advised a higher dosage of antibiotics as a long term treatment, which results in a suppression but not eradication of an infection.¹⁵ However, the antimicrobial drugs available that present high activity and resistance mechanisms against bacteria were developed for planktonic bacteria.¹⁵

Therefore, strategies for the prevention of the formation of biofilm and treatment of pre-existing biofilm in medical devices surfaces are crucial.¹⁵ However, the best approach is always through prevention. Various compounds have already been used, such as antibiotics, disinfectants, silver compounds, ammonium quaternary, antimicrobial peptides, Quorum sensing inhibitors and biosurfactants.^{18,29,41}

The combination of different types of antibiotics seems an effective strategy compared to the used of monotherapies, due to their synergy action.⁴² An example is the combination of a Ciprofloxacin (fluoroquinolone) and Azithromycin (macrolide) that lead to oxidative stress and cellular membrane disruption, respectively.⁴³

The use of antiseptic compounds by itself or in combination with other antimicrobial compounds has also been studied. An example is chlorhexidine-silver sulfadiazine, where the silver ions have bactericidal properties capable of killing Gram positive and Gram negative bacteria and chlorhexidine with antiseptic, disinfectant and biocidal properties.^{18,44,45}

The use of metallic molecules is another important strategy to fight medical devices-associated infections, the most used being silver based molecules. These molecules include silver and silver compounds that have been recognized for their antibacterial properties.^{28,46} Combination strategies using metallic molecules include titanium-doped iron, silver-doped phenyltriethoxysilane, silver-doped inorganic-organic hybrids, silver-doped titanium, silver-doped hydroxyapatite coatings and silver-doped silica films.¹⁸

Silver nanoparticles and other nanoparticles releasing nitric oxide, chlorhexidine and photocatalytic TiO₂ have been developed with bactericidal properties.^{28,47} Nanoparticles are an important strategy to load antibacterial molecules, since these compounds are not capable of maintaining effectiveness, due to elution with the body fluids and time of exposure. Therefore, the use of nanoparticles helps to control, maintain and prolong the release.^{28,47}

Another possible strategy is the use of quaternary ammonium molecules that have been recognized for their antibacterial properties, long-term stability and long-lasting effects.^{18,28} The interaction of positively charged nitrogen in the ammonium group and negatively charged acidic phospholipids present in the membrane cause disruption of both Gram-positive and Gram-negative bacteria.¹⁸ Quaternary ammonium salts and silver compounds have been used as a combinatory effective antibacterial strategy.¹⁸

The use of Antimicrobial peptides (AMPs) have been recognized as promising for the prevention of biofilm formation and represent an increasingly explored research area.⁴⁸ AMPs are small sized peptides (usually positively charged) composed by 15 to 30 amino acids naturally produced by eukaryotes and prokaryotes that possess antimicrobial properties against biofilms.^{18,49} AMPs interact electrostatically with bacteria by attraction with the negatively charged cell membrane resulting in membrane perturbation, creation of pores, attacking cytoplasm, metabolic function of the cells and disintegrating the membrane, leading to membrane disruption, once a minimum concentration is reached.^{18,49}

Other molecules that play a significant role in preventing biofilm formation are Quorum sensing inhibitors (QSI) by interference with the regulatory network of Quorum sensing.^{49,50} QSI interfere with the aggregation of bacteria and encapsulation in the biofilm, reducing bacterial virulence, resulting in phagocytosis and death.^{28,50} QSI are produced either chemically or naturally from fungal or herbal sources and include β -lactam ceftazidime, brominated furanones and cinnamaldehyde analogues that block the transduction cascade, enzymatic degradation (AHL lactonases, AHL acylases, AHL oxidases and reductases) and analogues of AHL.^{15,50}

Recently, Biosurfactants (BS), have gain recognition due to the unique properties they display, such as ability of dispersal, antiadhesive and antimicrobial and antibiofilm activities providing higher effectiveness, when compared to other pre-existent strategies.²⁸ BS are surface active agents that can interact with the hydrophobicity of the surface playing a direct role in the adhesion of the microorganism.²⁸ Therefore, it is important to research further these compounds and understand if they are possible effective candidates for the usage, we are proposing them for.

1.4. Using Biosurfactants as antimicrobial agents

1.4.1. Biosurfactants

Biosurfactants are surface-active compounds produced by microorganisms as secondary metabolites that are important for cellular metabolism, motion and protection with many applications.^{51,52,53} Characterized as an heterogeneous group of amphiphilic compounds, BS, present a characteristic chemical structure that consists of an hydrophilic head and an hydrophobic tail, which interacts with the interfaces between aqueous and nonaqueous systems, including air.^{51,54,55} The structure is responsible for the compounds' properties, such as reduction of surface tension, selectivity, specific activity in extreme environmental conditions, foaming, wetting and emulsification.^{51,52,54}

Additionally, BS present favourable properties when compared to other molecules, such as lower toxicity, higher biocompatibility, higher biodegradability and the possibility of maintaining effectiveness and stability against several environmental conditions, such as temperature, pH and high ionic strength.^{52,54,56,57} These are considered a new greener and environmental-friendly way to produce surfactants and offer an easy fast production, scale-up and more functional properties.^{55,58,59} Due to their unique properties, BS can be used in various applications, such as pharmaceutical, food, agriculture, petroleum and cosmetic industries.^{51,52}

BS are classified based on the producing microorganisms and chemical structure, but can be broadly divided in two groups: high molecular weight (HMW) molecules or polymeric BS (polysaccharides, lipopolysaccharides, and lipoproteins) and low molecular weight (LMW) molecules (glycolipids, lipopeptides and proteins).^{60,61} Nowadays, the low molecular weight glycolipids are recognized as the most promising.⁵⁴

Glycolipids are the most used BS due to the great surface-active properties they possess, such as dispersion, emulsion, foaming, solubilization, wetting and penetration.^{53,62} Glycolipids are composed by monosaccharide residues bound by a glycosidic linkage to a hydrophobic moiety.⁶³ Glycolipids comprise sophorolipids (SLs), rhamnolipids (RLs), trehalose lipids, cellobiose and mannosylerythritol (MELs), which are the most studied.^{53,64} In **Table 1.2.** are presented the most studied and important glycolipids as well as examples of microorganisms responsible for their production.

Table 1.2. Classification of the class of biosurfactants glycolipids, subclasses and producer microorganisms.^{53,55,60}

Glycolipids	Producer Microorganisms
Sophorolipids	<i>Candida bombicola</i> ; <i>Candida magnolia</i> ; <i>Candida apicola</i> ; <i>Candida bogoriensis</i> ; <i>Torulopsis petrophilum</i> ; <i>Wickerhamiella domercqiae</i>
Rhamnolipids	<i>Pseudomonas aeruginosa</i> ; <i>Pseudomonas chlororaphis</i> ; <i>Burkholderia thailandensis</i> ;
Trehalose Lipids	<i>Mycobacterium tuberculosis</i> ; <i>Nocardia sp.</i> <i>Corynebacterium sp.</i> ; <i>Rhodococcus erythropolis</i> <i>Micrococcus luteus</i>
Cellobiose	<i>Cryptococcus humicola</i> ; <i>Pseudozyma fusiformata</i> <i>Pseudozyma graminicola</i>
Mannosylerythritol	<i>Pseudozyma antarctica</i> ; <i>Pseudozyma aphidis</i> <i>Pseudozyma parantarctica</i> ; <i>Pseudozyma rugulosa</i> <i>Ustilago</i>

Despite all the different glycolipids we will be focusing on the two most promising, RLs and SLs, due to the promising and valuable properties that have been reported.⁶⁴

1.4.2. RLs

RLs can be described as a crystalline acid and are produced by many different species of microorganisms, such as fungi, bacteria or yeast.^{57,59} The species responsible for the main production of RLs, meaning higher yield is Gram negative bacteria *Pseudomonas aeruginosa*, when isolated from various environments like soil, plants or water.^{53,57,58} Something to note is that RLs are produced majorly by pathogenic strains, so there is a current need to find non-pathogenic strains that allow easy scale-up and have high production capacity.⁵⁹

Usually, RLs are produced by microorganisms as a mixture of congeners, that vary by the length of the fatty acid chain, degree of unsaturation of the fatty acid chains and differ on the number of rhamnose molecules.^{52,58,65} Generally, *P. aeruginosa* produces 60 congeners and homologues with one or two rhamnose moieties, consisting of mono and di-RLs, but the ratio of both can be controlled during production.^{58,59}

More specifically, the rhamnose moieties are linked through glycosidic bond to an hydrophobic group composed by one or two fatty acids moieties, which are saturated or unsaturated alkyl chains as illustrated in **Figure 1.3**.^{52,65,66}

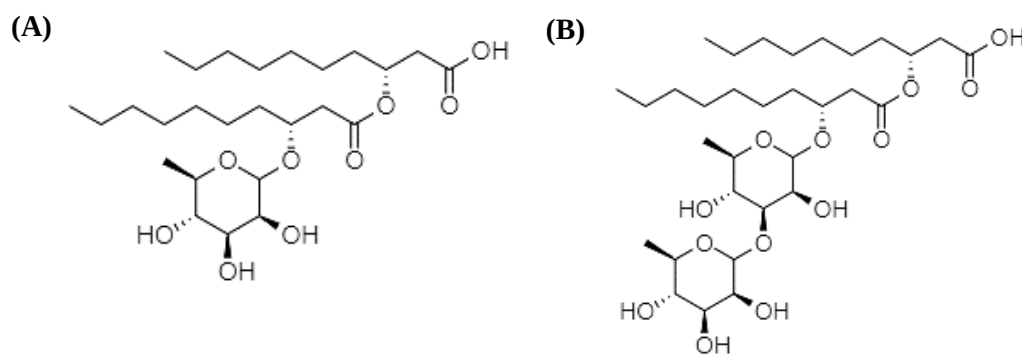


Figure 1.3. Illustration of chemical structure for mono-rhamnolipids (A) and di-RLs (B).

RLs are one of the most studied glycolipids, presenting the most researched publications and highest patents, due to their properties and high production quantities.^{58,59} While, performing research for the past three decades, RLs have acquired some recognition due to presenting some valuable characteristics, such as antifungal, antimicrobial against several microbes, antiviral and antimycoplasma activity.^{59,67} Therefore, some of these findings were summarized and displayed in **Table 1.3**, concerning the antimicrobial activity of RLs displayed against different bacteria, yeast and fungi.

Furthermore, RLs present antiadhesive properties that can be used as an antimicrobial strategy through antimicrobial-functionalization through coating of the surface of medical devices, causing changes to the surfaces' hydrophobicity.⁶⁸ This changes will result in an indirect interference to the adhesive properties of microorganisms as proven in various reports, such as M. Díaz de Rienzo *et al.* (2016)⁵⁶ and L. De Araujo *et al.* (2011)⁶⁹. RLs can also cause disruption of already formed biofilm by interacting with the microcolonies of cells that are encased in the EPS matrix, removing EPS and altering biofilm environment, which can be valuable.⁶⁸

The disadvantages of using RLs beyond the pathogenic producer strains, is the high cost of production, so researchers must focus in finding easy and cheaper ways of production with cheap substrates, appropriate production strains and using bioreactor technology.⁵⁹ However, currently some strategies are already put in place.^{58,68}

1.4.3. SLs

SLs are produced mostly by yeasts, such as *Candida bombicola*, *Torulopsis gropengiesseri*, *Torulopsis bombicola* and *Candida bogoriensis* and are produced as a mixture of congeners.^{56,64,67} However, *Starmerella bombicola* allows the production of SLs with the highest yield.⁷⁰ It has been reported that SLs can be produced with high production yields, such as 400 g/L and that substrate conversion is possible at 70%.⁶⁴ The carbon source provided allows the production of a given SL and dictates the length of the carbon chain and the fatty acid structure.⁵⁶

The mixture of congeners produced will differ on the chain length, unsaturation degree, and hydroxylation position of the constituent fatty acid.^{2,64} Furthermore, SLs can be produced by renewable resources (e.g. whey) and in higher amounts (300 g/L) than other biosurfactants.⁶⁷

The chemical structure of SLs consists of a sophorose linked to a hydroxyl fatty acid through the stabling of a glycosidic bond.⁶⁴ The sophorose can suffer acetylation processes in different positions and also esterified at positions 4', 6' or 6'' with the carboxylic end.^{2,70} Furthermore, SLs can be present in either an acidic (non-esterified) or lactonic (esterified) forms as illustrated in **Figure 1.4**.²

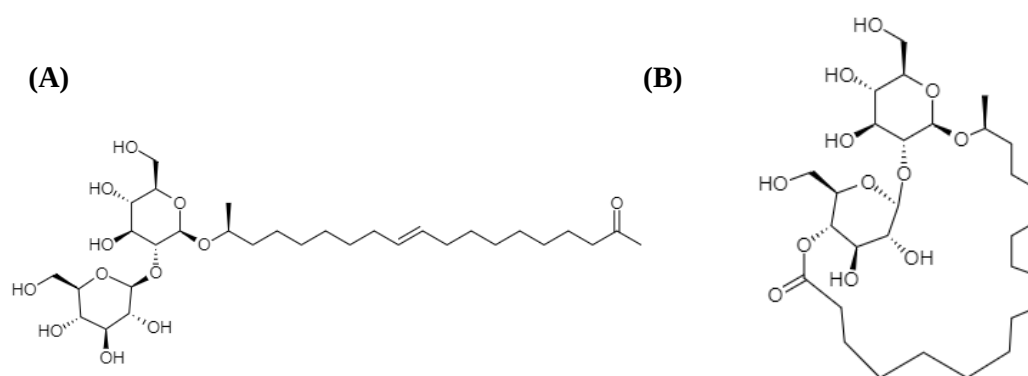


Figure 1.4. Illustration of the chemical structure of acidic (A) and lactonic (B) SLs.

SLs are commercially available glycolipids that are produced by non-pathogenic strains, an advantage in comparison with RLs, which is safer in the development of large-scale production.⁶⁸ Furthermore, these are produced in considerable amounts, which is why SLs interest peaked as promising BS.⁶⁴ These compounds present favourable characteristics, such as antibacterial, antiviral, antimycoplasma, antifungal and antialgal properties.⁷¹ Therefore, some of these findings were summarized and displayed in **Table 1.4**, concerning the antimicrobial activity of SLs displayed against different bacteria, yeast and fungi.

Normally, lactonic SLs present better characteristics, such as antimicrobial activity and better surface tension action.^{64,68} Lactonization of SLs results in easier isolation, because the lactone reduces the rotation freedom between atoms resulting in the formation of crystals instead of a viscous oil.^{64,68} However, acidic SLs display higher solubility and foaming characteristics.^{64,68}

Table 1.3. Antimicrobial Activity displayed by RLs.

Rhamnolipids Antimicrobial Activity					
Compounds	Rhamnolipids Producer	Target Species	Method	Results	Ref.
Mixture of 11 RLs homologues	<i>Pseudomonas aeruginosa</i> 47T2 NCIB 40044	A total of 31 strains	MIC: Solid medium Sabouraud agar plates, 2 h at 25°C, static. Concentration range 0.5 a 276 µg/mL.	MIC: 8 - 75 µg/mL	72
MR01and MASH1 mixtures RLs	<i>P. aeruginosa</i> MR01 and <i>P. aeruginosa</i> MASH1	15 strains and 5 fungi	MIC: Muller Hinton broth containing Ca ²⁺ , Mg ²⁺ , 37°C for 2 h, static. Concentration range 0.25 to 512 µg/mL.	MIC: 32- >512 µg/mL (Gram positive) >512 µg/mL (Gram negative) 64- >256 µg/mL (fungi)	73
		<i>E. coli</i> ATCC 25922, <i>P. aeruginosa</i> ATCC 27853, <i>S. aureus</i> ATCC 29213, <i>S. epidermidis</i> ATCC 12228, and <i>B. subtilis</i> ATCC 6051	Disc diffusion: Muller Hinton Agar Plate, 35°C (no CO ₂) for 16-18 h, static. Concentration range 10, 20, and 30 mg/mL.	Gram negative: 0 mm Gram positive: 14-30 mm	
Commercial rhamnolipid JBR599	<i>P. aeruginosa</i> PA1	5 strains: ATCC 7644, 15313, 19112, 19117, SCOTT A; 27 wild-type isolates	MIC: TSYE broth, 24 h a 37°C, static. Concentration range 4.9 to 2500 mg/mL.	MIC: 78.1 to 2500 mg/mL 90.6 % of the 32 strains were sensitive.	74
Crude RLs	<i>P. aeruginosa</i> PA1	15 strains of <i>L. Monocytogenes</i> ATCC 7644, 19112, 19117, 15313, Scott A, L01, L02, L03, L04, L05, L06, L07, L08, L09 and L10	Colony forming units: TSYE broth, 35°C, static. Concentration 0.1% w/v.	Inhibited 2.73 % (7644) and 4.57% (19112) for crude RLs and 100% (7644 and 19112) for purified RLs.	69
8 homologues RLs	<i>P. aeruginosa</i> 47T2 NCIB 40,044	<i>C. albicans</i> ATCC 10231 and methicillin-resistant <i>S. aureus</i> (MRSA) ATCC4 3300	MIC: Muller Hinton Broth, 37°C for 2 h, static. Concentration range 0.0244 to 256 mg/mL.	MIC: > 256 mg/mL.	75
44% mono-RLs and 29% di-RLs	<i>Pseudomonas aeruginosa</i> LBI	Bacteria, yeast, and fungi	MIC: Muller Hinton agar, 37°C (bacteria), Sabouraud Dextrose agar, 30°C (yeast and fungi), static. Concentration range 64 to 500 µg/mL.	MIC: 64 µg/mL for <i>Bacillus cereus</i> and <i>Mucor miehei</i> . 256 µg/mL for <i>Neurospora crassa</i> , <i>Staphylococcus aureus</i> and <i>Micrococcus luteus</i> , >500 µg/mL other.	76
Crude RLs	<i>P. aeruginosa</i> ST5 (SARCC 697)	20 Gram negative bacteria; 11 Gram positive bacteria and 10 Fungi.	Disc diffusion: Luria-Bertani Broth, Muller Hinton agar, 37°C for 24-48 h, static. Concentration 1 mg/mL.	9.8 to 29.3 mm (Gram negative) 10.7 to 22.3 mm (Gram positive) 11.3 to 18.3 mm (Fungi)	77
Crude RLs (7 homologues)	<i>P. aeruginosa</i> AT10	Gram positive, Gram negative, yeasts and fungi	MIC (2-fold serial dilution): Muller Hinton Broth, 37°C for 24 h for bacteria, Sabouraud agar plate, 25°C for 72 h for yeasts and fungi, static. Concentration range 8 to 256 µg/mL.	MIC: 16 to > 256 µg/mL for Gram positive; 8 to 256 µg/mL for Gram negative; >256 µg/mL yeasts and 16 to 128 µg/mL for fungi.	78

*Continuation of Table 1.3. Antimicrobial Activity displayed by RLs.

Rhamnolipids Antimicrobial Activity					
Compounds	Rhamnolipids Producer	Target Species	Method	Results	Ref.
RLs mixture	<i>P. aeruginosa</i> LBI	Gram positive, Gram negative, yeasts and fungi	MIC (2-fold serial dilution): Muller Hinton Broth, 37°C for 24 h for bacteria, Sabouraud agar plate, 25°C for 72 h for yeasts and fungi, static. Concentration range 4 to 250 µg/mL.	MIC: Best antimicrobial activity for bacteria at 4, 8 and 32 mg/mL.	79
Commercial RLs	<i>P. aeruginosa</i>	<i>Staphylococcus aureus</i> ATCC 8095, <i>Escherichia coli</i> ATCC 43895, <i>Listeria monocytogenes</i> ATCC 19112, <i>Bacillus cereus</i> ATCC 33018 and <i>Salmonella Enteritidis</i> ATCC C13076	MIC: Suspension in saline solution (NaCl 0.86%), Growth in TSYEA plates, 37°C for 24 h, static. Concentration range 4.9 to 2500 µg/mL.	MIC: Gram positive favoured at acidic conditions and Gram negative showed resistance at all pH levels (>2500 µg/mL).	80
			SEM: 2 h at 37°C, static. Concentration MIC values	Enhanced cell damage and accumulation of intracellular material.	
RLs	<i>P. aeruginosa</i> LBI using soybean oil as carbon source	<i>L. monocytogenes</i> ATCC 19112 and <i>S. aureus</i> ATCC 25923	Adhesion test with Crystal Violet: TSYE broth, 35°C for different intervals, static. Concentration 0.25%, 0.5% and 1.0% (w/v) aqueous solutions.	At 1.0% reduced 57.8% adhesion of <i>L. monocytogenes</i> and by 67.8% adhesion of <i>S. aureus</i> .	81
		<i>L. monocytogenes</i> ATCC 19112, <i>S. aureus</i> ATCC 25923, <i>Salmonella enterica</i> and <i>Enteritidis</i> PNCQ030	Disruption of biofilms: TSYE broth, 35°C for 24 h, static. Concentration 0.25% and 1.0% (w/v) aqueous solutions.	At 0.25% RLs removed 58.5% the biofilm of <i>S. aureus</i> , 26.5% of <i>L. monocytogenes</i> , 23.0% of <i>S. Enteritidis</i> and 24.0% the mixed culture.	
mono-RLs and di-RLs	<i>P. aeruginosa</i> ATCC 9027 and <i>Burkholderia thailandensis</i> E264	<i>Bacillus subtilis</i> BBK006	LIVE/DEAD BacLight Kit (Propidium iodide and Syto9): Nutrient Broth, 30°C for 48 h, static. Concentration 0.4 g/L.	More sensitive to di-RLs than the mono-RLs.	82
RLs	<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i> PAO1, <i>E. coli</i> NCTC 10418, <i>B. subtilis</i> NCTC 10400 and <i>S. aureus</i> ATCC 9144	MIC: Luria- Bertani Broth, 24 h at 37°C, static. Concentration range 0 to 10 % (v/v).	MIC: <i>B. subtilis</i> and <i>S. aureus</i> were inhibited at 0.5% v/v. <i>P. aeruginosa</i> and <i>E. coli</i> were not inhibited.	62
48.9% mono and 51.1% di-RLs	Urumqui Unite Bio-Technology Co. Ltd (Xinjiang, China)	<i>Pseudomonas aeruginosa</i> P60	Bacterial growth and cell viability: Luria-Bertani Broth, 24 h at 37°C, cell viability measured at 0 and 24 h and OD600 nm at 0, 6, 8, 12, and 24 h, static. Concentration 100 µg/mL.	Bacterial growth rate was not affected. Cell viability changed and dead cell ratio increased 8.3% after 24 h.	83

Table 1.4. Antimicrobial activity displayed by SLs.

Sphorolipids Antimicrobial Activity

Compound	Sphorolipids Producer	Target Species	Method	Results	Ref.
SLs (SSLA)	<i>Candida bombicola</i> ATCC 22214 with lauryl alcohol	<i>E. coli</i> ATCC 8739, <i>S. aureus</i> ATCC 6538, <i>B. subtilis</i> ATCC 6633, <i>P. aeruginosa</i> ATCC 9027 and <i>C. albicans</i> ATCC 2061	MIC/agar plate method: Nutrient Broth Medium/ Nutrient agar plates and MGPY (<i>C. albicans</i>), 48 h at 30°C for <i>C. albicans</i> , 6 h at 37°C for <i>E. coli</i> , and 28°C for others, static. Concentration range 1 to 75 µg/mL tested every 2 hours until 6 h was reached.	MIC: 30 µg/mL <i>E. coli</i> and <i>P. aeruginosa</i> , 6 µg/mL <i>S. aureus</i> , 1 µg/mL <i>B. subtilis</i> , 50 µg/mL <i>C. albicans</i> .	84
			SEM: Nutrient Broth Medium, same growth as previous. Concentrations 30 µg/mL at 2 h <i>E. coli</i> , 1 µg/mL <i>P. aeruginosa</i> at 4 h, 6 µg/mL <i>S. aureus</i> at 4 h, 1 µg/mL <i>B. subtilis</i> at 4 h and 50 µg/mL <i>C. albicans</i> at 4 h.	SSLA resulted in cells lysis (Gram positive) and merely caused shrinking or presence of irregularities (Gram negative).	
SL (95% acidic)	<i>Candida bombicola</i> ATCC 22214 production with canola oil	<i>B. subtilis</i> KCTC 1028, <i>S. xylosus</i> ATCC 35663, <i>S. mutans</i> ATCC 35668, <i>P. acne</i> ATCC 6919 and <i>E. coli</i> KCTC 1039	MIC: LB (LenoxL); LAB M + clostridial medium (<i>P. acne</i>), 37° C (CO ₂), 6-7 days for <i>P. acne</i> , and others 1-2 days, static. Concentration range 0.5 to 4 ppm.	MIC: <i>B. subtilis</i> 4 ppm, <i>S. xylosus</i> 1 ppm, <i>S. mutans</i> 1 ppm, <i>E. coli</i> >4 ppm, <i>P. acne</i> 0.5 ppm	67
SL1 and SL2	<i>Candida bombicola</i> ATCC 22214 for SL1 and commercially available SL2 (Sopholiance)	<i>Cupriavidus necator</i> ATCC 17699, <i>B. subtilis</i> BBK006 and <i>S. aureus</i> ATCC 9144.	MIC: LB broth, 21 h room temperature, static in vivo. Concentration 5% (v/v).	MIC: >5% v/v for <i>C. necator</i> and <i>B. subtilis</i> (3 h).	56
			SEM (Syto 9): nutrient broth 50% (w/v) 30 min room temperature, dynamic (200 rpm). Concentration 5% (v/v).	Disrupt biofilms at > 5% (v/v).	
SLs (free-acid and lactonic forms)	<i>S. bombicola</i> ATCC 22214 using palmitic, stearic and oleic acids	<i>E. coli</i> O157:H7 (RM1484, RM6535, RM7386, 06F00475, and Sakai)	Counting viable cells: TSA plates, 37°C for 24 h, static. Concentration range 0.1%, 0.5% and 1.0% with various levels of ethanol (1 to 20%).	Lactonic stearic and oleic SLs were more effective.	85
SLs mixture	<i>S. bombicola</i> CBS 6009	<i>S. aureus</i> ATCC 25923 and <i>E. coli</i> ATCC 25922.	MIC/MBC: Muller Hinton Broth, 37°C for 24 h, static. Concentration range 12.5 to 750 µg/mL.	MIC/MBC: 50/50 µg/mL <i>S. aureus</i> >750 µg/mL <i>E. coli</i>	2
			Crystal violet: BHI medium with 1 % (w/v) glucose, 37°C for 24 h, static. Concentration range 0.10 to 3 mg/mL.	Inhibited <i>S. aureus</i> and <i>E. coli</i> biofilm formation in an extension of 100 and 60% at >0.38 mg/mL.	
			Resazurin/Cell count: BHI medium/TSA plates, 37°C for 24 h, static. Concentration range 0.10 to 3 mg/mL.	Fluorescence reduction at 0.38 and 0.75 mg/mL and cell reduction >2 log units for <i>S. aureus</i> . Reduction of 50% for all tested concentrations for <i>E. coli</i> .	
Sls mixture	<i>Candida bombicola</i> ATCC 22144 using fat-chicken medium	<i>P. mirabilis</i> , <i>E. coli</i> , <i>Salmonella enterica</i> subsp., <i>Enterococcus faecium</i> , <i>S. aureus</i> and <i>Streptococcus mutans</i>	MIC by turbidity/ Cell viability (CFU): Tryptone Soy Broth/ Muller Hinton agar, 37°C for 18 to 24 h, static. Concentration range 7.8 to 2000 µg/mL.	MIC: Gram positive and negative at 500 and 2000 µg/mL. At 50 and 500 mg mL ⁻¹ led to a reduction in the cell viability.	86
			Disc diffusion: Muller Hinton Agar, 37°C for 18 to 24 h, static. Concentration 2 µg/mL.	Inhibition for all strains tested were observed at 2 µg.	

*Continuation Table 1.4. Antimicrobial activity displayed by SLs.

Sophorolipids Antimicrobial Activity

Compound	Sophorolipids Producer	Target Species	Method	Results	Ref.
SLs with sucrose and ethanol vehicles as carriers	<i>Candida bombicola</i>	<i>E. coli</i> , <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>S. pneumoniae</i> , <i>Proteus mirabilis</i>	MIC: cation-adjusted Mueller-Hinton broth, 18 to 24 h, static in vitro. Concentration range 0.5 to 512 µg/mL.	MIC: <i>E. coli</i> and <i>S. aureus</i> >512 µg/mL for most SL derivatives and >128 µg/mL for 2 SL derivatives in ethanol vehicle.	56
SLs ethyl ester diacetate derivative				MIC: >128 µg/mL SL derivatives tested had no significant antibacterial activity at the tested MICs.	
SLs	<i>Starmerella bombicola</i> (<i>Sopholiance</i>)	<i>Candida albicans</i> , <i>Candida tropicalis</i> , <i>F. neoformans</i> , <i>E. coli</i> , <i>Str. Salivarius</i> and <i>M. Luteus</i> .	MIC: Glucose-peptone medium, 28°C for 2 days (yeasts), liquid 5.5 medium B, 37°C (bacteria), dynamic/static. Concentration range 0 to 32 mg/mL at pH 4 and 6.	MIC: Yeasts: pH4- 15, 15 and 1.1 mg/mL and pH6-15, 15 and 2.2 mg/mL Bacteria: 32, 23, 23 mg/mL	87
SLs	<i>Rhodotorula bogoriensis</i> (C ₂₂ -SLs) and <i>Candida bombicola</i> (C ₁₈ -SLs)	<i>Propionibacterium acnes</i>	Plate assay method: Assay Plates, 37°C for 2-4 days, anaerobic, static. Concentration 40 mg/mL in discs (6 mm) of PHB/HV	C ₂₂ -SLs strongly inhibited the growth of <i>P. acnes</i> . C ₁₈ -SL of showed a weaker activity.	88
SLs	<i>Candida bombicola</i>	6 different strains	MIC: Muller Hinton Broth, 37°C for 24 h, static. Concentration range 0.006 to 6.25 mg/mL.	More effective against Gram positive than Gram negative bacteria.	89
SLs, SLs combined with tetracycline or cefaclor.	<i>Candida bombicola</i> ATCC 22214	<i>E. coli</i> ATCC 8739	MIC: Standard microdilution method, static. Concentration 100–1000 µg/mL SLa and 20–80 µg/mL cefaclor.	MIC: 200 µg/mL for cefaclor, 1000 µg/mL for SLs against <i>E. coli</i> .	90
		<i>S. aureus</i> ATCC 29737	MIC: Standard microdilution method, static. Concentration range 100–600 SLs µg/mL and 5–100 µg/mL tetracycline.	MIC: 150 µg/mL for tetracycline, 400 µg/mL for SLs against <i>S. aureus</i> .	
SLs	<i>Candida parapsilosis</i>	<i>Escherichia coli</i> MTCC No. 1687 and <i>Staphylococcus aureus</i> MTCC No. 96	MIC: Nutrient medium, 37°C overnight (aerobic conditions), static. Concentration range 5 to 20 mg/mL.	MIC: <i>E. coli</i> and <i>S. aureus</i> at 10 and 5 mg/mL.	91
			Agar diffusion: Nutrient Agar medium, 37°C for 24 h, static. Concentrations 0.3, 0.75, 1.50 mg/mL.	1.5 mg/mL at 2.87 cm for <i>E. coli</i> and 2.66 cm for <i>S. aureus</i> .	
SLs	<i>Candida bombicola</i>	<i>P. aeruginosa</i> PAO1, <i>E. coli</i> NCTC 10418, <i>B. subtilis</i> NCTC 10400 and <i>S. aureus</i> ATCC 9144	MIC: Luria- Bertani Broth, 24 h at 37°C, static. Concentration range 0 to 10 % (v/v).	MIC: <i>B. subtilis</i> was inhibited at 0.5% v/v and others at 1% v/v.	62
			Crystal Violet staining of biofilm LB medium, 24 h at 37°C, static. Concentration range 0.5 to 5% (v/v).	<i>P. aeruginosa</i> , <i>E. coli</i> , <i>B. subtilis</i> and <i>S. aureus</i> biofilm was disrupted at 5%.	

1.4.4. RLs and SLs Antimicrobial Mechanisms

As previously described, both RLs and SLs, possess antimicrobial activities, however their antimicrobial mechanism of action is not properly understood or described yet, but an hypothesized mechanism has been suggested.^{71,74,92} Therefore, it is important to keep investigating and determining the interaction between these molecules and microorganisms.

BS are molecules that have amphipathic structures, which allow the interaction with cellular membranes, such as the bilayer membrane.^{74,92} The interaction of the BS with the cellular membrane causes changes and perturbations that will lead eventually to the disruption of the cellular membrane and ultimately the release of the cytoplasmatic content of the cell and consequently important metabolites.^{57,92}

The cellular membrane of bacteria is characterized as a bilayer membrane composed by amphipathic molecules, namely phospholipids, and membrane proteins. Furthermore, the interaction will cause destabilization of the cell membrane and alteration of cell surface properties, such as cell surface hydrophobicity (CSH), cell surface charge (CSC), content of cell surface compounds, cells' surface structure, membrane permeability and fluidity.^{57,71,93} Accordingly, to the environmental conditions these properties will differ and will influence each other.⁵⁷ Cell surface properties of microorganisms will consequently affect the interaction of microorganisms and the external environment, being crucial to the growth, transportation and metabolism of microorganism.⁵⁷

The alteration of CSH seems to be the most characterized change, when biosurfactants interact with cellular membranes of bacteria. The change in hydrophobicity occurs due to changes in functional groups and chemical elements.^{56,92} The adsorption of the glycolipids onto to the cell surface and the modifications in the compounds present in the cell surface are the proposed mechanisms responsible for the hydrophobicity changes.⁵⁷ It has been reported a rapid increase in CSH, due to the adsorption of RLs at low concentrations and a stabilization or decrease of CSH at high RLs concentrations.⁵⁷ This reported difference is caused by different modes of action, monomer adsorption (low concentrations), which is more significant, and micelle adsorption (high concentrations).

Gram positive bacteria possess a thick peptidoglycan layer with polysaccharides, neutral acids and proteins. However, Gram negative bacteria are composed by an outer membrane layer with a high composition of fatty acids, such as lipopolysaccharides (LPS). These possess a neutral pH usually, but when the phosphate groups or the carboxylic groups are ionized, they can possess anionic charges, therefore conferring higher hydrophilicity. Therefore, SLs and RLs interacting

with the cellular membranes can cause a decrease of hydrophobicity of both effectively. However, because of the different compositions a more significant effect is present in more hydrophobic microorganisms, such as Gram positive bacteria.⁹²

The cellular membrane is generally negatively charged due to the presence of phosphates and carboxylic groups that possess anionic charges, as previously mentioned. The adsorption or incorporation of biosurfactants, such as RLs and SLs, can neutralize or enhance the original CSC. However, it has been found that when adsorbed these can bring some negative charge, which can decrease CSC.⁵⁷ These compounds can also remove intracellular compounds by increasing membrane permeability, such as proteins, polysaccharides, glycoproteins or LPS, which can also alter CSC. However, the surface charge can also be altered by release or removal of cell surface compounds, like negatively charged molecules, such as proteins or lipopolysaccharides causing a reduction in the negativity of cell surface charge.⁵⁷

The cellular membrane possesses two important characteristics, such as membrane permeability and fluidity that are responsible for the uptake in the cell.⁵⁷ Reports show that the interaction of BS to cell membrane can cause an increase of membrane permeability that is related to the ion concentrations present in extracellular and intracellular content.^{57,71} Normally, this increase can be due to the release of membrane proteins and the incorporation of biosurfactants in the cell membrane, causing the formation of transmembrane pores, ultimately leading to the outpour of intracellular content.⁵⁷

Closely related is membrane fluidity that is also influenced by the interaction with BS that affect the fatty acid and protein content and composition.⁵⁷ Membrane fluidity depends on the membrane lipid composition (e.g. ratio of saturated fatty acid to unsaturated fatty acid present in lipid), protein content, composition and average chain length of fatty acid present.⁵⁷ The release of proteins is also a common occurrence in the interference of compounds with cell membrane surfaces.⁵⁶

These surface changes can lead to alteration of this barrier causing cell damage that will eventually lead to membrane disruption, releasing metabolites, such as proteins, lipopolysaccharides and intracellular enzymes.^{57,93}

✓ **Chapter 2: Thesis Aim and Outline**

2. Thesis Aim and Outline

In the Healthcare sector the prevalence of medical device-associated infections is still a major issue. Any patient receiving healthcare is prone to develop these infections, due to the easy colonization of microorganisms and biofilm formation on the surface of medical devices. Therefore, there is a current need to develop new effective strategies to eradicate this concern.

Currently, a possible strategy is the improvement of medical devices by antimicrobial-functionalization, through the coating of the surface with antimicrobial agents. An interest has emerged on BS, mainly RLs and SLs, due to the antimicrobial and antibiofilm properties that have been reported.

The evaluation of their antimicrobial and antibiofilm activities was crucial, therefore it was important to verify if in the presence of these coated surfaces, biofilm formation was hindered as it has been previously investigated. The interaction of both RLs and SLs should be studied against relevant bacteria causing medical device-associated infections and against silicone-based materials mimicking the medical devices.

Furthermore, the characterization and illustration of the antimicrobial mechanism of these compounds was an important objective, since is not fully understood. Defining the antimicrobial mechanism will allow the development of more effective treatment strategies to hopefully reduce the incidence of the medical device-associated infections.

Consequently, this research work focussed on evaluating the antimicrobial and antibiofilm activities of glycolipids microbially produced. The main goals of this study were:

1. To biosynthesize and extract the glycolipid compounds, SLs, using oleic acid and glucose as the main sources.
2. Characterize the present mixtures of RLs and SLs.
3. Evaluate antimicrobial activity of the extracts of RLs and SLs against six different strains in the planktonic state.
4. Study the antimicrobial mechanism of RLs and SLs against planktonic bacteria. Evaluate bacterial cell surface hydrophobicity, bacterial surface charge, cellular viability, and membrane integrity in the presence of the biosynthesized mixtures of RLs and SLs.
5. Evaluate antibiofilm activity of RLs and SLs coated on PDMS specimens by adsorption against six different strains in the sessile state.
6. Evaluate the treatment of PDMS specimens with plasma and coated with the RLs mixtures.

✓ Chapter 3: Materials and Methods

3. Materials and Methods

3.1. Chemicals

Acetic acid glacial anhydrous 100% (ACS, ISO, Ph.Eur reagent), sulfuric acid 95-97% (EMSURE, ISO), potassium dihydrogen phosphate 99.5-100.5% (ISO reagent), dodecyl sulphate sodium salt $\geq 95\%$, vegetable oleic acid (Ph.Eur, BP, NF), 4-methoxybenzaldehyde and formaldehyde solution 35% were purchased from Merck (Darmstadt, Germany). Crystal violet, resazurin sodium salt, D(+)-glucose monohydrate $\geq 99.0\%$, sodium sulphate 99+% (ACS reagent), sodium phosphate dibasic $\geq 99\%$ (ACS reagent), potassium chloride 99.0-100.5% (ACS reagent), chloroform 99% (ISO for analysis), dichloromethane $\geq 99.0\%$ (ACS reagent, ISO), levofloxacin and 1-octanol 99+% (ACS reagent) were acquired from Sigma-Aldrich (St. Louis, USA). Ethanol absolute anhydrous 99%; ethyl acetate 99.9% (ACS, Ph.Eur, USP reagent) and acetonitrile 99.9% (ACS, Ph.Eur and USP reagent) were purchased from Carlo Erba Reagents (Val-de-Reuil Cedex, France). Methanol $\geq 99.8\%$ (ACS, ISO, Ph.Eur reagent) purchased from VWR BDH Prolabo Chemicals (Fontenay-sous Bois, France). Sodium chloride (PA-ACS-ISO) was purchased from PanReac (Barcelona, Spain). Ammonium acetate was purchased from Sharlau (Barcelona, Spain). Propidium iodide was purchased from Invitrogen Thermo Fisher Scientific (San Francisco, USA). Rhamnolipids 90% pure was purchased from AGAE Technologies (Corvallis, USA). Water was obtained by a Milli-Q purification system (Millipore).

3.2. Culture Media

Bacteriological agar type E, brain heart infusion broth (BHI), yeast extract, tryptone soy broth (TSB), müeller hinton broth (MHB), peptone water (indole free) and tryptone USP were purchased from Biokar Diagnostics (Allonne, France).

3.3. Culture Media Preparation

The culture media preparation was performed according to producer instructions. After, preparation culture media was sterilized (autoclave, Medline Italia) at 121 °C for 15 min.

3.4. SLs biosynthesis and extraction

SLs biosynthesis was performed accordingly to Pontes *et al.* (2016)² and Ribeiro *et al.* (2013, 2015)^{94,95} using *Starmerella bombicola* CBS 6009, obtained from the Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures). Seed cultures were performed on glucose, peptone, yeast extract and agar (GPYA) plates and incubated at 25°C for 72 h. Colonies from a fresh seeded culture of *S. bombicola* were inoculated in a 100 mL flask, containing 30 mL of GPY. The inoculated media was kept at 25°C under agitation (240 rpm) for 48 h and then

transferred to a 1 L flask with 300 mL of GPY media supplemented with vegetable oleic acid (2%v/v). After, 168 h at 25° C under agitation (240 rpm), the extraction of SLs was performed twice with ethyl acetate (1:1). The organic phase obtained was centrifuged at 3737 x g for 8 min (Alegra 6R Centrifuge- Beckam Coulter) at 25° C and dried with anhydrous sodium sulphate to remove any residual water. After filtration, the extracts were evaporated on a rotatory-evaporator (Büchi Rotavapor R-200) at 40°C, until dry. The dried mixture obtained was washed with n-hexane (3 vol.) with the purpose of removing any remaining lipid carbon source present and centrifuged at 6686 x g for 10 min (Centrifuge 5430 – Eppendorf). The residual n-hexane present in the extract was removed under nitrogen stream. The obtained SLs mixture was used in subsequent assays.

3.5. Glycolipids Isolation and Identification

3.5.1. Preliminary Glycolipids identification

Thin Layer Chromatography (TLC) was performed as a preliminary method to evaluate the glycolipids mixture using the conventional method of chloroform as the mobile phase.⁹⁶ TLC Silica gel 60 F₂₅₄ 20x20 for analytical chromatography from Merck (Darmstadt, Germany) was used as the stationary phase and a mobile phase of chloroform: methanol: water (16.5:3.75:0.5 (v/v)), was prepared to perform thin layer chromatography. The mixture of glycolipids was dissolved in ethyl acetate (5 mg/mL) and applied to the TLC plate. In order to perform the separation of the mixtures, the TLC was placed vertically in a chamber containing the mobile phase. The visualization of the compounds was obtained by spraying with a staining solution of acetic acid: sulfuric acid: 4-methoxybenzaldehyde (50:1:0.5) followed by heating at 110°C for 10 min.

3.5.2. Characterization and Identification of Glycolipids mixture

In order to further identify the compounds, present in the mixtures of SLs and RLs, an analysis of High-Performance Liquid Chromatography coupled to detector of Mass Spectrometry (HPLC-MS) was performed accordingly to Ribeiro *et al.* 2012(a)⁹⁶ and Ribeiro *et al.* 2012(b)⁹⁷. A reversed phase Waters™ Atlantis dC18 column (150 mm × 2.1 mm id, and 5 micrometer particle size) was used in a Waters™ Alliance 2695 HPLC Separation Module connected to a Waters™ Micromass Quatromicro API Tandem Quadrupole Mass Spectrometer equipped with a Waters™ electrospray ionisation (ESI) source. The flow rate was constant at 0.25 mL min⁻¹, injection volume was 4 µL and samples were maintained at 10 °C. Mobile phase consisted of water:formic acid (99.5%:0.5%) (eluent A) and acetonitrile (eluent B). The following elution program was used: isocratic 5 min at 50% of eluent B, gradient for 20 min until 60% of eluent B, gradient for 20 min until 100% of eluent B, isocratic 10 min of 100% of eluent B and equilibrium time of 10 min. The equipment was controlled by Waters Empower Software and operated in the

positive ion electrospray mode, ESI capillary voltage was optimized to 4 kV and cone voltage was set on 60 V. Source and desolvation temperature were adjusted to 120°C and 350°C, respectively. Nitrogen gas was generated from a nitrogen source (Gasman G4800) and used to supply cone (50 L h⁻¹) and desolvation gas (750 L h⁻¹). For fragmentation experiments, different collision energies values (10–85 eV) were used depending on the compound analysed. Data acquisition and analysis was performed with MassLynx Software 4.1.

3.6. Antimicrobial Activity

Antimicrobial activity of planktonic bacteria was evaluated with three different assays, accordingly to Pontes *et al.* (2016)² to determine Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Biofilm Inhibitory Concentration (MBIC).

3.6.1 Strains and Culture Conditions

The following strains were used in this study: methicillin-sensitive *Staphylococcus aureus* ATCC 25923, methicillin-sensitive *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 28319, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922 obtained from the American Type Culture Collection (ATCC, USA) and clinic methicillin-resistant *Staphylococcus aureus* (MRSA) kindly donated by Dr. Luísa Jordão (Instituto Ricardo Jorge, Portugal). Aliquots were collected from stocks stored at -80 °C to expand culture on tryptic soy agar (TSA) plates for 24 hours at 37 °C.

3.6.2. Minimum Inhibitory Concentration Assay

The antimicrobial activity of compounds can be studied by Minimum Inhibitory Concentration Assay determination using Broth Microdilution Method as described by the Clinical and Laboratory Standards Institute (CLSI).⁹⁸ A glycolipids stock solution (1.6 mg/mL) prepared in MHB media was applied in 96-well microtiter plates (MTPs) and two-fold dilutions were performed in MHB media reaching final concentrations ranging from 0.8 to 0.003 mg/mL. The inoculum was prepared by direct colony suspension in 3 mL of MHB media and obtaining 1x10⁸ CFU/mL by adjusting the sample's optical density (OD) at the wavelength of 600 nm (Ultrospec 10 Cell Density Meter, Biochrom). The final inoculum concentration in the wells of a 96-well MTP was correspondent to 5x10⁵ CFU/mL. Negative controls consisted of MHB media and positive controls consisted inoculated MHB media. The results for these experiments were obtained after a 24-hour period of incubation at 37°C, without agitation, by the measuring optical density at 595 nm in a Microplate Multimode Reader (Zenyth 3100, Anthos). The assays were performed in two independent experiments for three replicates of each concentration.

3.6.3. Minimum Bactericidal Concentration Assay

The antimicrobial activity of compounds was studied by Minimum Bactericidal Concentration (MBC). To obtain the MBC, 20 µl were transferred from the wells that revealed no growth in the MIC Assay and diluted in MHB media (1:10) in a new 96-well MTP. The MTP was incubated for 24 hours at 37°C, without agitation, and the optical density at 595 nm was measured in the Microplate Multimode Reader (Zenyth 3100, Anthos). The assays were performed in two independent experiments for three replicates of each concentration.

3.6.4. Minimum Biofilm Inhibitory Concentration Assay

Minimum Biofilm Inhibitory Concentration (MBIC) was observed in the 96-well microplate under MIC assay conditions. This assay was performed by removing the content of the wells of the MTP used in the MIC assay, washing them twice with sterile phosphate buffered saline (PBS) composed by 8 g NaCl, 0.27 g KH₂PO₄, 0.2 g KCl and 1.44 Na₂PO₄ in 1 L sterile water, with the purpose of removing non-adherent cells. After washing, the biofilm was fixed with 96% (v/v) ethanol. The biofilm on the MTP wells was stained with crystal violet staining solution 0.1% (v/v), and free stain was then removed by washing the wells three times with sterile distilled water and gently removed without disturbing the biofilm after each washing. Then, the wells content was re-suspended in 1% (v/v) acetic acid solution and optical density was measured at 595 nm in the Microplate Multimode Reader (Zenyth 3100, Anthos). The assays were performed in two independent experiments for three replicates of each concentration.

3.7. Antimicrobial mechanism of action of SLs and RLs

The six strains described in section 3.6.1 were used in the different studies to evaluate the antimicrobial mechanism of action of SLs and RLs against planktonic bacteria.

3.7.1. Membrane Integrity and Cellular Viability

To evaluate if cell membrane integrity was compromised in the presence of glycolipids the uptake of a red-fluorescent nucleic acid stain was used, propidium iodide (PI). PI is a cell membrane impermeable dye that enters cells with comprise membranes by binding to double-stranded DNA causing an enhancement of florescence.⁹⁹ Furthermore, cell viability was accessed by the reduction of resazurin to resorufin.^{100,101}

The inoculum was prepared by direct colony suspension in 3 mL sterile NaCl (0.9%) and adjusted at a wavelength of 600 nm (Ultrospec 10 Cell Density Meter, Biochrom) and further diluted in NaCl to obtain a final concentration of 5x10⁵ CFU/mL in a 96-well MTP. Then, the Glycolipids were added with 0.4 mg/mL of concentration. The controls consisted of culture in NaCl (0.9%) and culture in NaCl (0.9%) with sodium dodecyl sulphate (0.5 mg/mL).

After a 4-hour period of incubation of cells with glycolipids in solution at 37 °C propidium iodide (1.5 mM diluted in NaCl) and resazurin (50 mM diluted in 10 mM SDS at pH=7.4) assays were conducted. PI solution was added with final concentrations of 0.3 µM and resazurin at 2.5 µM. After a 30 min incubation period at 37°C fluorescence was measured with an excitation and emission wavelength of 535 nm and 595 nm, respectively in the Microplate Multimode Reader (Zenyth 3100, Anthos). The ratio of PI uptake was calculated as the ratio between the fluorescence intensities obtained from each sample and the average of the negative control samples as described by the following:

$$\frac{\text{Fluorescence Intensity (Sample)}}{\text{Average (Negative Control Samples)}} \quad (1)$$

Cellular viability by resazurin reduction was evaluated for planktonic bacteria after a 3 h period of incubation at 37°C by measuring the fluorescence with an excitation wavelength of 535 nm and emission wavelength of 595 nm in the Microplate Multimode Reader (Zenyth 3100, Anthos). The results were presented as percentages of the fluorescence intensity of each sample and the average of fluorescence intensity of the negative control samples (inoculum diluted in NaCl) as described by the following equation:

$$\frac{\text{Fluorescence Intensity (Sample)}}{\text{Average (Negative Control Samples)}} \times 100 \quad (2)$$

The results were expressed as mean and standard deviation (mean± SD) of two independent experiments.

3.7.2. Bacterial Surface Hydrophobicity

The bacterial surface hydrophobicity was evaluated with the method of partitioning of cells in the mixture of organic and aqueous solvent (1-octanol-water), according to Pontes *et al.* (2016)² and Sodagari *et al.* (2013)¹⁰². 8 colonies were removed from a bacterial culture and inoculated by direct resuspension in 10 mL of BHI medium (37°C, 200 rpm) overnight. In a first experiment the partition in organic and aqueous solvent of bacterial cells was evaluated. Consequently, 4 mL of the overnight bacterial culture were centrifuged at 12050.5 x g for 10 min (Centrifuge 5430 – Eppendorf), washed and resuspended in PBS twice. The cell suspension was adjusted to an optical density (OD) of 0.4 at 600 nm wavelength (Hitachi U-2000 Spectrophotometer) with a final volume of 2 mL. To the 2 mL of cell suspension (OD_{initial}=0.4), 4 mL of 1-octanol were added. The mixture of aqueous and organic phases was vortexed for 2

min and then left to rest to separate for 30 min. Finally, the optical density of the aqueous phase (OD_{final}) was measured at 600 nm wavelength (Hitachi U-2000 Spectrophotometer). In order to evaluate the effect of RLs and SLs on cell surface hydrophobicity 3 mL of the overnight grown cellular suspension were added of RLs and SLs to a final concentration of 0.4 mg/mL. The cell suspension with glycolipids remained agitating (480 rpm) at room temperature for a 3-hour period. After this period, the same procedure applied to the cell culture without glycolipids was applied. The two values, OD_{initial} and OD_{final}, allowed the partition coefficient to be obtained as described by the following equation:

$$\left(1 - \frac{[OD]_{final}}{[OD]_{initial}}\right) \times 100 \quad (3)$$

Two independent experiments were performed, and each concentration tested in three replicates. The results were expressed as mean and standard deviation (mean±SD). It is important to note that the solution of glycolipids should be removed in the process of repeated centrifugation and washing before the partition of phases was performed. However, a method to determine if any glycolipids remained bound to the cell surface was not executed.

3.7.3. Bacterial Surface Charge

Bacterial surface charge was evaluated in the presence of RLs and SLs accordingly to Pontes *et al.* (2016)² by zeta potential measurements, with slight modifications. The inoculum was prepared by direct colony suspension in 1 mL of MHB media and adjusted to 1x10⁸ CFU/mL by measurements of optical density at 600 nm wavelength (Ultrospec 10 Cell Density Meter, Biochrom). Formaldehyde was added to the bacterial suspension with a final concentration of 5%(v/v). After, a 24-hour incubation period (37°C, no agitation) bacterial cells were centrifuged at 12050 x g for 5 min (Centrifuge 5430 – Eppendorf), washed and resuspended in 0.9% (w/V) NaCl. Glycolipids solutions (RLs and SLs) at final concentrations of 0 and 0.4 mg/mL were mixed with the cell suspension obtaining a final inoculum concentration correspondent to 5x10⁵ CFU/mL. Lastly, an aliquot was dispersed in distilled sterile water and surface charge was analysed by electrophoretic light scattering (Malvern Nanosizer Z, Malvern Instruments). The assays were carried out in triplicate (n=3) and obtained results were expressed as mean and standard deviation (mean±SD).

3.8. Antibiofilm Activity on medical grade PDMS towards sessile bacteria

3.8.1. Functionalization of PDMS by adsorption

FDA medical grade Polydimethylsiloxane (PDMS) approved from Sove- Sociedade de Vedantes e Máquinas (Porto, Portugal) was cut into of 0.8x0.8 cm square pieces with approximately 0.5 mm thickness. PDMS pieces were then washed three times with deionized water. After being dried, the PDMS pieces were autoclaved at 121 °C for 15 min. The adsorption assays were conducted in 24-well MTPs as described in Pontes *et al.* (2016)². PDMS pieces were fixed in the 24-well MTP and an aqueous solution of RLs or SLs with concentrations ranging from 3 to 0.1 mg/mL were used to promote glycolipid adsorption on to the medical grade PDMS. The 24-well MTP was incubated for 24 hours at 25°C with agitation (240 rpm). After the 24 h the content was removed from the wells and the PDMS were used in subsequent assays.

3.8.2. Contact Angle Measurements

PDMS specimens previously dried in vacuum chamber with absorbed RLs at concentrations ranging from 0 to 3 mg/mL prepared as mentioned in section 3.8.1 were characterized by contact angle measurements. These measurements were performed by sessile drop method deposition of MilliQ water drops onto the PDMS surface. The measurements were obtained by assessing the drops contact angle at room temperature. The images were acquired using a digital microscope (Rohs USB Digital Microscope). The image acquisition and analysis were obtained 10 min after the drop deposition using the ADSA-P software (Axisymmetric Drop Shape Analysis-Profile). The experiments were performed in quadruplicates (n=4) obtaining four different measurements for each sample. The obtained results were expressed as mean and standard deviation (mean±SD).

3.8.3. Inoculum

PDMS specimens functionalized by adsorption with SLs and RLs were submitted to antibiofilm activity assays, such as crystal violet assay and the plate counting colony forming units' methods. Therefore, the strains were prepared according to Pontes *et al.* (2016)² by direct colony suspension. Briefly, the direct colony suspension was performed in 3 mL of BHI supplemented with glucose 1% (w/v) and the optical density was adjusted corresponding to 3×10^8 CFU/mL (Absorbance Ultrospec 10 Cell Density Meter, Biochrom). Then, diluted in BHI medium with glucose at 1% (w/v) obtaining a final inoculum concentration of 3×10^6 CFU/mL for each well of the 24-well MTP in a final total volume of 1 mL. The MTP was incubated for 24 h at 37°C, under static conditions. The assays were performed with positive controls, which consisted of untreated PDMS with inoculated media and negative controls were performed with plain PDMS samples.

3.8.4. Biofilm formation on glycolipids functionalized PDMS

The quantification of biofilms present in the functionalized PDMS specimens was performed using the crystal violet method accordingly to the section 3.6.4, with slight modifications. Once washed, each PDMS with the stained biofilm was removed from the 24-well MTP and solubilized in 500 µL acetic acid solution 1% (v/v). The samples were placed in the ultrasound for 10 min to detach the biofilm. Then, 200 µL of the solubilized stained biofilm from each sample were transferred to a 96-well MTP. to measure the optical density at 595 nm in a Microplate Multimode Reader (Zenyth 3100, Anthros).

Biofilm formation (%) was assessed by the following equation:

$$\frac{([OD]_{\text{sample}} - [OD]_{\text{negative control}})}{([OD]_{\text{positive control}} - [OD]_{\text{negative control}})} \times 100 \quad (4)$$

where [OD]_{sample} is the optical density of the sample itself, [OD]_{negative control} is the optical density of the non-treated PDMS biofilm and the [OD]_{positive control} is untreated PDMS surface with inoculum. The results for the relative biofilm formation (%) were expressed as mean and standard deviation (mean ± SD) of two independent experiments.

3.8.5. Biofilm cellular viability on glycolipids functionalized PDMS

Cellular viability of biofilm in PDMS specimens was evaluated using plate counting colony forming units (CFU), according to Pontes *et al.* (2016).² The 24-well MTP from the functionalized PDMS specimens was inoculated as described previously and incubated for 24 h at 37°C, under static conditions.

After the incubation period the wells were washed twice with phosphate buffered saline (PBS) with the purpose of removing non-adherent cells. The PDMS samples were transferred to tubes containing in 1mL of MHB media and subjected to ultrasounds and vortex for 4 minutes each to release the adhered bacteria from the PDMS layers. After, performing the intended dilutions, 20 µl of the biofilm cell suspension were spread in TSA media plates. The plates were incubated for 24 h at 37°C and colonies grown were counted to obtain CFU/cm². The results were obtained by applying the following equation (5):

$$\left(\frac{N^{\circ} \text{ Colonies} \times \text{DF}}{V_{\text{Sample}}} \right) / A_{\text{PDMS}} \quad (5)$$

DF corresponding to Dilution Factor, V_{sample} corresponding to the volume of sample taken of 0.02 mL and A_{PDMS} corresponding to Area of the PDMS specimens of 0.64 cm². Results were expressed as mean and standard deviation (mean ± SD) of two independent experiments.

3.9. Plasma Functionalization

3.9.1. Functionalization of PDMS with RLs after treatment with Plasma

The functionalization by plasma consisted of submitting PDMS specimens to plasma treatment with plasma cleaner (PDC-002, Harrick plasma Ithaca, New York, USA), frequency=50 Hz, Max power= 200 W, Max RF power = 30 W for 5 to 10 min. The silicone specimens were submitted to an air plasma discharge, which results in the formation of reactive groups in their surface.^{103,104} Then, ethanolic solutions of RLs (i.e. 0, 15, 30, 60 and 120 mg/mL) were sprayed onto the PDMS specimens surface. The silicone specimens were left to dry and used in subsequent assays.

3.9.2. Contact Angle measurements

The PDMS specimens were characterized by contact angle measurements. These measurements were performed as mentioned in section 3.8.2. The experiments were performed in quadruplicates (n=4) obtaining four different measurements for each sample. The obtained results were expressed as mean and standard deviation (mean±SD).

3.9.3. Fourier transform infrared spectroscopy- Attenuated Total Reflectance

The adsorption of RLs on PDMS specimens pre-treated with plasma was also assessed by Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR, Bruker Alpha II). The functionalized specimens with RLs were evaluated by placement on the ATR diamond crystal that provided an incidence angle of 42°. Spectra were obtained with an average of 32 s collected with a resolution of 4 cm⁻¹ ranging from 4000 to 400 cm⁻¹. Then, for the purpose of spectra acquisition OPUS 7.8 Software was used.

3.9.4. Antibiofilm Activity towards Sessile bacteria

The PDMS specimens functionalized as described in section 3.9.1 were submitted to an antibiofilm activity assay, the plate counting colony forming units (CFU) assay. The inoculum was prepared by direct colony suspension of *S. aureus* ATCC 25923 in 3 mL of BHI supplemented with glucose 1% (w/V) with a final inoculum concentration of 1x10⁷ CFU/mL obtained in the 24-well MTP containing the functionalized PDMS specimens. After a 3-hour incubation period at 37°C under static conditions the wells were treated as previously mentioned in the section 3.8.5. The assays were performed with positive controls which consisted in untreated PDMS with inoculated media and negative controls were performed with plain PDMS specimens. Assays were performed at least in two independent experiments and presented as described previously.

3.10. Statistical Data Analysis

Analysis was conducted with GraphPad Prism 6.01 software. Data normality was determined by the Shapiro-Wilk test to access the difference between groups. For normal data sets, one-way ANOVA was performed, followed by multiple comparisons using Turkey's test. For non-parametric data sets, Kruskal-Wallis test was performed, followed by multiple comparisons using Dunn's test. Obtained p values < 0.05 were considered as significant.

✓ Chapter 4: Results and Discussion

4. Results and Discussion

4.1. RLs Characterization

With the purpose of characterizing the purchased mixture of RLs that was further used in the present work, preliminary TLC analysis and High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis were performed (**Figure 4.5**). The analysis allowed the identification of the main RLs present in the mixture.

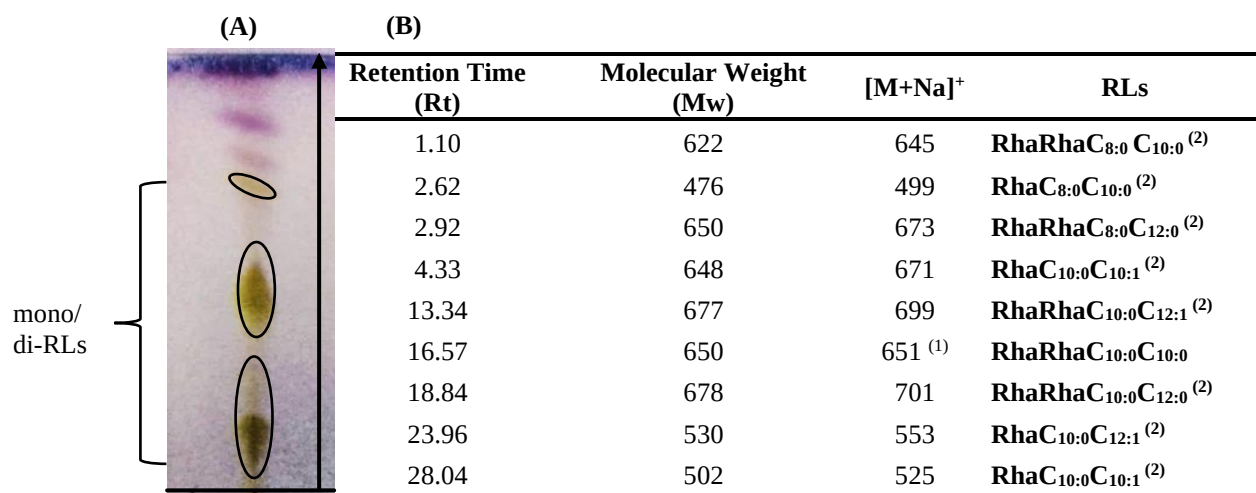


Figure 4.5(A). TLC separation of RLs mixture produced by *Pseudomonas aeruginosa* and **Figure 4.5(B).** Identified RLs presented in the mixture produced by *Pseudomonas aeruginosa*. *Note: (1) isomers are presented (2) Molecular ion occurred with a proton [M+H]⁺.

The preliminary analysis performed by TLC (**Figure 4.5(A)**) of the RLs mixture produced by *Pseudomonas aeruginosa* allowed an initial characterization of the compounds present. The TLC analysis allowed the identification of 3 distinct brown bands corresponding to different RLs, corresponding to mono and di-RLs. Furthermore, last bands presented more affinity to the stationary phase.^{105,106}

The HPLC-MS method allowed the identification of the present RLs in the mixture produced by *Pseudomonas aeruginosa*, with the correspondent retention times and molecular weight as presented in **Figure 4.5(B)**. The most predominant peaks of mono-RLs were identified by HPLC-MS analysis in the mixture as RhaC_{10:0}C_{12:1} and di-RLs as RhaRhaC_{10:0}C_{10:0}, as observed of the two predominant bands in the HPLC chromatogram.

It is known that *Pseudomonas aeruginosa* is responsible by mostly producing RLs in two forms, mono-RLs and di-RLs.⁵² The identification of mixtures of RLs as already been performed and the most prominent RLs produced are generally RhaC₁₀C₁₀ and RhaRhaC₁₀C₁₀.^{66,107}

4.2. SLs Biosynthesis and Characterization

The biosynthesis and extraction of SLs, produced by *Starmerella bombicola*, in GPY medium supplemented with oleic acid was achieved with a yield of 3.3 ± 0.6 g/L. The biosynthesis process of SLs was performed in a smaller scale with oleic acid and glucose as the building blocks, so the yield obtained for these conditions of production was appropriate to the yield of 6.8 g/L obtained by Ribeiro *et al.* (2015)⁹⁵. The biosynthesized mixture of SLs was preliminarily analysed by TLC analysis and identified by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis. The analysis allowed the identification of the SLs present in the mixture as displayed in **Figure 4.6**.

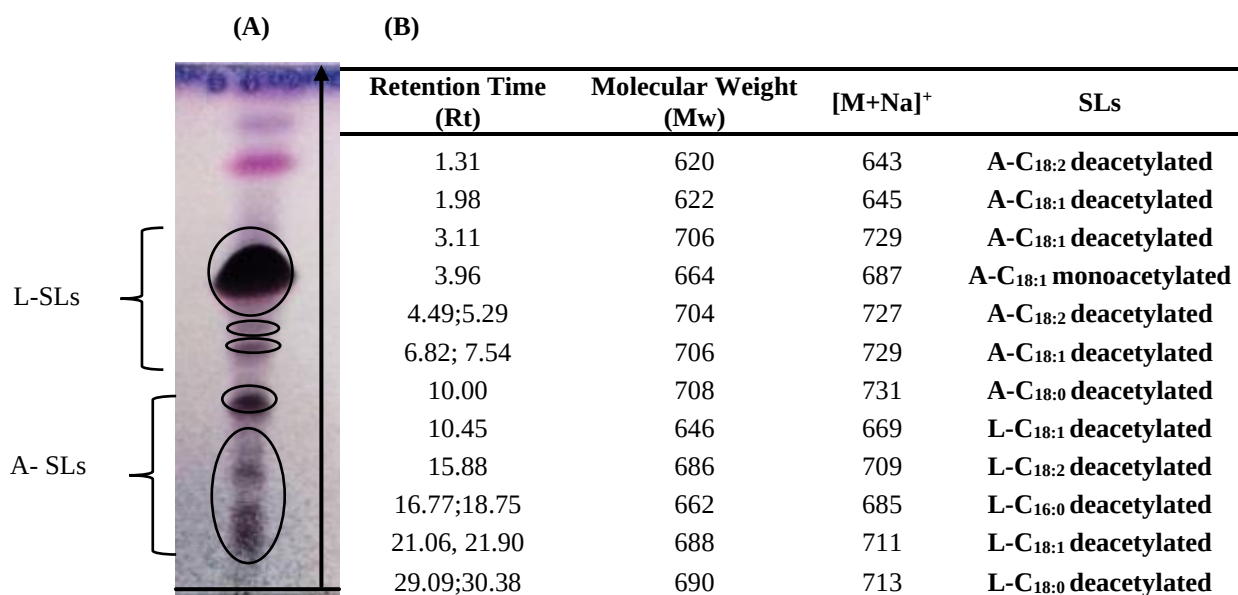


Figure 4.6(A). TLC separation of SLs mixture produced by *Starmerella bombicola* supplemented with oleic acid and **Figure 4.6(B).** Identified SLs presented in the mixture produced by *Starmerella bombicola* supplemented with oleic acid. *Note: The molecular ion occurred as [M+Na]⁺ for acidic (A) and lactonic (L) SLs.

A preliminary analysis of the SLs mixture by *Starmerella bombicola* was performed using the TLC method (**Figure 4.6(A)**). In the preliminary analysis it was possible to identify distinct purple spots, correspondent to lactonic and acidic SLs.

The HPLC-MS analysis allowed the characterization of the mixture of SLs and the first diluted correspond mostly to diacetylated acidic SLs.⁹⁶ The last correspond mainly to lactonic deacetylated SLs that present higher affinity to the stationary phase. The main SLs produced by *Starmerella bombicola* identified through HPLC-MS were diacetylated lactonic SLs, namely SLs comprising a hydroxy fatty acid chain with 18 carbon atoms and different unsaturation grade, i.e. C_{18:0}, C_{18:1} and C_{18:2} (**Figure 4.6(B)**). It is known that *Starmerella bombicola* is responsible by

mostly producing SLs in the lactonic deacetylated form, comprising a fatty acid of 16 or 18 carbon atoms (C16 and C18), which was congruent.^{92,108} Previously, the identification of SLs present in a mixture (i.e. obtained with oleic acid supplementation) was identified in work developed at our laboratory Ribeiro *et al.* (2015)⁹⁵, Ribeiro *et al.* (2012)⁹⁶ and Pontes *et al.* (2016)², using similar experimental conditions. The results obtained are in accordance with the previous work developed.

4.3. Antimicrobial Activity towards planktonic bacteria

The antimicrobial activity of the glycolipid biosurfactants, SLs and RLs, was tested with three different assays against the five different strains i.e. *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 28319, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922 and methicillin-resistant *Staphylococcus aureus* (MRSA). The MIC value for SLs against *Staphylococcus aureus* ATCC 25923 was previously obtained in Pontes *et al.* (2016)² as 50 µg/mL, therefore it was not evaluated.

The antimicrobial activity of RLs and SLs to planktonic cells to was assessed visually by turbidity change for the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). A preliminary assay to assess Minimum Biofilm Inhibitory Concentration (MBIC) was performed. The glycolipids used were the mixtures of produced by *Starmerella bombicola* for SLs and *Pseudomonas aeruginosa* for RLs.

The results for the three assays are presented in **Table 4.5** of the evaluation of the antimicrobial activity, of SLs and RLs, against the tested microorganisms.

Table 4.5. Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC) and Minimum Biofilm Inhibitory Concentrations (MBIC) obtained from the three assays performed with SLs and RLs against six strains.

	MIC (µg mL ⁻¹)		MBC (µg mL ⁻¹)		MBIC (µg mL ⁻¹)	
	RLs	SLs	RLs	SLs	RLs	SLs
<i>S. aureus</i> ATCC 6538	100/100	50/50	800/800	>800/>800	100/100	50/50
<i>S. epidermidis</i> ATCC 28319	200/200	400/400	200/200	>800/>800	100/100	400/400
<i>E. coli</i> ATCC 25922	>800/>800	>800/>800	NE	NE	NP	NP
<i>P. aeruginosa</i> ATCC 15442	>800/>800	>800/>800	NE	NE	>800/>800	>800/>800
MRSA	400/400	>800/>800	400/400	400/400	400/400	>800/>800

*Note: NE- Not-evaluated and NP- Not-produced. The two values obtained were determined by visual observation/optical density measurement.

Overall, it was possible to conclude that the most vulnerable bacteria to both biosurfactants, at the evaluated concentrations, were *S. epidermidis* ATCC 28319, *S. aureus* ATCC 6538 and *MRSA*, due to the lower concentrations obtained for the MIC, MBC and MCBI. Since the three strains were identified as the most vulnerable to the action of these compounds these will be used in following experiments for evaluating antibiofilm activity in sections 4.5. and 4.6.

Both glycolipids presented a low activity against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15422. At the highest concentration of 800 µg/mL growth inhibition, killing of bacterial cells and inhibition of biofilm was not observed. The low effect of glycolipids against these two strains can be due to the difficult penetration by antimicrobial compounds, in Gram negative bacteria, such as *E. coli* and pathogenic bacteria *P. aeruginosa*. Since, the outer lipopolysaccharide membrane of Gram negative bacteria is present, compared to Gram positive bacteria.^{109,110}

Antimicrobial activity has already been studied for SLs and RLs against different strains, as previously mentioned. In Pirog *et al.* (2019)¹¹¹ it was shown that for RLs and SLs against different strains the MIC ranged from 50–500 µg/mL, and 10–200 µg/mL, respectively. Moreover, MIC value of 60 µg/mL for RLs against *S. aureus* ATCC 6538 was obtained, also in agreeance with our results.¹² The MIC of 50 µg/mL for *S. aureus* ATCC 25923 and >750 µg/mL for *E. coli* ATCC 25922 was determined for SLs, similarly to the obtained results.² Furthermore, Pontes *et al.* (2016)² results showed that no growth inhibition was observed for SLs against *E. coli* ATCC 25922 at 750 µg/mL, in agreeance to the results obtained.

The use of Levofloxacin as control in the microdilution assay guaranteed the method suitability. Since, levofloxacin MIC was determined as 0.5 mg/L, which was in accordance with >0.25 mg/L and 0.25-0.5 mg/L determined previously by A. Matos *et al.* (2015)¹¹² and D. S. North *et al.* (1998)¹¹³.

4.4. Antimicrobial mechanism of SLs and RLs

Planktonic bacteria were submitted to different tests to elucidate SLs and RLs antimicrobial mechanism. Membrane integrity, cell viability, bacterial surface hydrophobicity and bacterial surface charge were evaluated for the six different strains.

4.4.1. Method Optimization

A previous test of optimization of different parameters such as inoculum final concentration, inoculation media, glycolipids concentration, incubation time and measuring times for propidium iodide (PI) uptake and resazurin (Rz) reduction was performed. The optimized parameter values were 5×10^5 CFU/mL, 0.9% NaCl, 0.4 mg/mL of glycolipids, 4 hours, 30 min and 3 h, respectively.

4.4.1.1. Optimization of Parameters

The first method used consisted of incubating the bacterial cells in MH medium for 24 h with a final inoculum concentration of 1.8×10^4 CFU/mL in the 96-well MTP. After an incubation period of 24 h, the glycolipids (0.05 mg/mL) and SDS were added. Then, PI and Rz assays were performed. The results from three separate measurements for *S. aureus* ATCC 25923 are presented in **Table 4.6**.

Table 4.6. Three separate assays for PI uptake ration and Rz reduction percentages obtained for *S. aureus* ATCC 25923.

Microorganism	Method	Samples				
		Control	RLs	SLs	SDS	
<i>S. aureus</i> ATCC 25923	Assay 1	Membrane Integrity	1.0 ± 0.1	1.0 ± 0.1	0.96 ± 0.1	0.4 ± 0.0
		Cellular Viability	100 ± 23.7	90.2 ± 22.8	63.6 ± 9.0	69.5 ± 6.5
	Assay 2	Membrane Integrity	1.0 ± 0.4	1.4 ± 0.3	1.1 ± 0.2	1.2 ± 0.1
		Cellular Viability	100 ± 27.8	48.5 ± 9.6	51.2 ± 7.7	125.3 ± 13.7
	Assay 3	Membrane Integrity	1.0 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	0.8 ± 0.1
		Cellular Viability	100 ± 22.8	20.5 ± 0.1	20.5 ± 0.0	103.9 ± 21.9

The results obtained for the three experiments were inconsistent for both the Membrane Integrity and Cellular Viability studied methods as observed in **Table 4.6**. The observable variability of the presented results was associated to the growing method for the inoculum and to the concentration of glycolipids used, therefore the optimization of these parameters was performed as mentioned in section 4.4.1.

4.4.1.2. Optimization of incubation time

Other parameters, such as, the incubation time for PI uptake was also evaluated with the purpose of improving the obtained results. Three different incubation times were accessed for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, incubated as previously mentioned in section 4.4.1.1., for 30 min, 1 h and 3 h as presented in **Table 4.7**.

Table 4.7. PI uptake ratios obtained at 3 different times (i.e. 30 min, 1 h and 3 h) for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922.

Microorganisms	Measuring Times	Method	Samples		
			Control	RLs	SDS
<i>S. aureus</i> ATCC 25923	30 min	Membrane Integrity	1.0 ± 0.0	1.5 ± 0.1	1.1 ± 0.0
	1 h	Membrane Integrity	1.0 ± 0.0	1.4 ± 0.1	1.1 ± 0.0
	3 h	Membrane Integrity	1.0 ± 0.2	1.3 ± 0.1	1.1 ± 0.0
<i>E. coli</i> ATCC 25922	30 min	Membrane Integrity	1.0 ± 0.0	1.5 ± 0.0	1.7 ± 0.1
	1 h	Membrane Integrity	1.0 ± 0.0	1.4 ± 0.0	1.5 ± 0.1
	3 h	Membrane Integrity	1.0 ± 0.2	1.4 ± 0.0	1.4 ± 0.0

Observing **Table 4.7** it was possible to conclude that the best incubation time for PI uptake was at 30 min, since slightly higher ratios were obtained for the two studied bacteria. The incubation time for Rz was fixed at 3 hours for all the experiments.

4.4.2. Propidium Uptake and Resazurin reduction

After the optimization, the two methods for PI uptake and Rz reduction were performed to evaluate the six strains (**Table 4.8**).

Table 4.8. PI uptake ratio and resazurin reduction percentages obtained for the six different strains with the ideal conditions.

Microorganisms	Method	Samples			
		Control	RLs	SLs	SDS
<i>S. aureus</i> ATCC 25923	Membrane Integrity	1.0 ± 0.0	2.5 ± 0.1*	1.1 ± 0.0	3.3 ± 0.1 *
	Cellular Viability	100.0 ± 9.9	83.1 ± 10.0	74.0 ± 5.7 *	82.1 ± 9.9
<i>S. aureus</i> ATCC 6538	Membrane Integrity	1.0 ± 0.0	2.5 ± 0.1 *	1.2 ± 0.0	3.2 ± 0.6 *
	Cellular Viability	100.0 ± 3.6	41.3 ± 6.2	32.8 ± 1.0 *	32.0 ± 2.9 *
MRSA	Membrane Integrity	1.0 ± 0.0	2.5 ± 0.1 *	1.2 ± 0.2	3.1 ± 0.1 *
	Cellular Viability	100.0 ± 13.8	77.8 ± 5.1	59.0 ± 6.9 *	65.4 ± 1.8 *
<i>S. epidermidis</i> ATCC 28319	Membrane Integrity	1.0 ± 0.0	2.4 ± 0.1 *	1.2 ± 0.0	2.9 ± 0.5 *
	Cellular Viability	100.0 ± 6.2	80.9 ± 5.1	71.0 ± 4.1 *	70.8 ± 7.6 *
<i>E. coli</i> ATCC 25922	Membrane Integrity	1.0 ± 0.0	2.5 ± 0.1*	1.1 ± 0.0	3.5 ± 0.1 *
	Cellular Viability	100.0 ± 21.1	165.0 ± 0.0	136.9 ± 0.0	88.3 ± 5.9
<i>P. aeruginosa</i> ATCC 15442	Membrane Integrity	1.0 ± 0.0	2.4 ± 0.0 *	1.2 ± 0.1	2.8 ± 0.3 *
	Cellular Viability	100.0 ± 14.0	34.5 ± 9.5 *	30.5 ± 10.1 *	27.8 ± 6.1 *

*Note: Statistically Significant (p value ≤ 0.05), when compared to the control.

The ratios of PI uptake obtained (**Table 4.8**) due to the action of RLs were always higher (2.4 and 2.5) than for action of SLs (1.1 and 1.2). RLs were able to significantly affect membrane integrity for all the strains, suggesting that RLs had higher membrane-damaging activity, when compared to SLs.

The percentages of Rz reduction were lower in the presence of SLs, suggesting higher effect on the metabolic activity of cells, when compared to RLs. Statistically, we can infer that SLs had significant action on all strains cell's viability, except for *E. coli* ATCC 25922. It was possible to conclude that *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442 were the most susceptible to both RLs and SLs. However, *E. coli* ATCC 25922 was not affected by both compounds.

We can conclude from the results obtained that RLs affected membrane integrity more actively and SLs affected cellular viability in a higher extent for all studied strains.

RLs can disrupt the cellular membrane through pore formation, influencing membrane integrity as it could be observed by the data obtained for PI uptake. This is in agreement with other studies that describe that RLs intercalates in the cytoplasmatic membrane resulting in changes to the cell surface charge and disruption of membrane fluidity, which leads to pore formation, therefore increasing membrane permeability.^{114,115} The pore formation can allow the rapid influx or uptake of antibiotics that were ineffective, due to antibiotic resistance.¹¹⁶ Radlinski *et al.* (2019)¹¹⁷ found that RLs promoted the cellular uptake of aminoglycosides antibiotics, tobramycin, that otherwise are proton motive force-dependent and the entrance is hindered in antibiotic resistant bacteria.

The technique described by P. Stiefel *et al.* (2015)¹¹⁸ was followed in this work, but the evaluation of both parameters membrane integrity (uptake PI) and cell viability (reduction of Rz) to evaluate the activity of RLs and SLs, had never been executed, as far as we know. Therefore, not a lot of studies were presented to support the results obtained. The assay used to evaluate membrane integrity and cell viability is generally applied with a combination dye BacLight live/dead of propidium iodide and Syto9.⁹⁹ Some studies have demonstrated that both RLs and SLs affect membrane integrity and cellular viability.

Such as Sotirova *et al.* (2012)⁹⁹ that reported that RLs (10 µg/mL) had an effect on bacteria shown by SEM as shown by surface infolds and small blebs on *B. subtilis*, however no changes in membrane permeability were verified. The results obtained suggest that the RLs at this concentration did not affect membrane integrity. However, only when RLs were combined with MTS (50 µg/mL) a dramatic permeability shift for propidium iodide was observed in vegetative cells, proving to have a synergistic action.

Furthermore, SLs also were reported by V. Dengle-Pulate *et al.* (2014)⁸⁴ to change bacterial cells of *E. coli* (30 µg/mL at 2 h), *P. aeruginosa* (1 µg/mL at 4 h), *S. aureus* (6 µg/mL at 4 h), *B. subtilis* (1 µg/mL at 4 h,) and *C. albicans* (50 µg/mL at 4 h) shown by morphological and topographic changes in SEM analysis. Lysis of cells causing extrusion of the cell contents were observed for Gram positive bacteria, while in the case of Gram negative bacteria it merely caused shrinking of the cells or the presence of irregularities on the cell surface.

4.4.3. Bacterial surface hydrophobicity

With the purpose of elucidating further the antimicrobial mechanism, by which these compounds affect bacterial cells' surface, a method described by Rosenberg *et al.* (1980)¹¹⁹ was used to evaluate bacterial cell surface hydrophobicity (CSH). The method was based on the partitioning of bacterial cell suspensions with or without prior contact with 0.4 mg/mL of RLs or SLs for 3 h. The measurements of the OD from the water phase initial and final were used as an index of cell hydrophobicity by calculating the partition coefficient (P%), therefore possible to evaluate the affinity of the cells to the interface created. The partition assay was performed using 1-octanol instead of n-hexadecane as described by Sodagari *et al.* (2013)¹⁰² and the results were obtained and expressed as an average of the partition coefficient percentage (P%) presented in **Table 4.9**.

Table 4.9. Bacterial surface hydrophobicity obtained as Partition Coefficient (P%) for the six different strains without biosurfactants, in the presence of RLs or in the presence of SLs.

Bacterial Strains	Partition Coefficient (P%)		
	Biosurfactant-free	RLs (0.4 mg/mL)	SLs (0.4 mg/mL)
<i>S. aureus</i> ATCC 25923	24.8 ± 3.3	74.7 ± 2.4*	41.7 ± 0.3
<i>S. aureus</i> ATCC 6538	51.6 ± 2.2	76.1 ± 2.2*	59.7 ± 18.9
<i>MRSA</i>	24.1 ± 5.2	43.9 ± 4.1*	29.7 ± 4.4
<i>S. epidermidis</i> ATCC 28319	18.4 ± 7.8	19.0 ± 5.3	4.7 ± 1.5*
<i>P. aeruginosa</i> ATCC 15442	25.0 ± 8.3	55.9 ± 18.0*	38.3 ± 10.7
<i>E. coli</i> ATCC 25922	34.5 ± 2.0	35.1 ± 1.1	22.2 ± 4.7*

*Note: Statistically Significant (p value ≤ 0.05), when compared to the control (Biosurfactant-free).

The obtained data for partition coefficient (**Table 4.9**) showed that *S. aureus* ATCC 6538 present the highest hydrophobicity corresponding to 51.6 ± 2.2 %. In the presence of RLs, bacterial cell's hydrophobicity increased for *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *MRSA* and *P. aeruginosa* ATCC 25922, increases ranging from 49.9 to 19.8 %. However, *S. epidermidis* ATCC 28319 and *E. coli* ATCC 25922 were not significantly affected by the presence of RLs.

S. aureus ATCC 25923, *S. aureus* ATCC 6538, *MRSA* and *P. aeruginosa* ATCC 25922 present an increase of Partition Coefficient (P%) from 16.9 to 5.6 %, in the presence of SLs. However, the hydrophobicity of *S. epidermidis* ATCC 28319 and *E. coli* ATCC 25922 decreased 13.7% and 12.3%, respectively.

With the obtained results it was possible to conclude that both glycolipids affected bacterial cell's hydrophobicity, however RLs had higher activity. Additionally, it was determined that *S. epidermidis* ATCC 28319 and *E. coli* ATCC 25922 bacterial cells were less vulnerable to both compounds.

Several studies have shown that RLs, cause a decrease in cell surface hydrophobicity for primary hydrophobic cells.⁶⁰

However, studies have reported different and contradictory effects on CSH. Sodagari *et al.* (2013)¹⁰² discovered that RLs (1 g/L) increased the hydrophobicity of *P. aeruginosa* PAO1 and *E. coli*, decreased the hydrophobicity of *P. putida* and *S. epidermidis*, and had no clear effect on *B. subtilis*. Zhang and Miller *et al.* (1994)¹²⁰ reported that RLs (6 mM) increased the hydrophobicity of *P. aeruginosa* ATCC 9027 and NRRL 3198, however did not have an effect on fast degraders *P. aeruginosa* ATCC 15442 and ATCC 27853. Still, a consensus was found that the decrease of CSH was observed at high concentrations of RLs and increase at lower concentrations.

Ma Zhao *et al.* (2018)¹²¹ reported an increase, when 0.1 mg/mL of RLs was used on the strain *Pseudomonas sp.* Ph6. Another article by Zhong *et al.* (2007)¹²² concluded that the adsorption, at low concentrations of RLs (2.5 mg/mL) caused an increase of the CSH, however when higher concentrations (25 or 250 mg/mL) are provided it has been reported a stabilization or decrease for *P. aeruginosa*.⁵⁷ These findings are in agreeance with the performed experiments, since lower concentrations of 0.4 mg/mL were used.

This was further proven by Kaczorek *et al.* (2008)¹²³ that observed that RLs at 0 to 10 mg/mL slightly increased CSH for *Candida maltosa*. However, the hydrophobicity of yeasts (*Candida maltosa* and *Yarrowia lipolytica*) was not affected, when RLs were below 75 mg/L and decreased for higher RLs concentrations.

Furthermore, the increase in hydrophobicity may also be due to the adsorption of RLs in the surface and the modification of cell compounds presented in the cell surface.⁵⁷ This was evaluated by Al-Tahhan *et al.* (2000)¹²⁴ that discovered that the presence of RLs (6 mM) caused the cell surface of *Pseudomonas aeruginosa* ATCC 27853 and ATCC 9027 to become hydrophobic through release of LPS. This effect was also verified by A. Sotoriva *et al.* (2009)¹²⁵, RLs at concentrations 0 to 100 µg/mL increased slightly the cell surface hydrophobicity of *P. aeruginosa* and maintained from 100 to 300 µg/mL. It was proposed that this increase was also due to a reduction of the total cellular LPS content.

4.4.4. Bacterial Surface Charge

As previously reported, it has been discovered that glycolipids, RLs and SLs, affect cell surface charge.^{56,57} Therefore, Zeta potential measurements were performed for characterization of bacterial surface charges modifications in the presence of RLs and SLs at concentrations of 0 and 0.4 mg/mL. The zeta potential (mV) measurements of the six different strains evaluated are presented in **Table 4.10**.

Table 4.10. Bacterial surface charge accessed by zeta potential (mV) measurements obtained for the six different strains with RLs or with SLs at 0 and 0.4 mg/mL.

Samples	Biosurfactants Concentration (mg/mL)	RLs	SLs
		Zeta Potential (mV)	Zeta Potential (mV)
Bacteria free Biosurfactant Solution	0.4	-21.2 ± 1.4	-47.3 ± 6.7
<i>S. aureus</i> ATCC 25923	0	-9.2 ± 0.3	-9.2 ± 0.3
	0.4	-15.6 ± 3.2*	-23.3 ± 2.7*
<i>S. aureus</i> ATCC 6538	0	-25.6 ± 0.6	-25.6 ± 0.6
	0.4	-20.2 ± 3.7	-29.0 ± 3.5
<i>MRSA</i>	0	-34.2 ± 0.8	-34.2 ± 0.8
	0.4	-35.7 ± 2.8	-39.5 ± 2.0
<i>S. epidermidis</i> ATCC 28319	0	-28.3 ± 1.8	-28.3 ± 1.8
	0.4	-28.6 ± 4.1	-33.4 ± 3.0
<i>P. aeruginosa</i> ATCC 15442	0	-29.5 ± 1.7	-29.5 ± 1.7
	0.4	-32.2 ± 1.5	-31.6 ± 0.6
<i>E. coli</i> ATCC 25922	0	-50.6 ± 1.5	-50.6 ± 1.5
	0.4	-50.0 ± 1.1	-50.6 ± 3.2

*Note: Statistically Significant (p value ≤ 0.05), when compared to the control (Bacteria with biosurfactant 0 mg/mL).

Firstly, the results obtained demonstrated that the samples of glycolipids, at 0.4 mg/mL, without added bacteria conferred negative charge to the zeta potential measurements. Furthermore, it was possible to conclude that SLs presented higher negative charge (-47.3 ± 6.7), when compared to RLs (-21.2 ± 1.4).

A low increase or maintenance of negative charge of all the bacterial cells was observed in the presence of RLs for all studied strains ranging from 0 to 6.4 mV. However, RLs were able to decrease significantly the bacterial surface charge of *S. aureus* ATCC 25923, by increasing 6.4 mV the negative charge.

SLs caused an increase of negative charge of all bacterial strains, except for *E. coli* ATCC 25922 that maintained the zeta potential value of -50.6 mV. The most affected bacteria was once again *S. aureus* ATCC 25923, increasing 14.1 mV the negative charge. The other strains increased the measured negative charge ranging from 0 to 5.3 mV.

Comparing the two glycolipids it was possible to conclude that SLs were able to increase bacterial negative charge more significantly than RLs, however the increase can be due to the negative charge contribution that the compound confers to the samples.

Nevertheless, in previous reports it was found that RLs and SLs affected bacterial surface charge.^{92,57,56} The decrease in zeta potential can be due to the adsorption of the anionic RLs (0.5 to 20 mg/mL) caused by the negative charges it provides the surface of the cells on *P. aeruginosa*, according to Ishigami *et al.* (1987)¹²⁶. This effect was observed in our results, though the concentrations used in our experiments were lower.

However, the increase of zeta potential was also reported by Liu *et al.* (2012)⁹³ due to the pre-treatment of *P. simplicissimum*, with RLs (0.5 g/L). The release of cell surface compounds, such as proteins and LPS caused by RLs (0 to 10 mM) was reported by Al-Tahhan *et al.* (2000)¹²⁴. This can cause the increase of the zeta potential, due to the negative charge these confer to the cell surface and favor electrostatic interactions with cations, reducing the negative charge of the cell.

4.5. Antimicrobial Activity of RLs towards Sessile bacteria

4.5.1. Surface characterization of PDMS specimens by contact angle measurements

To proceed with the assessment of glycolipids antibiofilm activity, when adsorbed onto PDMS specimens, it is important to first characterize the surface. Therefore, contact angle measurements were performed to study the wettability properties of PDMS specimens functionalized with RLs adsorbed to their surface. The contact angle measurements of the PDMS specimens functionalized with concentrations of RLs ranging from 0 to 3 mg/mL after 10 minutes are presented in **Figure 4.7**.

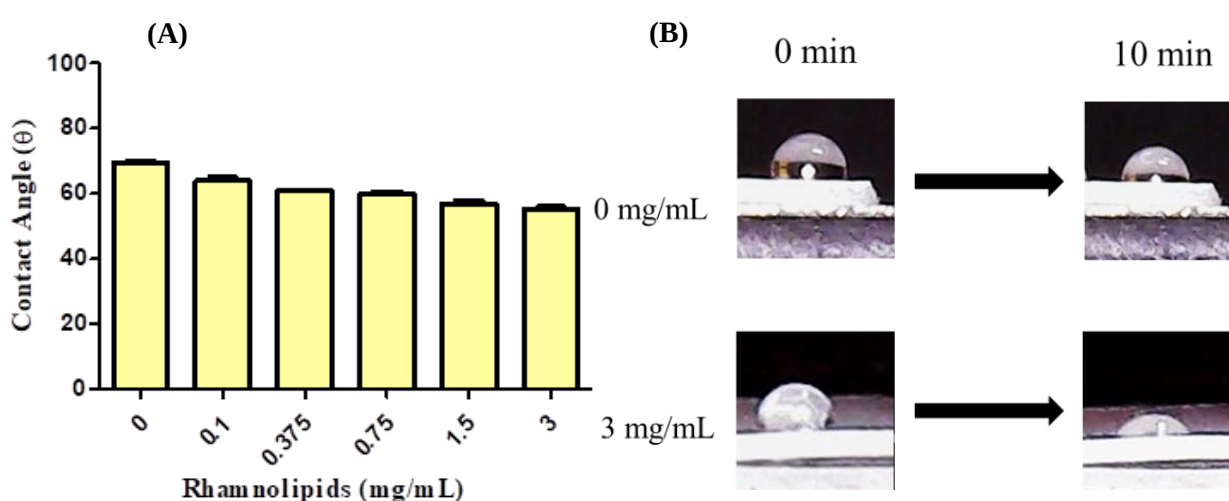


Figure 4.7. Surface wettability of PDMS specimens. Contact angle measurements (A) of PDMS specimens functionalized with RLs at concentrations of 0, 0.1, 0.375, 0.75, 1.5 and 3 mg/mL adsorbed to the surface after 10 min. (B) Images of Mili-Q water drops in contact with different PDMS specimens (PDMS-RLs0.0 and PDMS-RLs3.0 at t=0 and t=10 min.

As the concentrations of RLs absorbed to the surface of the PDMS specimens increased the contact angle decreased, ranging from approximately $71 \pm 5^\circ$ to $55 \pm 1^\circ$. The decrease in the contact angle was indicative of higher wettability properties of the functionalized PDMS specimens. The images of the water drops in the PDMS specimens covered with RLs at the initial time of 0 min and after 10 min (**Fig. 4.7B**) also demonstrate the wettability changes in the surfaces by the appearance of the droplets.

Therefore, it was possible to conclude that the method of functionalization created a less hydrophobic surface on the PDMS material surface. Furthermore, the change in surface properties will influence bacteria adhesion.² A more hydrophilic surface hinders the attachment of microorganisms, which will consequently avoid biofilm formation.

Contact angle measurements were already performed previously with different surfaces covered with SLs (*Starmerella bombicola* CBS 6009), RLs (*P. aeruginosa* LBI) and biosurfactant (*Lactobacillus spp.*) at different concentrations, such as glass, PVC, PET, PDMS and others to study their wettability properties.^{2,127,128}

A study has shown that adding RLs (0 to 1000 mg/L) to cover a surface of PDMS specimens decreased the contact angle at higher concentrations than 51.5 mg/L (Critical Micellar Concentration-CMC), which was congruent with the obtained results.¹²⁷ Rodrigues *et al.* (2006)¹²⁹ also observed this improvement of wettability properties on silicone rubber, due to the decrease in the contact angle when using RLs at 4 mg/mL or 2 mg/mL.

Consequently, we can conclude that the PDMS specimens functionalized by adsorption with RLs at the studied concentrations can be used in the evaluation of glycolipids antibiofilm activity. The surface characterization of PDMS specimens covered by SLs has already been previously reported, hence why it was not evaluated.²

4.5.2. Biofilm Quantification

A preliminary assay had already been performed to evaluate biofilm inhibition (MBIC), as previously presented in section 4.3. Nevertheless, it was essential to understand if biofilm formation was inhibited in the presence of glycolipids adsorbed onto the medical grade PDMS specimens. Because the results previously obtained were only evaluated for biofilm formation on the bottom of the MTP, instead of the PDMS surface. Therefore, crystal violet stain was used to evaluate and quantify biofilm formation for the four strains of microorganisms previously identified as vulnerable. Firstly, biofilm quantification was evaluated for PDMS specimens functionalized with RLs concentrations ranging from 0 to 3 mg/mL as presented in **Figure 4.8**.

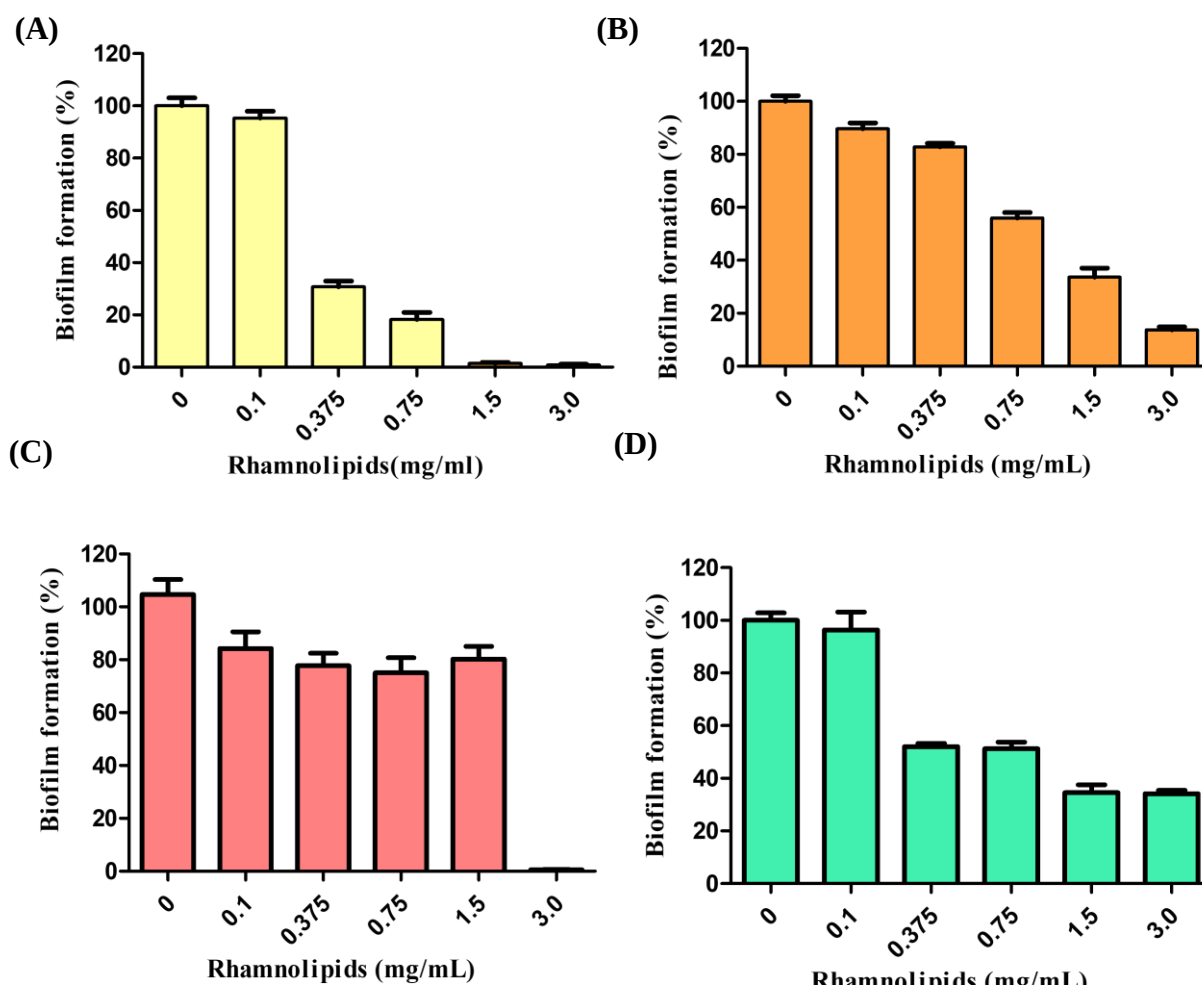


Figure 4.8. Biofilm formation assessed through crystal violet staining on RLs functionalized PDMS specimens. Biofilm formation of *S. aureus* ATCC 25923 (A), *S. aureus* ATCC 6538 (B), *S. epidermidis* ATCC 28319 (C) and MRSA (D) was produced on medical grade PDMS specimens. Different RLs solutions (0.10; 0.375; 0.75; 1.50 and 3.00 mg/mL) were used to promote RLs adsorption.

Firstly, a decrease in biofilm formation was perceptible, for the tested strains, as the concentrations of adsorbed RLs in the PDMS specimens increased. However, this effect was not observed against *S. epidermidis* ATCC 28319 that maintained a reduction, only reducing significantly at 3 mg/mL.

Overall, when concentrations of 0.75 mg/mL of RLs were used to functionalize PDMS specimens, a considerable inhibition of biofilm formation for the other three strains was obtained, ranging from 44 to 80% of biofilm inhibition. Nevertheless, the concentration of 3 mg/mL was the most effective against biofilm formation of all strains. Furthermore, RLs had higher antibiofilm activity against *S. aureus* ATCC 25923 and MRSA, since considerable inhibition of 70 and 48% was observed, respectively, at 0.375 mg/mL.

4.5.3. Cellular Viability

Crystal violet stains all the biofilm present in the surface of the PDMS specimens allowing the quantification of the biofilm formation. Therefore, another method was needed to quantify the viable cells in the surface of the PDMS specimens, such as colony forming units (CFU) counting. Counting colony forming units of PDMS specimens functionalized by adsorption with RLs was performed using the same concentrations ranging from 0 to 3 mg/mL. The same four strains were evaluated, and the results are displayed in Figure 4.9.

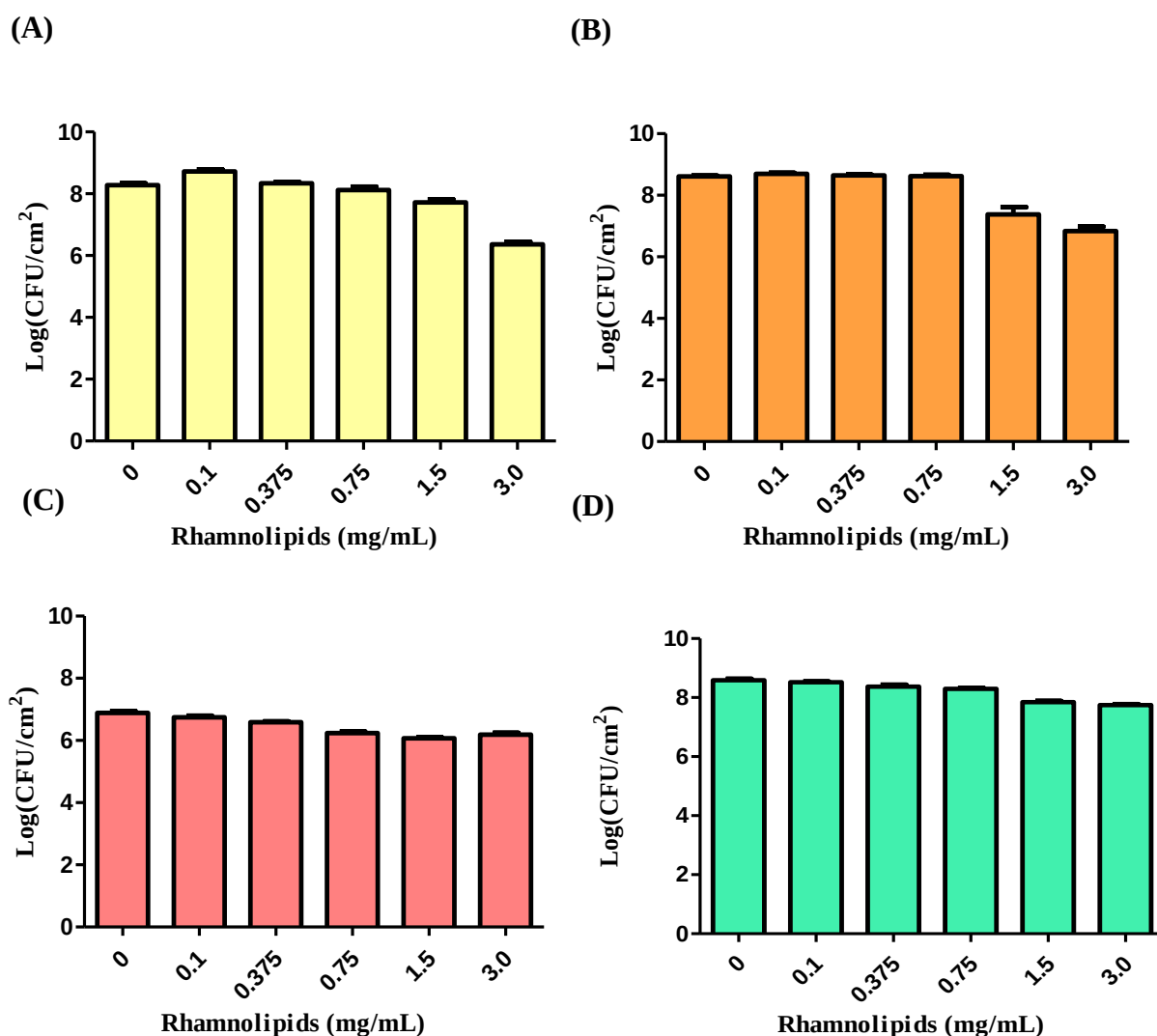


Figure 4.9. Biofilm formation assessed through colony forming units (CFU) counting on RLs functionalized PDMS specimens. Biofilm formation of *S. aureus* ATCC 25923 (A) *S. aureus* ATCC 6538 (B), *S. epidermidis* ATCC 28319 and *MRSA* (D) was produced on medical grade PDMS specimens. Different RLs solutions (0.10; 0.375; 0.75; 1.50 and 3.00 mg/mL) were used to promote RLs adsorption.

Overall, a decrease of biofilm formation was observed for all studied strains as the concentrations of RLs previously applied increased. Substantial reduction for all tested strains was obtained especially at concentrations 1.5 and 3 mg/mL.

In accordance with crystal violet assay, higher antibiofilm activity was obtained for RLs against *S. aureus* ATCC 25923. The number of colonies reduced substantially, achieving a reduction of 2 log units at concentration of 3 mg/mL, when compared to the positive control. A similar behaviour was obtained against *S. aureus* ATCC 6538 where a reduction of 1.8 log units was reached. As previously observed in section 4.5.2. the observed reduction was not as substantial as for *S. aureus* ATCC 25923, which was congruent.

Biofilm formation inhibition for *MRSA* reached a reduction lower than 1 log unit at 0.8, proving to be less affected than the strains described above. This behavior was congruent to the previously observed, because at concentration of 3 mg/mL biofilm formation in section 4.5.2. was still high compared to other strains.

The reduction in viable cells for *S. epidermidis* ATCC 28319 was not very substantial, proving once more that this strain was less vulnerable to the presence of RLs as described previously. The reduction of the biofilm formation was lower than 1 log unit at 0.7 log units.

The results obtained allowed to conclude that RLs were capable of decreasing biofilm formation on PDMS specimens. The antibiofilm activity can be due to the decrease in hydrophobicity of this material observed previously in the contact angle measurements.

4.6. Antimicrobial Activity of SLs towards Sessile bacteria

4.6.1 Biofilm Quantification

Biofilm quantification was performed using the crystal violet staining on PDMS specimens functionalized with SLs at concentrations ranging from 0 to 3 mg/mL presented in Figure 4.10.

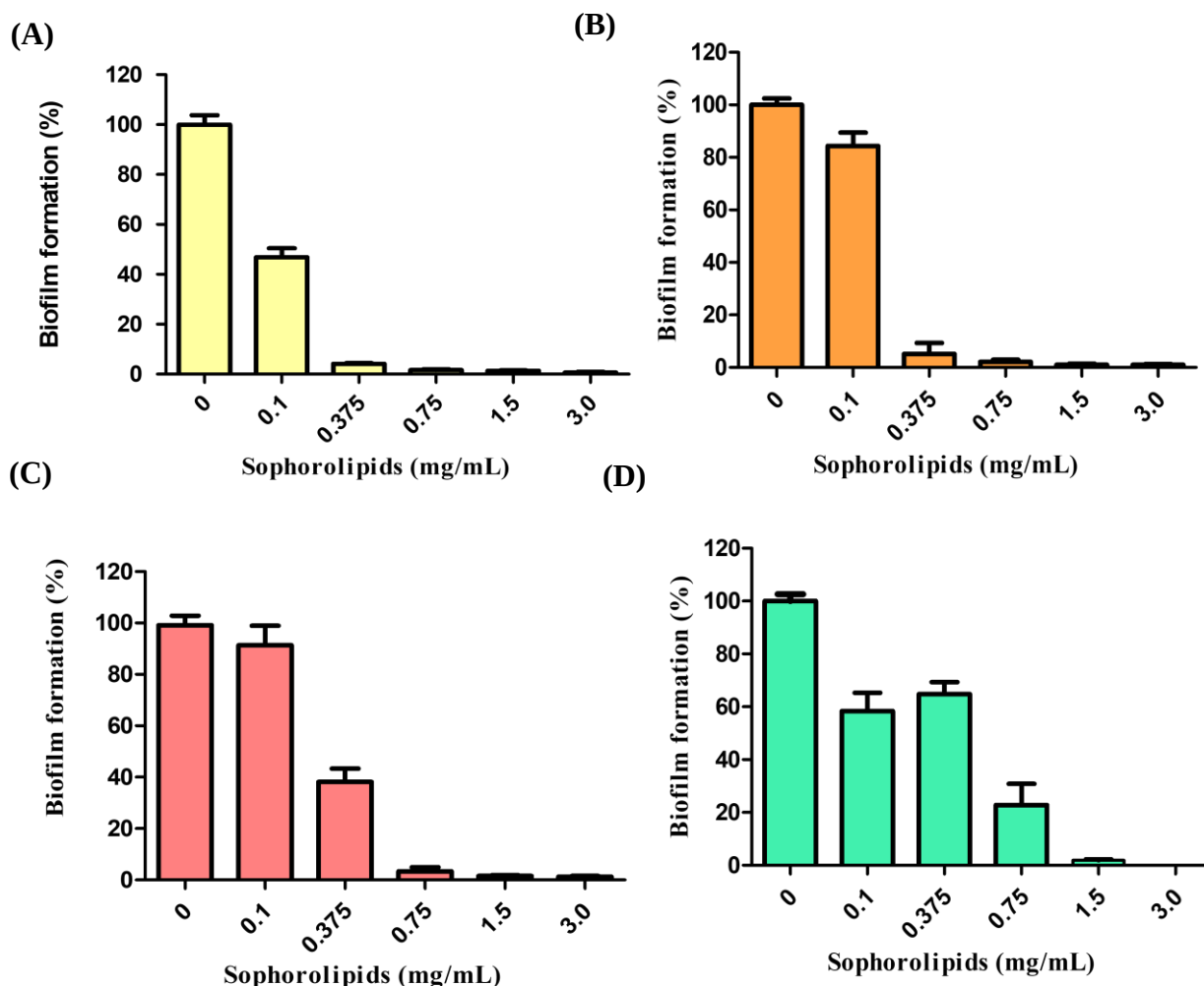


Figure 4.10. Biofilm formation assessed through crystal violet staining on SLs functionalized PDMS specimens. Biofilm formation of *S. aureus* ATCC 25923 (A), *S. aureus* ATCC 6538 (B), *S. epidermidis* ATCC 28319 (C) and MRSA (D) was produced on medical grade PDMS specimens. Different SLs solutions (0.10; 0.375; 0.75; 1.50 and 3.00 mg/mL) were used to promote RLs adsorption.

Overall, as the concentrations of adsorbed SLs in the PDMS specimens increased a decrease in biofilm formation was observed for all studied strains. Biofilm inhibition ranged from 98 to 100% at the highest concentration of 3 mg/mL. Generally, when concentrations of 0.75 mg/mL of SLs were used to functionalize PDMS specimens, a considerable inhibition of biofilm formation was obtained for all strains, ranging from 77 to 98%. Therefore, concentration of 0.75 mg/mL can be identified as

the most effective against the four strains. However, at that concentration *MRSA* displayed less biofilm inhibition, when compared to other strains.

SLs presented higher antibiofilm activity against *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 28319, since considerable inhibition of 96, 95 and 62% was observed, respectively, at 0.375 mg/mL.

4.6.2 Cellular Viability

Antibiofilm Activity of SLs was also evaluated with the counting colony forming units' method of PDMS specimens functionalized by adsorption with SLs and the obtained results are presented in **Figure 4.11**.

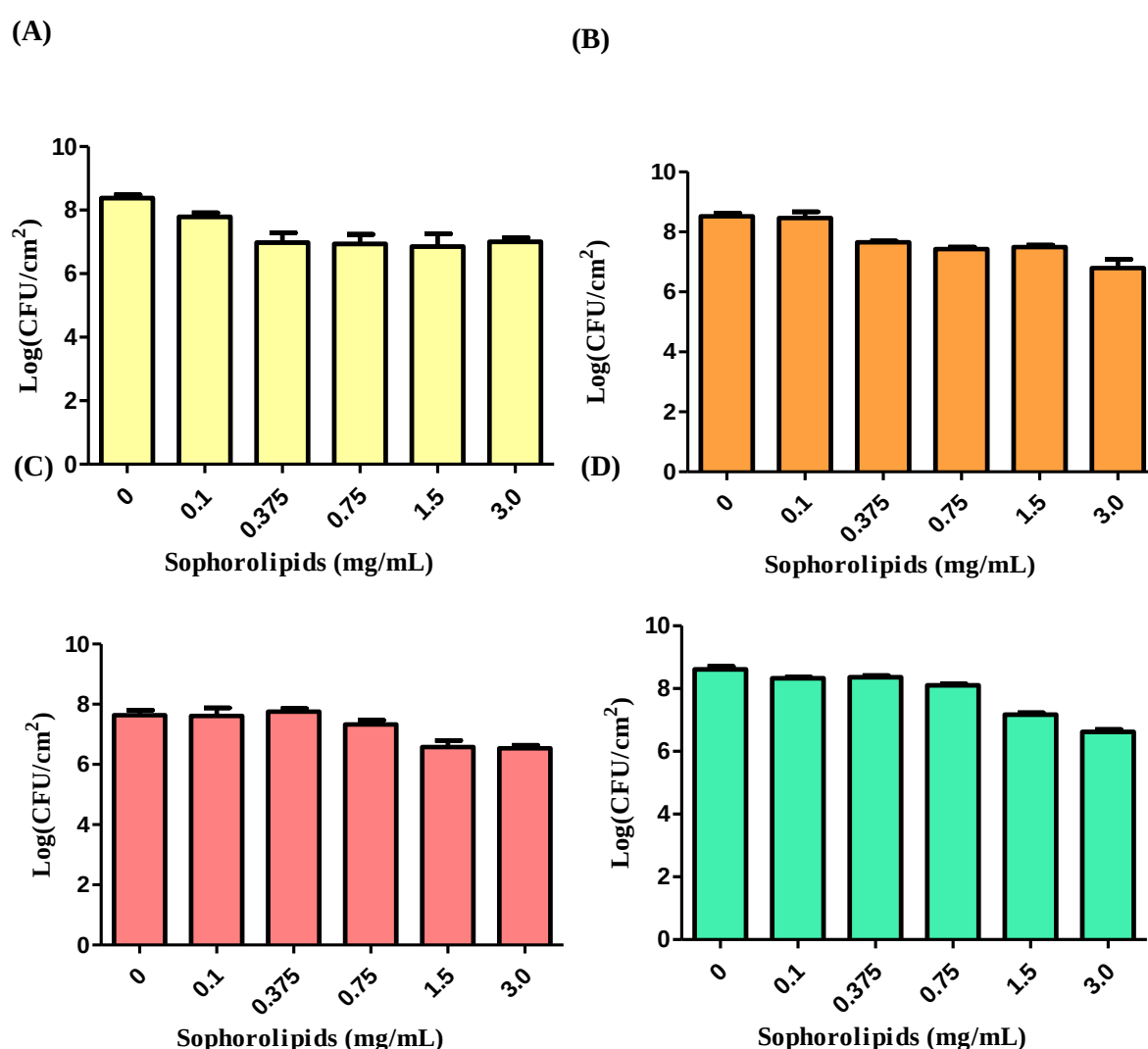


Figure 4.11. Biofilm formation assessed through colony forming units (CFU) counting on SLs functionalized PDMS specimens. Biofilm formation of *S. aureus* ATCC 25923 (A) *S. aureus* ATCC 6538 (B) and *MRSA* was produced on medical grade PDMS specimens. Different SLs solutions (0.10; 0.375; 0.75; 1.50 and 3.00 mg/mL) were used to promote SLs adsorption.

Overall, the number of colonies reduced as the concentrations of SLs applied to the PDMS specimens increased. The reduction of the number of colonies was prominent especially at concentrations of SLs of 0.375 mg/mL for *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and 1.5 mg/mL for *S. epidermidis* ATCC 28319 and *MRSA*. However, substantial reduction for all tested strains was obtained especially at concentrations of 3 mg/mL of SLs.

A reduction of 2 log units was obtained for *MRSA*, a reduction of 1.4 log units was observed for *S. aureus* ATCC 25923 and 1.7 log units for *S. aureus* ATCC 6538, however a reduction of 1.1 log unit was obtained for *S. epidermidis* ATCC 28319 proving to be less vulnerable.

The behaviour displayed by *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538 was similarly described in section 4.6.1, where substantial reduction of the number of colonies and biofilm formation was obtained at 0.375 mg/mL.

Some reports have already examined the antibiofilm activity of SLs and RLs.^{2,56,51} Nevertheless, the role that these biosurfactants play in biofilm inhibition has not been heavily studied. An article by Pontes *et al.* (2016)² evaluated biofilm formation with crystal violet and colony forming units of SLs at concentrations ranging from 0 to 3 mg/mL against *S. aureus* ATCC 25923. With the increasing of SLs concentrations, biofilm inhibition of *S. aureus* ATCC 25923 increased, in agreeance with our results. A percentage of 100% of biofilm inhibition was obtained at 0.38 mg/mL, similarly to the 96 % biofilm inhibition obtained in our results. Furthermore, a reduction of 3 log units was obtained, when evaluating CFU counts at a concentration of SLs of 1.5 mg/mL against *S. aureus* ATCC 25923.

4.7. Plasma treatment and RLs functionalization on PDMS Specimens

After, evaluating the antibiofilm activity of glycolipids adsorbed on the surface of PDMS specimens it was important to explore other methods of functionalization. The functionalization of PDMS specimens with glycolipids by adsorption is not a permanent method, because these compounds lose their properties due to time and dilution in fluids. Therefore, it would be beneficial to maintain their antibiofilm activity as a long-term property.¹³⁰

Plasma treatment is the proposed method that reportedly can create a more hydrophilic surface, resulting in a more long-lasting bond of a certain compound to a surface.^{131,132} The functionalization of PDMS specimens with plasma treatment is not a widely studied topic with glycolipids, so the findings will not be heavily supported by bibliography and more supplementary studies should be performed for this treatment to be optimized.

4.7.1. Characterization of PDMS specimens by Contact Angle Measurements

Firstly, surface characterization was performed by contact angle measurements on PDMS specimens adsorbed with RLs at concentrations of 15, 30, 60 and 120 mg/mL previously subjected to plasma treatment. The contact angle measurements were obtained for the PDMS specimens after 5 and 10 min of plasma treatment as displayed in Figure 4.12.

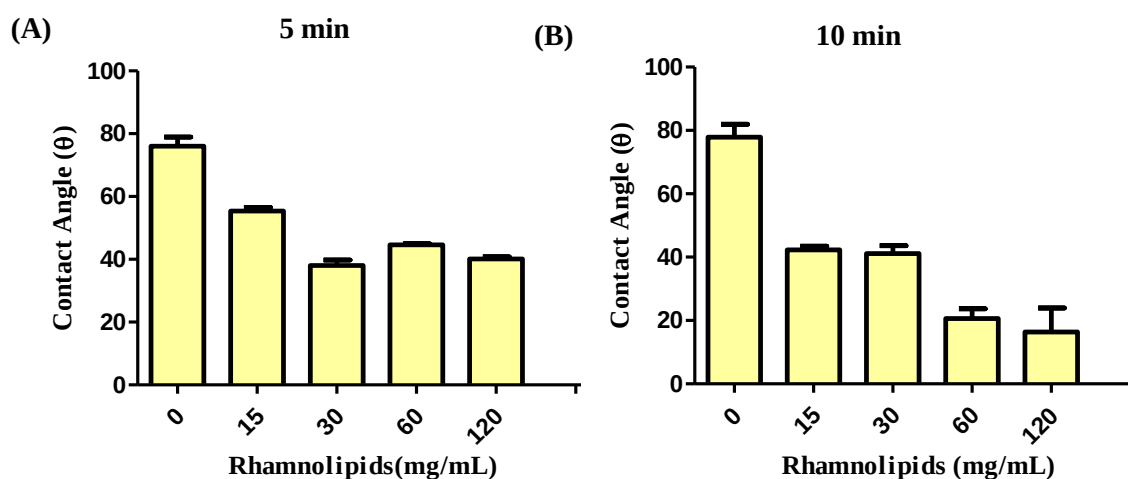


Figure 4.12. Surface wettability of PDMS specimens treated with plasma. Contact angle measurements performed for 5 (A) and 10 min (B) of plasma treated PDMS specimens functionalized with RLs at concentrations of 15, 30, 60 or 120 mg/mL.

The measurements allowed to perceive the functionalization of PDMS specimens with RLs at different concentrations, previously treated with plasma for 5 or 10 min. For both treatments it was possible to conclude a reduction of the contact angle as the concentrations increased, concluding that the surface wettability properties increased.

For plasma treatment after 5 min (**Figure 4.12A**) it was observable a reduction of approximately 20° at 15 mg/mL from $76 \pm 7^\circ$ to $55 \pm 2^\circ$, when compared to the control. As the concentration increased to 30 mg/mL the contact angle decreased further, at approximately $38 \pm 3^\circ$. However, the reduction stabilized at approximately 40° , not reducing further as the concentrations increased to 60 and 120 mg/mL.

After 10 min (**Figure 4.12B**) we can conclude the contact angle measurements were similar between concentrations 15 and 30 mg/mL, reducing from approximately 78 to $43 \pm 2^\circ$ and $41 \pm 6^\circ$, respectively. As the concentrations increased to 60 and 120 mg/mL the reduction of contact angle became more prominent reducing to approximately 20° and 16° , respectively.

It was possible to conclude that the functionalization of the PDMS specimens with RLs was successful due to the contact angle reduction observed for both treatments. However, the plasma treatment of 10 min (B) allowed a higher reduction of the contact angle, when compared to 5 min(A).

4.7.2. Fourier Transform Infrared Spectroscopy- Attenuated total reflectance

FTIR-ATR analysis was performed to evaluate qualitatively the RLs adsorbed onto medical grade PDMS, previously treated with plasma. The analysis is achieved by identifying representative bands belonging to the PDMS specimens and RLs chemical groups. The obtained spectra are representative of the PDMS specimens previously treated with plasma (A), PDMS specimens covered by RLs (B) previously treated with plasma and RLs solution (C) presented in **Figure 4.13** with a range of 4000-400 cm^{-1} .

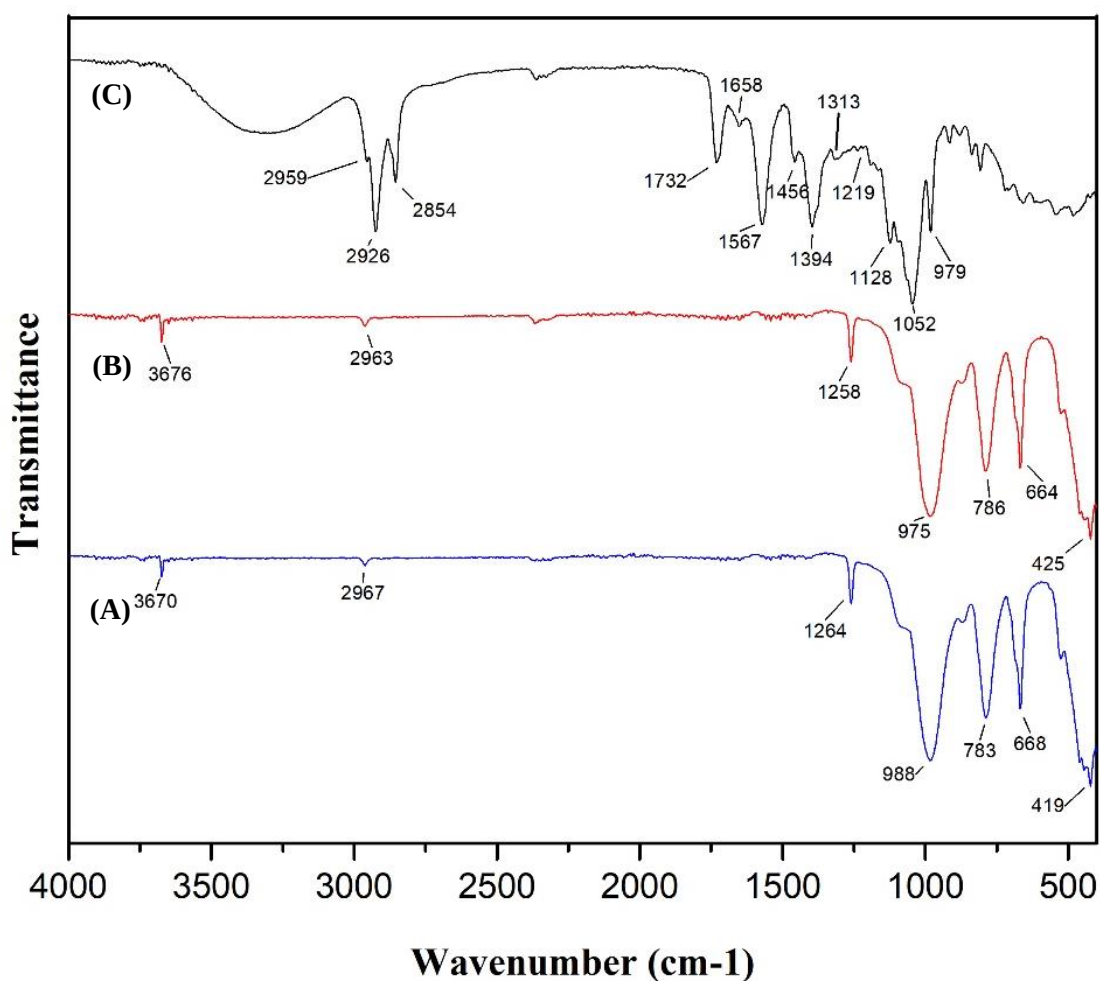


Figure 4.13. FTIR-ATR spectra obtained for PDMS specimens previously treated with plasma (A), PDMS specimens previously treated with plasma adsorbed with RLs by spraying method (B) and RLs solution (C).

The characteristic bands of PDMS are observed at 1264, 1098 and 988 cm^{-1} , associated to Si-CH₃, Si-C and Si-O stretching bonds, respectively, showing typical FTIR-ATR spectrum of PDMS (**Figure 4.13(A)**). The characteristic bands of PDMS were already described in previous reports as Pontes *et al.* (2016)² and Kim *et al.* (2012)¹³³.

The RLs characteristic bands are also presented in **Figure 4.13(C)** that were previously described by F. Leitermann *et al.* (2008)¹³⁴ and P. Rhaman *et al.* (2010)¹³⁵. The easily identifiable wide/broad bands at 3676 cm^{-1} and 1658 cm^{-1} were correspondent to the presence of the hydrogen bonding and O-H stretching of residual water.

The vibrational double bands identified at 2926 and 2854 cm^{-1} correspond to symmetric C- H stretching modes of aliphatic groups, more specifically of hydroxydecanoic acid chain tails of rhamnolipid. At 1732 cm^{-1} it was possible to identify the C=O stretching mode characteristic of carboxylic acid groups and ester bonds. The peak at 1567 cm^{-1} is attributed to C=O vibration of the carboxylic groups.

Furthermore, the peak at 1456 cm^{-1} corresponds to C-H deformation, 1394 cm^{-1} to O-H deformation and 1313 cm^{-1} to O-H deformation, accordingly to F. Leitermann *et al.* (2008)¹³⁴.

Although it was not possible to observe RLs characteristic band on the PDMS specimens previously treated with plasma adsorbed with RLs by FTIR-ATR (**Figure 4.13(B)**), from the contact angle analysis it is possible to conclude that surface had been successfully modified.

4.7.3. Cellular Viability towards sessile bacteria

The PDMS specimens were functionalized by adsorption with RLs at concentrations of 0, 15, 30, 60 and 120 mg/mL and submitted to either 5 or 10 min of plasma treatment for evaluating antibiofilm activity against *S. aureus* ATCC 25923 as presented in **Figure 4.14**.

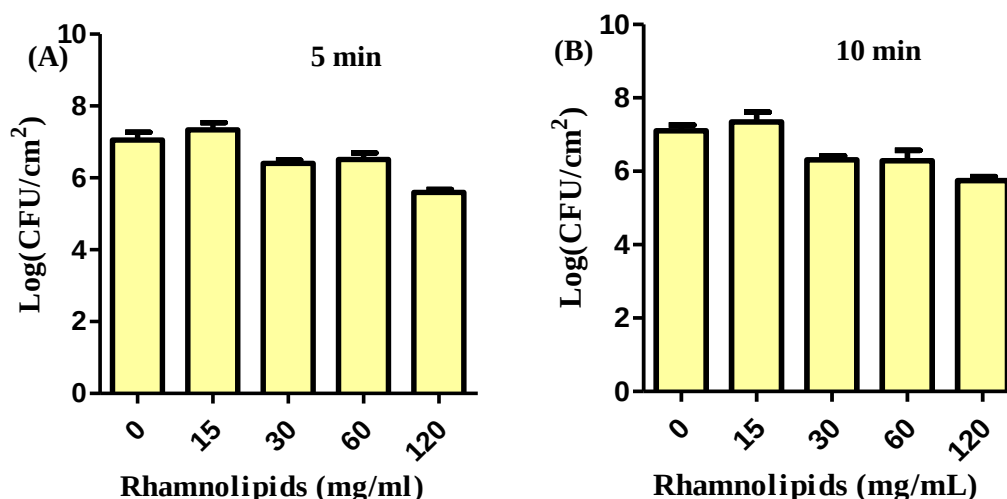


Figure 4.14. *S. aureus* ATCC 25923 colony forming units (CFU) count for inhibition of biofilm formation evaluation of PDMS specimens functionalized with RLs at concentrations of 0, 15, 30, 60 and 120 mg /mL and subjected to plasma treatment for 5 (A) and 10 min (B).

Firstly, it was observable a reduction in the CFU for all experimented conditions as the concentrations of RLs used to functionalize the PDMS surface increased. Reduction of the number of colony forming units was only observable when using concentrations of 30 mg/mL and higher for both duration of plasma treatments. A reduction of the colony forming units was obtained as over 1 log unit for both 5 (**Figure 4.14(A)**) and 10 min (**Figure 4.14(B)**) of plasma treatment, when compared to unfunctionalized PDMS.

It was possible to conclude that the most effective treatment was obtained when using the RLs at concentrations of 120 mg/mL, since the highest reduction was obtained. Furthermore, the treatments with plasma for 5 or 10 min did not have substantial differences, due to the similar results obtained.

In the future more experiments should be performed to evaluate this new treatment of using plasma to create a more lasting functionalization.

✓ Chapter 5: Conclusions and future work

5. Conclusions and future work

Currently, the presence of biofilm on the surface of implanted medical devices is a global issue that can lead to medical device-associated infections, which can endanger a patient's life. Many microorganisms can adhere, multiply and form biofilm onto the surface of medical devices hindering the effectiveness of the available treatment, such as antibiotics. The loss of effectiveness is due to the antimicrobial protection of the biofilm and the possibility of emergence of antibiotic resistance. Therefore, there is a need of novel strategies to fight this problem that can effectively prevent and treat the formation of biofilm on these surfaces.

Recently, BS have gained some interest demonstrating potential for the treatment of these infections, due to research showing their antimicrobial and antibiofilm activities. The aim of this work was to evaluate the antimicrobial and antibiofilm activities of mixtures of congeners of RLs and SLs against relevant microorganisms. Furthermore, it was important to investigate the antimicrobial mechanism of these glycolipids since it is still not fully understood.

The composition of the mixtures of congeners of RLs produced by *P. aeruginosa* and SLs mixture produced by *S. bombicola* (obtained from the supplementation with oleic acid) were characterized preliminarily by TLC analysis and posteriorly identified by HPLC-MS analysis. The RLs mixture was composed by mono and di-RLs and the main compounds were identified as RhaC_{10:0}C_{12:1} and RhaRhaC_{10:0}C_{10:0}. In the SLs mixture it was identified a mixture of acidic and lactonic SLs and the most prominent were identified as diacetylated lactonic SLs, with an hydroxy fatty acid chain containing 18 carbon atoms and different unsaturation degrees, namely C18:0, C18:1 and C18:2.

After the identification of the mixtures of glycolipids, the antimicrobial activity of RLs and SLs was evaluated against planktonic bacteria by obtaining the MIC, MBC and MBIC. It was concluded the best activities against Gram positive bacteria *S. aureus* ATCC 6538, *S. epidermidis* ATCC 28319 and *MRSA*. The ranges of MIC values obtained were 100 to 400 µg/mL for RLs and 50 to >800 µg/mL for SLs. However, Gram negative bacteria *P. aeruginosa* ATCC 15422 and *E. coli* ATCC 25922 were less vulnerable with higher MIC values obtained of >800 µg/mL, hypothesized to be due to differences in the bacterial cell wall that offer antimicrobial protection.

Since, the antimicrobial mechanism of action of RLs and SLs is still not fully understood it was important to evaluate the antimicrobial mechanism of these mixtures against planktonic bacteria.

Membrane integrity by propidium iodide uptake and cellular viability by resazurin reduction were evaluated through fluorescence intensity measurements in MTPs.

It was concluded that the RLs mixture had higher effect on membrane integrity allowing propidium iodide uptake more easily, due to the higher ratios of 2.4 and 2.5 obtained for all studied strains. Consequently, SLs seemed to have less activity on membrane integrity obtaining lower ratios correspondent to 1.1 and 1.2.

However, the SLs mixture had higher activity on perturbing cellular viability, obtaining lower percentages ranging from 30.5 to 74.0% and RLs mixture ranging from 34.5 to 83.1%. *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 6538 were the most affected by the presence of both glycolipids. Furthermore, *E. coli* ATCC 25922 was not affected by both compounds.

The antimicrobial mechanism of action of the mixtures was also assessed through alterations on bacterial cell surface hydrophobicity (CSH). It has been reported that biosurfactants can alter the bacterial CSH, therefore a method described by Rosenberg *et al.* (1980)¹¹⁹ was used to evaluate these changes on planktonic bacteria. The method was based on cell partition in a water-octanol interface and the removal of cells from the interface was indicative of cell hydrophobicity. An increase of P(%) was observed for the samples treated with the mixtures, however RLs had higher activity showing increases ranging 49.9 to 19.8 %, when compared to SLs ranging from 16.9 to 5.6 % for *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 25922, *S. aureus* ATCC 6538 and *MRSA*. Additionally, it was determined that *S. epidermidis* ATCC 28319 and *E. coli* ATCC 25922 bacterial cells were less vulnerable to both glycolipids, maintaining CSH in the presence of RLs and decreasing 13.7% and 12.3% for SLs.

Furthermore, BS were also reported to perform changes on cell surface charge (CSC), when interacting with the cellular membrane. Consequently, zeta potential measurements were performed for characterization of bacterial CSC on planktonic bacteria. It was concluded that SLs had higher activity on cells' surface charge, however both glycolipids reduced the cell surface charge of bacteria. The higher contribution of SLs in the decrease of CSC was attributed to the presence of the compound itself. However, the decreases were only significant for *S. aureus* ATCC 25923 for both glycolipids.

The mixtures of congeners RLs and SLs were used to coat the surface of PDMS specimens with concentrations ranging from 0 to 3 mg/mL to evaluate their antibiofilm activity against sessile bacterial species.

Firstly, characterization of the surface of the PDMS specimens was important to determine if the surface was correctly functionalized. Contact angle measurements were performed and a decrease in the contact angle ranging from approximately $71 \pm 5^\circ$ to $55 \pm 1^\circ$ was

observed. The decrease in the contact angle was indicative of higher wettability properties of the functionalized PDMS specimens. Therefore, it was possible to conclude that the method of functionalization created a less hydrophobic surface on the PDMS material surface proving that the RLs were successfully adsorbed.

Secondly, the antibiofilm activity of both glycolipids previously used to coat the surface of PDMS specimens was evaluated using two different methods. The evaluation was performed by quantification of biofilm present in the PDMS specimens using crystal violet dye. The second was by determining only the viable cells present in the biofilm using the colony forming units (CFU) counting.

The results demonstrated that as the concentrations of RLs previously used to functionalize the surface of PDMS specimens increased, the biofilm formation (%) and number of viable cells (CFU/cm²) decreased. Higher antibiofilm activity of RLs was obtained against *S. aureus* ATCC 25923 reaching a reduction of 99 % and 2 log units and for *S. aureus* ATCC 6538 with a reduction of 86 % and over 1.8 log unit.

Overall, as the concentrations of SLs used to coat the surface of PDMS specimens increased, a decrease in biofilm formation (%) and number of viable cells (CFU/cm²) was perceptible for all studied strains. SLs had higher antibiofilm activity against *S. aureus* ATCC 25923 reaching a reduction of 99 % and 1.4 log units, *S. aureus* ATCC 6538 of 99 % and 1.7 log units and for *MRSA* 100 % and 2.0 log units.

Seemingly, when comparing both glycolipids it is possible to conclude that SLs had higher antibiofilm activity against all strains, since higher biofilm inhibition was observed.

Other methods of functionalization were explored, since the functionalization method by adsorption of glycolipids to a surface is not a permanent method there is a need to explore more effective and long-lasting methods. Therefore, the surface of PDMS specimens was treated with plasma for 5 or 10 min and after the treatment was combined with functionalization by adsorption with RLs at concentrations of 15, 30, 60 and 120 mg/mL. The surface wettability properties of the PDMS specimens increased, concluded by the observable decrease of the contact angle for both treatments. However, using 10 min for the plasma treatment was more efficient reaching a higher reduction of contact angle at 120 mg/mL of RLs.

Furthermore, FTIR-ATR analysis was also performed to confirm the RLs adsorption to the PDMS specimens previously treated with plasma. However, it was not possible to observe RLs characteristic band by FTIR-ATR, from the contact angle analysis it was possible to conclude that surface had been successfully modified.

Lastly, cellular viability was evaluated for sessile bacteria in the PDMS specimens previously treated with plasma and combined with functionalization by adsorption with RLs. A decrease of the number of colonies was observed as the concentrations of RLs previously adsorbed increased, reaching a reduction of over 1 log unit for both 5 and 10 min treatments. The coating of RLs proved to hinder biofilm formation, even with the previous treatment with plasma, which can be recognized as optimistic results for this new strategy.

In the future the mixtures of SLs and RLs should be purified to then functionalize medical grade PDMS against relevant microorganisms and determine the most active compounds.

Combination strategies can also be used to enhance the antimicrobial and antibiofilm activities of these compounds. This strategy seems to have potential due to being performed successfully by researchers in recent years, such as combining RLs with SLs, RLs with caprylic acid or RLs with MTS.^{62,99,136} Therefore, the combination of other antimicrobial compounds, should also be explored onto the surface of medical grade PDMS against sessile bacteria.

The antimicrobial mechanism of these glycolipids should be further studied since it is still not fully understood. Since, it was proposed that these cause modifications to the cell membrane surface, other properties of the membrane can be a focus.

Since, long-lasting is an important property for the functionalization of medical grade PDMS with these antimicrobial compounds more experiments using the plasma treatment should be performed. Furthermore, other methods of functionalization can also be explored to obtain a permanent functionalization of these antimicrobial compounds to the surface of the medical grade PDMS.

Since the functionalization of PDMS surface with the mixtures of RLs and SLs were successful in the prevention biofilm formation, the biocompatibility of the biomaterial medical grade PDMS should be evaluated. Therefore, the effect of the medical grade PDMS functionalized with RLs should be studied against immortalized human cell lines. This evaluation as already been performed for medical grade PDMS functionalized with SLs against HCaT cells by Pontes *et al.* (2016)² and the results were optimistic. Cell viability, apoptosis and reactive oxygen species can be the different end points examined.

✓ Chapter 6: Bibliography

6. Bibliography

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