Integrated Master in Bioengineering

Repurposing ibuprofen to control *Staphylococcus aureus* growth

Dissertation for Master's degree in Biological Engineering

Isabel Maria Pereira de Oliveira

Supervisors:

Manuel José Vieira Simões

Anabela Portela Borges

Department of Chemical Engineering

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UNIVERSIDADE DO PORTO

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ABSTRACT

The emergence of multidrug-resistant (MDR) bacteria in persistent biofilm infections represents a significant public health threat. An interesting approach to overcome these issues is the repositioning of existing drugs. Drug repurposing has several advantages, including reduction of time and cost of the drug development process. The purpose of the present work was to evaluate the action of ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), against *Staphylococcus aureus* growth in planktonic and sessile states.

The antibacterial activity and mode of action of ibuprofen were studied using different bacterial physiological indices: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), propidium iodide (PI) uptake, intracellular potassium (K^+) release and physicochemical surface properties. It was confirmed that ibuprofen has antibacterial activity against *S. aureus* with a MIC of 500 µg/mL and a MBC of 1400 µg/mL. Bacterial cells treated with ibuprofen showed permeation to PI, release of intracellular K^+ and changes on the physicochemical surface properties, which indicated the action of ibuprofen on cytoplasmic membrane destabilization. Preliminary checkerboard method was also performed to analyse the activity of ibuprofen in combination with conventional antibiotics. The combination of ibuprofen with ciprofloxacin, erythromycin or tetracycline resulted in predominant antagonism interaction against the collection strain *S. aureus* CECT 976 and three antibiotic resistant strains (*S. aureus* SA1199B, *S. aureus* RN4220 and *S. aureus* XU212). To understand the activity of ibuprofen on factors that control initial bacterial adhesion distinct methods were performed, namely bacterial motility assay, thermodynamic prediction of adhesion by calculation of free energy of adhesion and comparison with bacterial adhesion to polystyrene (PS) microtiter plates. The effects on biofilm formation and control of monolayer adhered cells (2 h incubation) and biofilms (24 h incubation) were analysed using a microtiter plate assay and quantification of biomass, metabolic activity and culturability. The effect on bacterial motility was not conclusive. Ibuprofen did not prevent initial bacterial adhesion (within 2 h) but affected biofilm formation mainly by inactivation, with metabolic reductions between 60-80%. The thermodynamic approach revealed to be inappropriate for the prediction of *S. aureus* adhesion to PS, either in the absence or presence of ibuprofen. Monolayer adhered cells and 24 h old biofilms showed metabolic reductions up to 80% and total loss of culturability after treatment with ibuprofen. However, the drug only had moderate ability to remove attached cells and biofilms, with biomass reduction ≤ 40%. Therefore, this work emphasizes that ibuprofen may be a good candidate for repurposing as an antimicrobial and anti-biofilm agent, nevertheless further research is still needed to understand its full potential against pathogenic bacteria.

RESUMO

O aparecimento de bactérias multirresistentes associadas a biofilmes recalcitrantes representa uma grave ameaça para a saúde pública. Uma abordagem interessante para ultrapassar este problema é o reposicionamento de fármacos. Este método tem várias vantagens, como por exemplo a redução do tempo e custo do processo de desenvolvimento de novos fármacos. Assim, o principal objetivo do presente trabalho foi avaliar a ação inibitória do ibuprofeno, um fármaco anti-inflamatório não esteroide, no crescimento em estado planctónico e séssil de *Staphylococcus aureus*. Neste trabalho, a atividade antibacteriana e o modo de ação do ibuprofeno foram estudados utilizando diferentes índices fisiológicos microbianos: concentração mínima inibitória (MIC), concentração mínima bactericida (MBC), absorção de iodeto de propídio (PI), libertação de potássio intracelular (K^+) e propriedades físico-químicas da superfície bacteriana. Confirmou-se que o ibuprofeno possui atividade antibacteriana contra *S. aureus* com MIC e MBC iguais a 500 e 1400 µg/mL, respetivamente. Após tratamento com ibuprofeno, *S. aureus* apresentou permeabilização ao PI, libertação de K^+ intracelular e alterações nas propriedades físico-químicas da sua superfície, o que comprova a sua ação na desestabilização da membrana celular. Também foi realizado um teste preliminar para analisar a atividade conjunta do ibuprofeno com antibióticos convencionais. A combinação de ibuprofeno com ciprofloxacina, eritromicina ou tetraciclina contra a estirpe de coleção *S. aureus* CECT 976 e três estirpes resistentes (SA1199B, RN4220 e XU212) resultou predominantemente em interações de antagonismo. Para uma compreensão da atividade do ibuprofeno em fatores que controlam a adesão inicial bacteriana, foram realizados diferentes métodos, nomeadamente, teste de motilidade bacteriana, a previsão termodinâmica da adesão bacteriana pelo cálculo da energia livre de adesão e a comparação com a adesão bacteriana em microplacas de poliestireno (PS). A ação na formação de biofilmes e no controlo de células aderidas em monocamada (2 h) e de biofilmes (24 h) foi analisada com recurso a ensaios em microplacas e posterior quantificação da biomassa acumulada, atividade metabólica e culturabilidade em meio sólido. Os efeitos sobre a mobilidade bacteriana não foram conclusivos. O ibuprofeno não impediu a adesão inicial bacteriana, mas interferiu com a formação dos biofilmes, principalmente por inativação metabólica com reduções entre 60-80%. A abordagem teórica termodinâmica revelou-se inadequada para a previsão da adesão de *S. aureus* ao PS, tanto na ausência como na presença do fármaco. As células aderidas em monocamada e os biofilmes apresentaram reduções metabólicas até 80% e perda total de culturabilidade após tratamento com o ibuprofeno. No entanto, o fármaco apenas mostrou remoção moderada das células aderidas e dos biofilmes, com reduções de biomassa acumulada ≤ 40%. Deste modo, este trabalho enfatiza que o ibuprofeno pode ser um bom candidato para reposicionamento como agente antimicrobiano e de propriedades anti-biofilme. No entanto, reconhece-se que ainda são necessários mais estudos para entender todo o seu potencial contra os principais agentes patogénicos.

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NOMENCLATURE

Abbreviations

CFU	colony forming units
CIP	ciprofloxacin
CV	crystal violet
DMSO	dimethyl sulfoxide
ERY	erythromycin
FICI	fractional inhibitory concentration index
LB	Luria-Bertani broth
MBC	minimum bactericidal concentration
MDR	multidrug-resistant
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
MIC_{50}	MIC at which 50% of the microorganisms are inhibited
MIC_{90}	MIC at which 90% of the microorganisms are inhibited
MRSA	methicillin-resistant *S. aureus*
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
PI	propidium iodide
PS	polystyrene
QS	quorum-sensing
SD	standard deviation
TET	tetracycline
VISA	vancomycin intermediate-resistant *S. aureus*
VRE	vancomycin-resistant *Enterococci*
VRSA	vancomycin-resistant *S. aureus*
WHO	World Health Organization

Indexes

-	Electron donor parameter of the Lewis acid-base component (γ^{AB})
+	Electron acceptor parameter of the Lewis acid-base component (γ^{AB})
AB	Lewis acid-base component
iwl	interaction between substances *i* and *l* that are immersed in *w*
LW	Lifshitz-van der Waals component

s	surface
sws	interaction between two entities of a given *s*, when immersed in *w*
Tot	Total
w	water

Measure units

ΔG	Free energy of interaction	mJ/m^2
θ	Contact angle	º
γ	Surface tension parameters	mJ/m^2

Chapter 1

INTRODUCTION

1.1 PROJECT PRESENTATION AND OBJECTIVES

The emergence of multidrug-resistant (MDR) bacteria and problems with the use of conventional antibiotics to treat infections caused by bacterial biofilms have prompted clinicians and scientists to consider that we are approaching a post-antibiotic era (Brown & Wright 2016; Worthington & Melander 2013). Although the development of new antibiotics is one approach for the treatment of MDR bacterial biofilm infections, the number of new classes of antibiotics in the drug development pipeline has been continuously declining (Ventola 2015). The decreased interest of pharmaceutical industries to search and develop new antimicrobials is mainly due to the current costly and time-consuming paradigm of drug discovery and the inherently low rate of return for antibiotics compared to drugs targeted at chronic diseases (Spellberg et al. 2007).

Unlike *de novo* drug discovery, repurposing old drugs with known pharmacological and toxicological profiles greatly reduces time, cost and risk associated with antibiotic innovation (Chong & Sullivan 2007). The concept of repurposing drugs to find new applications for older drugs has been gaining popularity in recent years and has been applied in a number of disease areas (Oprea et al. 2011). Following this, drug repurposing arises as an interesting alternative for the treatment of recalcitrant bacterial infections. The strategy of finding new uses for existing drugs has begun to produce results in this line of research, since antibacterial properties have been discovered for well-known anticancer, antipsychotic, anthelmintic and anti-inflammatory drugs (Rangel-Vega et al. 2015; Das et al. 2016). The potential compounds to be repurposed as alternatives to face bacterial infections are termed 'non-antibiotics' (Nishimura 1986).

Ibuprofen represents an example of anti-inflammatory drug that has shown to possess antimicrobial activity against several microbial pathogens. Elvers & Wright (1995) reported that ibuprofen inhibited the growth of Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis* and *Staphylococcus epidermidis*) with its antibacterial activity being more effective at values below pH 7. Naves et al. (2010) investigated the potential inhibitory effects of human serum albumin, ibuprofen and N-acetyl-L-cysteine on biofilms produced by *Escherichia coli*, and ibuprofen diminished biofilm development by five out of seven strains.

Moreover, ibuprofen reduced the production of quorum-sensing (QS)-regulated virulence factor elastase in *Pseudomonas aeruginosa* by 28-47%, compared to the untreated cultures (Ulusoy & Bosgelmez-Tinaz 2013). However, some aspects of the mode of antimicrobial and anti-biofilm activity of ibuprofen are not fully understood and need further investigation.

S. aureus is a Gram-positive bacteria and it is a commensal microorganism that colonizes the nares, axillae, vagina, pharynx or damaged skin surfaces (Noble et al. 1967). Staphylococci produce numerous toxins and enzymes that induce pro-inflammatory changes, destroy biological tissues and cause diseases such as toxic shock syndrome and staphylococcal scalded skin syndrome (Lowy 1998). The invading staphylococci are either removed by the host innate immune response or attach to host extracellular matrix proteins and form a biofilm (Archer et al. 2011). Although, host immune responses against persistent biofilm infections are largely ineffective. In the past *S. aureus* infections were historically treatable with common antibiotics such penicillin, but the emergence of drug-resistant strains is now a major concern. In the 1960s the methicillin-resistant *S. aureus* (MRSA) was endemic in hospitals but it appeared rapidly and unexpectedly in communities in the 1990s, and now it is prevalent worldwide (Deleo & Chambers 2009). Vancomycin has been used as a drug of last resort for the treatment of Gram-positive bacterial infections. However, clinical isolates of MRSA strains with decreased susceptibility to vancomycin (vancomycin intermediate-resistant *S. aureus* - VISA) were first reported in Japan in 1997, and the first vancomycin-resistant *S. aureus* (VRSA) was reported in the United States in 2002 (Gardete & Tomasz 2014). Therefore, due to its resistance to antibiotics and evasion to host immune system, *S. aureus* has re-emerged as a clinically relevant pathogen.

Consequently, the main aim of this research project was to evaluate the action of ibuprofen against *S. aureus* growth in planktonic and sessile states.

In the first stage of this study, the mechanisms involved in the antimicrobial activity of ibuprofen against *S. aureus* were investigated using several physiological indices. To understand the mode of action of ibuprofen, it was performed the physicochemical characterization of bacterial surface (surface tension and hydrophobicity) and the assessment of bacterial cytoplasmic membrane integrity by propidium iodide uptake and K^+ release. This drug was also analysed in terms of its ability to act synergistically with selected antibiotics to control bacterial growth.

Finally, ibuprofen was tested in order to evaluate its effect on sessile *S. aureus* cells. Bacterial motility, free energy of adhesion of *S. aureus* to PS, and bacterial adhesion to 96-well PS microtiter plates were analysed to understand the activity of ibuprofen on factors that control initial bacterial adhesion. The effects of ibuprofen on biofilm prevention and control of

monolayer bacterial adhesion (2 h incubation) and biofilms (24 h incubation) were also assessed using 96-well polystyrene microtiter plates.

1.2 THESIS ORGANIZATION

This dissertation is divided in five chapters. Chapter 1 presents the context, motivations and the main goals for the development of this study. It serves as a guideline for the overall work presented in the further chapters.

Chapter 2 provides a brief review of the literature. The relevance of bacterial biofilms infections and the global antibiotic resistance problem were discussed. It also focuses on the importance of drug repurposing and its positive outputs for the treatment of bacterial biofilm infections. Non-antibiotic drugs with antimicrobial activity against planktonic cells and biofilms were reported, as well the known combinatorial activity with conventional antibiotics and their mechanism of action. The existent literature about ibuprofen as a promising drug for therapeutic use against microbial infections was also addressed in this chapter, as well its side effects and benefices.

Chapter 3 focuses on the antibacterial activity and mode of action of ibuprofen against *S. aureus*. The minimum inhibitory and bactericidal concentrations of ibuprofen were determined. The physicochemical characterization of bacterial surface and the bacterial cytoplasmic membrane integrity were evaluated to understand the mode of action of ibuprofen. The combination of ibuprofen with some antibiotics was also performed to assess the possible synergistic interaction.

Chapter 4 provides the study of the effect of ibuprofen on sessile *S. aureus* cells. The action of ibuprofen on some aspects related with biofilm formation, namely initial bacterial adhesion (based both on the assessment of free energy of adhesion according to a thermodynamic approach and bacterial adhesion assay) and bacterial motility (motility assay) were examined. In this chapter, it was also analysed if ibuprofen inhibits biofilm formation and/or eradicate monolayer adhered bacteria (2 h incubation) and biofilms (24 h incubation).

The main conclusions and perspectives for further research were summarized in Chapter 5, giving an overview of the developed work.

Chapter 2

LITERATURE REVIEW

2.1 BACTERIAL BIOFILM INFECTIONS AND THE CHALLENGE OF ANTIBIOTIC RESISTANCE

A biofilm can be defined as a microbially derived sessile community, constituted by cells that are attached to a substratum, interface, or to each other, embedded in a matrix of extracellular polymeric substance, and that exhibit an altered phenotype with regard to growth, gene expression and protein production (Donlan & Costerton 2002). Biofilm-embedded microorganisms benefit from a number of advantages over planktonic cells. The extracellular matrix is capable of sequestering and concentrating environmental nutrients such as carbon, nitrogen and phosphate. On the other hand, microorganisms in the biofilm have the ability to evade multiple clearance mechanisms produced by host and synthetic sources, such as antimicrobial agents. The mechanisms responsible for biofilm resistance are the altered growth rate of biofilm organisms, the physiological changes due to the biofilm growth, and delayed penetration of the antimicrobial agent through the biofilm matrix. In particular, Singh et al. (2010) showed that the penetration of the antibiotics oxacillin, cefotaxime and vancomycin was significantly reduced through *S. aureus* and *S. epidermidis* biofilms.

The spectrum of biofilm infections is wide. The catheter-associated urinary tract infection and prosthetic joint infections are two of the most common biofilm-associated foreign body infections, but central line-associated blood stream infections and ventilator-associated pneumonia are also of significant concern (Romling et al. 2014). Soft tissue infections and mucosal infections are the main biofilm-associated diseases of today, although historically the most prominent biofilm-associated disease might be *P. aeruginosa*-mediated lung infection in cystic fibrosis patients (Høiby et al. 2010). Specifically, some of *S. aureus* biofilm-related diseases are osteomyelitis (Lew & Waldvogel 2004), indwelling medical device infection (Baldoni et al. 2009), periodontitis and peri-implantitis (Cuesta et al. 2010), chronic wound infection (Gjødsbøl et al. 2006), chronic rhinosinusitis (Wolcott et al. 2010), endocarditis (Christensen et al. 1985), ocular infection (Leid et al. 2002) and polymicrobial biofilm infections (Archer et al. 2011).

Biofilm infections are difficult to treat because the biofilm matrix and phenotypic characteristics of colonizing bacteria confer resistance to the host immune response and the action of antimicrobial drugs. Therefore long-term treatment with high doses and often using

a combination of antibiotics with different killing mechanisms are applied to overcome biofilm infections (Wu et al. 2014). In addition, the emergence of MDR pathogens is rapidly increasing, forcing the World Health Organization (WHO) to warn that the antimicrobial resistance is one of the most important problems for human health (Brooks & Brooks 2014). The molecular mechanisms of antibiotic resistance in biofilms have been described and can be summarised as: i) prevention of access to target by reduced permeability to antibiotics or increased efflux through overexpressed efflux pumps; ii) changes in antibiotic targets by mutation; iii) modification and protection of targets; and iv) direct inactivation of antibiotics by hydrolysis or transfer of chemical group (Blair et al. 2015). The ESKAPE pathogens (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa*, and *Enterobacter* spp.) are especially important due to their role in many infections, the frequency of antibiotic resistance amongst them and the lack of alternative antibiotics to combat these pathogens (Rice 2008).

Along with the increased resistance to existing agents, there is a lack of new antibiotics being developed. The decreased interest of pharmaceutical industries to search and develop new drugs to treat infectious diseases is mainly due to the time-consuming process and increased costs of putting a new drug on the market. Moreover, there is insufficient investment in this market due to the poor economic returns for the pharmaceutical sector and the lack of incentives by the medical and scientific communities, since the excessive use of antibiotics is considered one of the principal reasons for the emergence of resistance (Chong & Sullivan 2007).

2.2 DRUG REPURPOSING – AN ALTERNATIVE TO FACE BACTERIAL BIOFILM INFECTIONS

Drug repurposing is the process of finding new uses outside the scope of the original medical indication for existing drugs and it is also known as redirecting, repositioning and reprofiling (Ashburn & Thor 2004). There are different methodological approaches to the identification of new repurposing opportunities for existing drugs (Cavalla 2013). Experimental approaches involve the generation of new knowledge and include *in silico* methods such as molecular modelling and gene profiling, laboratory experiments such as *in vitro* and *in vivo* screening and methods based on analysis of human exposure, either from clinical trial information or from prescription data.

The advantage of using drugs that are already on the market is mainly because they have already been tested in humans (Aubé 2012). The safety profiles and pharmacokinetics of existing drugs are known and are often approved by regulatory agencies for human use, therefore, some stages of clinical trials can be bypassed (Chong & Sullivan 2007; Oprea & Mestres 2012). This is more likely to be the case for drugs being repurposed at similar or lower

dosage compared to the maximum dose that has already been approved by regulatory agencies. As a consequence, the cost and time associated with carrying out trials for newly identified molecules may be reduced (Ribeiro et al. 2016; Chong & Sullivan 2007) and thereby drug repurposing arises as an interesting alternative approach for the treatment of recalcitrant bacterial infections.

The potential compounds to be repurposed as alternatives to face bacterial infections are termed 'non-antibiotics' (Nishimura 1986) and there are several properties that these drugs could possess (Figure 1). First, non-antibiotics could have direct antibacterial activity, even if they are not used clinically as antibiotics, and they are defined as 'antimicrobial non-antibiotics'. Second, they could increase the efficacy of antibiotics and/or combat antibiotic resistance mechanisms, termed 'helper compounds'. Finally, non-antibiotics could help to treat the infection by interacting with host targets to activate host defence mechanisms and these non-antibiotics can be defined as 'host cell modulators' (Brown 2015; Martins et al. 2008).

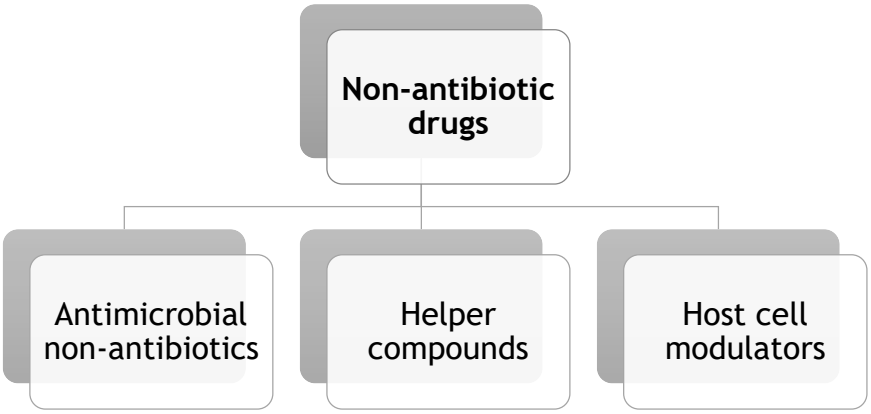

Figure 1 – Subgroups of non-antibiotic drugs.

There are a large number of non-antibiotic drugs that have antibacterial activity and/or with positive interaction with antibiotics. Moreover, it has been reported that some of these drugs can exhibit anti-biofilm activity (see Table A.1 in annexes).

2.2.1 Non-antibiotic drugs: antimicrobial activity and effects on biofilms

Potential antimicrobial activity and anti-biofilm effect have been found among drugs as anti-cancer, antipsychotic and anti-inflammatory pharmaceuticals. Such drugs classes have been tested against bacterial infections through *in vitro* and *in vivo* models.

Some compounds that were originally developed as anti-neoplastic are effective at attenuating growth of microbial pathogens, and some of them have shown promising results in animal models and clinical trials. The gallium compounds, 5-fluorouracil and floxuridine are some examples of such drugs (Bonchi et al. 2014; Imperi et al. 2013a). The antibacterial activity

of gallium compounds both *in vitro* and *in vivo* has been tested against *P. aeruginosa* (Kaneko et al. 2007) and *A. baumannii* pathogens (Antunes et al. 2012; De Léséleuc et al. 2012). In addition, Kaneko et al. (2007) showed that the transition metal gallium at low concentration prevented *P. aeruginosa* biofilm formation and stimulated detachment of adhered bacteria. The action of gallium worked in part by decreasing bacterial iron (Fe^{3+}) uptake and interfering with iron signalling by the transcriptional regulator *pvdS*. The uracil analogue 5-fluorouracil has potent antimicrobial effects against several bacterial pathogens. Gieringer et al. (1986) tested the effect of 5-fluorouracil on growth of *S. aureus* and *S. epidermidis* and confirmed that this compound inhibited the growth with an MIC_{50} lower than 0.8 µg/mL. Later, it was found that 5-fluorouracil inhibited *S. epidermidis* biofilms at levels below its MIC (Hussain et al. 1992) and decreased *E. coli* biofilm formation by around 5-fold at 25 µM (Attila et al. 2009). Additionally, Ueda et al. (2009) found that 5-fluorouracil abolished QS phenotypes in *P. aeruginosa* and reduced virulence. Floxuridine, an analogue of 5-fluorouracil, showed bactericidal activity with MIC_{90} of 0.0039 mg/L, against MDR *Staphylococcus* spp. isolates including MRSA, VRSA and VISA (Younis et al. 2015).

The antimicrobial activity of neuroleptics, especially the phenothiazines and chemically related compounds employed for the management of psychosis, has been the subject of some studies. Thioridazine and trifluopromazine presented consistent antimicrobial activity against methicillin-susceptible *S. aureus* and MRSA with MIC between 16 to 32 mg/L (Hendricks et al. 2003; Hendricks 2006). In addition, an *in vitro* cell study conducted by Ordway et al. (2002) demonstrated that phenothiazine thioridazine at a concentration of 0.1 mg/L inhibited intracellular growth of *S. aureus* in human macrophages. On the other hand, although chlorpromazine has limited inhibitory activity on *S. aureus* (Hendricks 2006), it exhibits a wide array of changes to its cell wall that are very similar to those produced by ß-lactam antibiotic oxacillin (Kristiansen & Amaral 1997). The effects of chlorpromazine on the morphology of *E. coli* also mimic the effects of ampicillin since both cause significant elongation of the organism (Amaral & Lorian 1991).

The statins, a lipid-lowering agents with anti-inflammatory activities, have also been investigated for their antibacterial effects. Masadeh et al. (2012) revealed that atorvastatin, simvastatin and rosuvastatin were able to induce variable degrees of antibacterial activity against diverse standard bacterial strains and bacterial clinical isolates. Simvastatin and atorvastatin were the most potent ones and these statins induced susceptibility to *S. aureus*, *Enterococci*, *A. baumannii*, *S. epidermidis, Enterobacter aerogenes* and resistant strains of MRSA and vancomycin-resistant *Enterococci* (VRE). The influence of statins on motility *of P. aeruginosa* was studied by Hennessy et al. (2013) and the results showed that swarming motility was decreased by simvastatin, lovastatin and mevastatin at 100 µM. In addition, simvastatin

had antimicrobial activity against *S. aureus* biofilms, reducing their formation, viability and extracellular polysaccharide production (Graziano et al. 2015).

Ebselen is another anti-inflammatory drug that has shown potent bactericidal activity, in an applicable clinical range, against MRSA, VRSA and VISA. The MIC at which 90% of clinical isolates of *S. aureus* were inhibited was 0.25 mg/L (Younis et al. 2015). Given the potent antibacterial activity of ebselen against planktonic MDR strains, Thangamani et al. (2015b) considered the possibility that this drug would also be able to reduce established staphylococcal biofilms. Actually, ebselen at 16 × MIC reduced the biofilm mass of *S. aureus* and *S. epidermidis*, approximately by 60% and 50%, respectively.

The non-steroidal anti-inflammatory drug celecoxib exhibited broad-spectrum antimicrobial activity against Gram-positive pathogens from a variety of genera and its topical application significantly reduced the mean bacterial count in a mouse model of MRSA skin infection (Thangamani et al. 2015a).

Along with the antineoplastic, antipsychotic and anti-inflammatory drugs there are other drug classes that also have antimicrobial activity, such as terfenadine, ivacaftor and auranofin. Terfenadine is an antihistaminic used for the treatment of allergic rhinitis, hay fever, and allergic skin disorders. Recently, it was found to possess antimicrobial activity *vs* the planktonic, biofilm and small-colony variant forms of *S. aureus* (Jacobs et al. 2013; Perlmutter et al. 2014). Treatment of 48 h old biofilms with 10 × MIC terfenadine elicited a 1.1-log reduction in biofilm cell viability, which was comparable to the activity of ciprofloxacin under the same assay conditions. Ivacaftor is prescribed for the treatment of cystic fibrosis because it improves pulmonary function since it stimulates chloride ion influx through cell membrane channels. Because ivacaftor structurally resembles quinolone antibiotics, the team of Reznikov et al. (2014) tested the hypothesis that ivacaftor possessed antibacterial properties. In fact, ivacaftor was active against *S. aureus* and *Streptococcus pneumoniae*. Auranofin is an oral gold-containing drug in clinical use for the therapy of rheumatoid arthritis and it has been reported to produce remarkable bactericidal effects against *Staphylococcus* sp. with MIC ranging from 0.0625 to 0.5 μg/mL (Cassetta et al. 2014; Thangamani et al. 2016). Moreover, auranofin significantly disrupted established *in vitro* biofilms of *S. aureus* and *S. epidermidis*.

2.2.2 Non-antibiotic drugs: synergy between conventional antibiotics and reversal of resistance

When the antimicrobial activity shown by a combination of drugs against a given microorganism is greater than the sum of the individual activities of each member of the combination, it is considered synergistic interaction. Synergy does not always require that the compounds of the combination employed against a given microorganism be active at the concentrations used. So, it is possible to use a drug that has no antimicrobial activity but, when

it is present, it increases the activity of a second antibiotic to which the organism was previously resistant (Kristiansen & Amaral 1997).

The synergy between conventional antibiotics and non-antibiotics has been reported by a number of authors (Kristiansen & Amaral 1997; Gunics et al. 2000; Worthington & Melander 2013). The synergic activity of non-antibiotics has been studied with ß-lactam antibiotics, tetracyclines, aminoglycosides, cephalosporins and other classes of antibiotics.

The phenothiazines and tricyclic antidepressants are two types of non-antibiotics that have produced synergy between conventional antibiotics. Promethazine has limited inhibitory activity against *E. coli* and *S. epidermidis*. However, the combination of promethazine with either tetracycline or erythromycin produce significant synergistic activity. The tricyclic antidepressant clomipramine also has synergistic interaction with the same two antibiotics against resistant *S. epidermidis* (Gunics et al. 2000). Combination of prochlorperazine reduced the resistance of MRSA to oxacillin in a range of 16 to 128 fold and it was observed a 128 fold reduction of MRSA resistance with the combination of oxacillin and thioridazine (Hendricks 2006). Additionally, chlorpromazine, trans-chlorprothixene and amitriptyline are able to reduce or reverse resistance of Gram-positive and Gram-negative bacterial strains to penicillin, methicillin, tobramycin and cefuroxim (Kristiansen et al. 2010).

Antunes et al. (2012) demonstrated that gallium nitrate, besides being active alone, also synergizes with colistin *in vitro* against both colistin-sensitive and –resistant *A. baumannii* isolates. Colistin has been a limited option and it has been used as a 'last-line' therapy for infections caused by MDR Gram-negative bacteria, in particular *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, when essentially no other options are available (Nation & Li 2009). Therefore, the combination of gallium nitrate-colistin could represent a promising therapeutic option against pan-resistant *A. baumannii*, since it would provide the benefit of reducing the colistin dosages required to treat the infections and reducing the probability of the emergence of resistant strains to this antibiotic.

Ejim et al. (2011) screened a collection of drugs to identify compounds that augment the activity of the antibiotic minocycline and found synergistic drug combinations that exhibited *in vitro* and *in vivo* activity against bacterial pathogens. Disulfiram and benserazide alone had weak antibacterial activity against *S. aureus* and *P. aeruginosa*, respectively, but these drugs showed stronger synergistic interactions with minocycline. The loperamide-minocycline pair revealed synergistic growth inhibition against *P. aeruginosa, A. baumannii, E. coli* and *K. pneumoniae* as well, despite loperamide alone had not detectable antibacterial activity. Additionally, loperamide decreased swimming motility of *P. aeruginosa* at 32 mg/mL.

Others non-antibiotics that increase the efficacy of conventional antibiotics are ivacaftor and celecoxib. Besides being active alone, ivacaftor displayed positive interactions with

ceftriaxone, vancomycin, trimethoprim-sulfamethoxazole, moxifloxacin and linezolid antibiotics against *S. aureus* and *S. pneumoniae* (Reznikov et al. 2014). Kalle & Rizvi (2011) demonstrated that celecoxib increased the sensitivity of bacteria to the antibiotics ampicillin, kanamycin, chloramphenicol and ciprofloxacin by accumulating the drugs inside the cells.

2.2.3 Modes of action

Although the antimicrobial activity of non-antibiotics and the potentiation of conventional antibiotics by combination with non-antibiotics have been reported, their mechanisms of action are not completely understood. Depending on the drug it could trigger different modes of action.

The inhibition of protein synthesis and the subsequent inhibition of toxin production are great advantages of some drugs, such as ebselen and celecoxib. The primary antibacterial mechanism of action of ebselen in *S. aureus* is the inhibition of protein synthesis, at a concentration equivalent to the MIC. However, additional secondary effects on DNA, RNA and lipid synthesis are observed at higher concentrations (Thangamani et al. 2015b). Celecoxib inhibit the synthesis of DNA, RNA, and protein in *S. aureus* at concentrations significantly below the MIC (Thangamani et al. 2015a).

The primary effects of phenothiazines are on the cytoplasmic membranes of prokaryotes and eukaryotes. The components of cytoplasmic membrane affected are efflux pumps, energy providing enzymes, such as ATPase, and genes that regulate and code for the permeability aspect of a bacterium (Dastidar et al. 2013). Phenothiazines inhibit the NorA-mediated fluoroquinolone efflux of *S. aureus* (Kaatz et al. 2003) and the same derivatives reduce oxacillin resistance involving target alteration (Kristiansen et al. 2007). Eilam (1984) reported that phenothiazines cause a substantial increase in the membrane potential and strongly inhibit the activity of the plasma membrane ATPase. Phenothiazines are also recognised as effective antiplasmid agents (Wolfart et al. 2006; Amaral et al. 2004; Molnár et al. 2003), which render the bacterial carrier of the plasmid sensitive to antibiotics.

Some studies have explored the possibility to use iron mimetics as antibacterial agents, which would interfere with iron metabolism and likely display pleiotropic effects. Actually, iron is an essential nutrient for almost all bacterial cells because it is a cofactor for crucial enzymes involved in DNA synthesis, metabolism and oxidative stress response. Therefore, the use of iron mimetics can decrease the iron uptake and cause antimicrobial effects. Gallium is an example of these agents because the solution and coordination chemistries of Ga^{3+} are very similar to those of Fe^{3+}, but Ga^{3+} is irreducible under physiological conditions (Bernstein 1998). It is generally assumed that gallium is able to repress the activity of iron-containing enzymes by substitution of Fe^{3+} in their active site (Bonchi et al. 2014). Bacteria exposed to gallium led to a decrease in enzyme activity of catalase, iron containing superoxide dismutase with an

increased susceptibility to H_2O_2, ribonucleotide reductase (a key enzyme in DNA replication) and aconitase (Olakanmi et al. 2010; Olakanmi et al. 2013).

Therefore, the modes of action of antimicrobial non-antibiotics are analogous with the mechanisms of conventional antibiotics. According with their target structures in the bacterial cell they can inhibit DNA replication, transcription elements, protein synthesis by interacting with the ribosomes and disrupting translation, lipid biosynthesis enzymes, cell wall and nucleic acid synthesis and disrupt cytoplasmic membrane structure and function (Madigan et al. 2012).

By contrast, the non-antibiotics bellowing to the subgroup of helper compounds that increase the efficacy of antibiotics and/or combat antibiotic resistance display other modes of action. These drugs may not kill or halt bacterial growth but modify the pathogenic bacteria to produce a phenotype that is susceptible to the antibiotic. Helper compounds can inhibit the modified target sites of antimicrobial action, inhibit the bacterial enzymes that inactivate antibiotics, increase the membrane permeability to exogenous products or inhibit the efflux pumps (Abreu et al. 2012).

2.3 THE PARTICULAR CASE OF IBUPROFEN

2.3.1 Pharmacology

Ibuprofen, 2-(4-isobutylphenyl)-propionic acid, is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, antipyretic and anti-inflammatory properties. It was developed as an anti-rheumatic drug in the 1960s and currently is one of the most common over-the-counter drugs (Davies 1998). The chemical structure of ibuprofen is demonstrated in Figure 2.

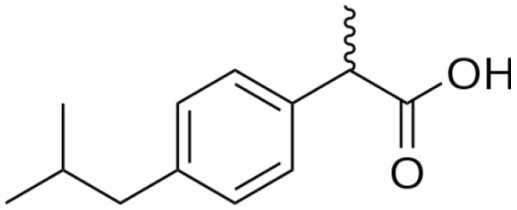

Figure 2 – Chemical structure of ibuprofen.

Like many NSAIDs, ibuprofen produce its therapeutic activities through non-selective inhibition of cyclooxygenase (COX) enzymes, COX-1 and COX-2, which are the primary enzymes of the prostaglandin (PGs) biosynthesis. COX-1 is constitutive and is responsible for the production of PGs that control a range of physiological functions, such as vascular, blood flow, gastric and renal functions. COX-2 is induced by inflammatory stimuli and produces PGs that contribute to the pain, inflammation and fever mediators (Vane & Botting 1998; Rainsford 2009).

Ibuprofen has the usual recommended adult daily dosage of 200 to 400 mg every 6 hours. Higher prescription doses at a maximum of 3200 mg/day are employed long-term for the treatment of rheumatic and other severe musculoskeletal conditions. The absorption of ibuprofen is rapid, with peak plasma or serum drug concentrations observed at approximately 1-2 h with some variations according to pharmaceutical formulation (Janssen et al. 1985; Davies 1998; Rainsford 2009).

The principal toxic effects of ibuprofen are gastrointestinal ulceration and bleeding. Along with several other NSAIDs, adverse effects of ibuprofen also include renal syndromes, allergic and hypersensitivity reactions, liver injury and cardiovascular risks. However, the relative doses required for acute and chronic gastrointestinal ulceration are often lower than with some more potent ulcerogens (e.g. aspirin, diclofenac, naproxen) and, at non-prescription doses, ibuprofen has little prospect of developing renal and associated cardiovascular events (Rainsford 2015; Henry et al. 1999; Murray & Brater 1999; Miwa & Jones 1999).

The most potentially serious drug-drug interactions include the use of NSAIDs with lithium, warfarin, oral hypoglycemics, high dose methotrexate, anti-hypertensives, angiotensin converting enzyme inhibitors and diuretics (Bushra & Aslam 2010). Significant drug interactions have been demonstrated for aspirin as well (Davies 1998).

2.3.2 Antimicrobial and anti-biofilm activity of ibuprofen

To the best of the author's knowledge, the first studies about the antimicrobial activity of ibuprofen were carried out by Hersh et al. in 1991 and by Sanyal et al. in 1993. Hersh et al. (1991) investigated the antibacterial activity of ibuprofen against common periodontal pathogens (*Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis, Bacteroides intermedius, Eikenella corrodens, Fusobacterium nucleatum* and *Wolinella recta*), whereas Sanyal et al. 1993 demonstrated its antifungal activity against dermatophyte fungi (*Trichophyton rubrum, Trichophyton metagrophytes, Trichophyton tonsurans, Microsporum fulva, Epidermophyton floccosum* and *Mucor* sp.). Later, Elvers & Wright (1995) investigated the antibacterial activity of ibuprofen against six bacterial species (*S. aureus, M. luteus, B. subtilis*, *S. epidermidis, E. coli* and *P. fluorescens*). The authors verified that ibuprofen inhibited the growth of the Gram-positive species and its activity was affected by pH. At pH 7, the MIC value for *S. aureus* exceeded 600 µg/mL, while at pH 6 the value was 350 µg/mL.

Antifungal activity of ibuprofen in combination with fluconazole against *Candida* species was investigated by Scott et al. (1995) and Pina-vaz et al. (2000). The combination of ibuprofen with fluconazole resulted in synergic activity, including for fluconazole-resistant strains. In 2005, Pina-Vaz et al. demonstrated that the synergic effect between ibuprofen and azoles resulted from blockade of efflux pumps.

The anti-biofilm properties of ibuprofen have also been of interest to some investigations. A modest prevention of *Candida albicans* biofilms on catheter disk model system was assessed by Alem & Douglas (2004). Prado et al. (2010) and Naves et al. (2010) investigated the effects of ibuprofen against biofilm formation by *S. pneumoniae* and pathogenic *E. coli* strains, respectively. *S. pneumoniae* biofilm was reduced in five out of eleven strains at a concentration of 128 μg/mL, and ibuprofen diminished biofilm development by five out of seven *E. coli* strains with reductions ranging from 37.2% to 44.8%. In 2015, Reśliński et al. verified that diclofenac and ibuprofen inhibited biofilm formation by *S. aureus* and *E. coli* on the surface of monofilament polypropylene mesh, a biomaterial that is often applied in hernia surgery.

Chapter 3

ANTIBACTERIAL ACTIVITY AND MODE OF ACTION OF IBUPROFEN

3.1 INTRODUCTION

Given the increasing prevalence of MDR bacteria and the limited efficacy of current available antibiotics, new approaches for the treatment of bacterial infections are needed. The repositioning of drugs that have previously been approved for other indications is an attractive approach to face the problems with recalcitrant bacterial infections, because these drugs have known toxicological and pharmacological profiles. Studies in this line have indicated that several compounds from different drug classes, including antihistamines, antineoplastic, antipsychotic and anti-inflammatory drugs, possess moderate to powerful antibacterial activity.

Non-antibiotic drugs can act by different mechanisms on microbial growth. They can have direct antimicrobial activity (antimicrobial non-antibiotics), increase the efficacy of antibiotics and/or combat antibiotic resistance mechanisms (helper compounds) or activate host defence mechanisms (host cell modulators) (Brown 2015; Martins et al. 2008). In particular, the action of antimicrobial non-antibiotics can be through various modes, such as membrane damage, metabolic alterations, DNA intercalations, adhesion suppression, among others.

The antimicrobial activity of ibuprofen has been known for more than 20 years (Hersh et al. 1991; Sanyal et al. 1993; Elvers & Wright 1995), however, some aspects of its specific mode of antibacterial action have not been explored. Therefore, this chapter aims to analyse the mechanisms involved in the antimicrobial activity of ibuprofen against *S. aureus*, using several physiological indices. First, the MIC and MBC of ibuprofen were confirmed by broth microdilution method. Then, the assessment of bacterial cytoplasmic membrane integrity by PI uptake and K^+ release, as well the physicochemical characterization of bacterial surface (surface tension and hydrophobicity) were performed in order to understand the mode of action of ibuprofen. Preliminary checkerboard method was also executed to analyse the ability of ibuprofen to act synergistically with tetracycline, ciprofloxacin and erythromycin against the collection strain (*S. aureus* CECT 976) and resistant strains of *S. aureus* (SA1199B, RN4220 and XU212) that overexpress efflux pumps.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and culture conditions

The main microorganism used in this study was the Gram-positive bacterium *S. aureus* CECT 976, obtained from the Spanish Type Culture Collection. Three additional strains, *S. aureus* SA1199B, *S. aureus* RN4220 and *S. aureus* XU212 resistant to ciprofloxacin (CIP), erythromycin (ERY) and tetracycline (TET), respectively, were used to assess the combinatorial activity of ibuprofen with these antibiotics. The bacteria at -80 ºC were transferred onto Mueller-Hinton (MH; Oxoid, UK) agar plate and grown for 24 h at 37 ºC. Then the bacterial strains were inoculated into MH broth and grown overnight at 37 ºC and 150 rpm. Finally, the cell cultures were standardized in fresh MH broth to an optical density of 0.1 ± 0.02 at 600 nm. Alternatively, for the experiments with incubation in 0.85% (w/v) saline (NaCl) solution, after the overnight growth, the bacterial cells were harvested by centrifugation (Eppendorf centrifuge 5810R; Eppendorf AG, Germany) at 3772 *g* for 15 min and washed once with NaCl solution. Then, optical density at 600 nm was adjusted to 0.1 ± 0.02 with NaCl solution.

3.2.2 Drugs, antibiotics and other reagents

Ibuprofen and piroxicam were obtained from Alfa Aesar GmbH & Co KG (Germany). The stock solutions of ibuprofen and piroxicam were prepared in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). The antibiotics TET and CIP were obtained from Sigma-Aldrich (USA) and ERY from AppliChem GmbH (Germany). The stock solutions of antibiotics were prepared in distilled water (CIP) and in DMSO (TET and ERY), and frozen at -18 ºC.

3.2.3 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth microdilution method was used for determination of MIC of each drug according to Borges et al. (2013). Briefly, 96-well PS microtiter plates were filled with 20 µL of each drug at a range of different concentrations and 180 µL of cell culture. The range of concentrations selected was 6.25-1000 µg/mL for ibuprofen and 6.25-2000 µg/mL for piroxicam. MIC was defined as the lowest concentration of the drug that prevented the bacterial growth after 24 h of incubation at 37 ºC and 150 rpm. The bacterial growth was determined at 600 nm using a microplate reader (Synergy HT; Biotek Instruments, USA). At least two independent experiments were performed for each drug. The highest concentration of DMSO remaining after dilution was 10% (v/v). After MIC determination, a volume of 10 µL of cell suspension was directly removed from the wells containing drug concentrations equal to and above the MIC and placed out on MH agar. Plates were incubated at 37 ºC for 24 h and the growth was visually

inspected. The MBC was recorded as the lowest concentration in which total growth inhibition was observed (Lopez-romero et al. 2015).

3.2.4 Dose response curves

Dose response curves were performed according to Borges et al. (2012) with some modifications. Considering the MIC and MBC previously determined, different amounts of ibuprofen from sub-inhibitory up to bactericidal concentrations (0, 125, 250, 500, 950 and 1400 µg/mL) in NaCl solution and in MH broth. Aliquots of 900 µL of each cell suspension were incubated with 100 µL of ibuprofen solutions for 1 h at 37 ºC and 150 rpm. After bacterial exposure to ibuprofen, seven-fold serial dilutions in NaCl solution were performed and 10 µL of each dilution was placed on MH agar plates. The plates were incubated at 37 ºC for 24 h. The number of colony forming units (CFU) was visually counted for plates containing 10 < CFU < 300 colonies and expressed as Log (CFU/mL). Cell suspensions without ibuprofen and with 10% (v/v) DMSO were used as controls. At least two independent experiments were performed for each condition.

3.2.5 Assessment of membrane integrity due to propidium iodide (PI) uptake

The Live/Dead *Bac*Light™ kit (Invitrogen, USA) was applied to assess membrane integrity by selective stain exclusion (Borges et al. 2013). The kit comprises two nucleic acid-binding stains, SYTO 9 and PI. Green fluorescing SYTO 9 is able to enter all cells, whereas red fluorescing PI enters only in cells with damaged cytoplasmic membranes. Thus, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red (Invitrogen, 2004). Three hundred microliters of each diluted (1:10) bacterial suspensions, from the dose response experiment, and 1 mL of NaCl solution were filtered through a Nucleopore ® (Whatman, UK) black polycarbonate membrane (pore size 0.22 µm) and then stained with 250 µL of SYTO 9 and 50 µL of PI. The dyes were left to react for 7 min in the dark. The membrane was then mounted in a microscope slide in a sequence of steps comprising one drop of *Bac*Light mounting oil, membrane, one drop of *Bac*Light mounting oil and a cover glass (24 x 24 mm). The microscope used for observation of stained bacteria was a LEICA DMLB2 epifluorescence microscope (LEICA Microsystems, Germany) coupled with a LEICA DFC300 FX camera and a 100× oil immersion fluorescence objective. To acquire images, LEICA IM50 Image Manager, Image processing and archiving software were used.

3.2.6 Potassium (K^+) release

Flame emission and atomic absorption spectroscopy were used for K^+ titration in bacterial suspensions treated with ibuprofen, according to Borges et al. (2013). After the overnight growth, the bacterial suspension was centrifuged at 3772 *g* for 15 min (Eppendorf centrifuge

5810R; Eppendorf AG, Germany) and washed once with ultrapure water. The optical density at 600 nm was adjusted to 0.1 ± 0.02. The samples were incubated with ibuprofen at ¼ MIC, ½ MIC, MIC and MBC to a final volume of 30 mL for 1 h at 37 ºC and 150 rpm. After the contact with ibuprofen, the samples were filtrated using a Fisherbrand (USA) syringe filter pore size 0.22 µm and added to 0.25 g of NaCl to neutralize the interference of other loads. Afterwards, the filtrates were analysed in a GBC AAS 932plus device using GBC Avante 1.33 software. Two independent experiments were performed for each condition. Cell suspensions with 10% (v/v) DMSO and cell suspensions without ibuprofen were used as controls.

3.2.7 Physicochemical characterization of bacterial surfaces

The physicochemical properties of the bacterial surface exposed and not exposed to ibuprofen were determined by the sessile drop contact angle measurement on bacterial lawns, prepared as described Busscher et al. (1984). Briefly, bacterial suspensions in NaCl solution were incubated with ibuprofen at ¼ MIC, ½ MIC and MIC to a final volume of 30 mL for 1 h at 37 ºC and 150 rpm. Each bacterial suspension was filtered using a sterile cellulose nitrate membrane filter, pore size 0.45 µm (Pall Corporation, USA) and the membrane was cut into six pieces and fixed on glass slides. Determination of contact angles was performed automatically using a Contact Angle System OCA 15 Plus (Dataphysics Instruments, Germany) video-based optical measure instrument, allowing image acquisition and data analysis. The measurements were carried out at room temperature (23 ± 2 ºC) using three different liquids: water, the polar formamide and the apolar α-bromonaphtalene (Sigma, Portugal). At least two independent experiments were performed for each condition. Cell suspension with DMSO and cell suspension without ibuprofen were used as controls.

Hydrophobicity was evaluated after contact angles measurements, following the van Oss et al. (1987; 1988; 1989) approach where the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w), ΔG_{sws} (mJ/m^2). If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{sws} < 0$, the material is considered hydrophobic. In contrast, if $\Delta G_{sws} > 0$, the material is hydrophilic. ΔG_{sws} can be calculated through the surface tension components of the interacting entities, according to Equation 1:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

Where, γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are the electron acceptor and electron donor parameters of the Lewis acid-base component (γ^{AB}), respectively, with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components of the surface can be obtained by measuring the contact angles of the three pure liquids, with known surface tension components, followed by the simultaneous resolution of three equations of the type of Equation 2, one for each liquid.

$$(1 + cos\ \theta)\gamma_w^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^{+}\gamma_w^{-}} + \sqrt{\gamma_s^{-}\gamma_w^{+}}\right) \quad (2)$$

Where, θ is the contact angle and $\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$. The liquids surface tension components were obtained from the literature (Janczuk et al. 1993).

3.2.8 Checkerboard microdilution assay

Checkerboard assay allowed the evaluation of a possible interaction between ibuprofen and the antibiotics TET, CIP and ERY, and it was performed in 96-well microtiter plates according with Chan et al. (2011) with some modifications. Serial 2-fold dilutions of ibuprofen and antibiotics were prepared and 10 µL of each solution were added to a 96-well microtiter plate so that each row and column contained a fixed concentration of one agent and increasing concentration of the second agent. The concentration of each compound ranged from 1/32 to 2 × MIC. The MIC values of the antibiotics against *S. aureus* strains (CECT 976, SA1199B, RN4220 and XU212) were obtained from the work of Abreu et al. (2014). The resulting plate presented a pattern in which every well contained a unique combination of concentrations between the two molecules. Then, each microtiter well was inoculated with 180 µL of bacterial suspension with an optical density of 0.1 ± 0.02 at 600 nm, and the plates were incubated at 37 ºC and 150 rpm for 24 h. Cell suspension with DMSO and cell suspension without drugs were used as controls. The bacterial growth was determined at 600 nm using a microplate reader (SPECTROstar Nano; ABMG LABTECH, Germany). At least two independent experiments were performed for each test and the highest concentration of DMSO remaining after dilution was 10% (v/v).

MIC were determined for each antibiotic at each ibuprofen concentration and for ibuprofen at each antibiotic concentration. MIC values obtained for a given combination were used to evaluate the effects of combination between ibuprofen and antibiotics by calculating the fractional inhibitory concentration index (FICI) according to Equation 3:

$$\sum \mathrm{FICI} = \mathrm{FICI_A} + \mathrm{FICI_B} = \frac{\mathrm{MIC_{AB}}}{\mathrm{MIC_A}} + \frac{\mathrm{MIC_{BA}}}{\mathrm{MIC_B}} \quad (3)$$

Where MIC_A and MIC_B are the MICs of drugs A (antibiotics) and B (ibuprofen) when acting alone and MIC_{AB} and MIC_{BA} are the MICs of drugs A and B when acting in combination. Off-scale MICs were converted to the next lowest doubling concentration. “Synergy” was defined when FICI values was ≤ 0.5, while values between 0.5 < FICI ≤ 4.0 were classified as indifferent, whereas FICI values > 4.0 were defined as antagonism (MacKay et al. 2000).

3.2.9 Statistical analysis

Both mean and standard deviation (SD) within samples were calculated for all cases. At least two independent experiments were performed for each condition tested. One-way ANOVA with Bonferroni test was performed for data assuming a normal distribution. Other data were statistically analysed by the non-parametric Wilcoxon test. Statistical analysis was based on a confidence level ≥95% ($p < 0.05$ was considered statistically significant).

3.3 Results and Discussion

3.3.1 Minimum inhibitory and bactericidal concentrations of ibuprofen and piroxicam

The MIC was defined as the lowest concentration that prevented visible microbial growth, whereas the MBC was interpreted as the lowest concentration that eliminated the capacity of the bacterial cells to proliferate in solid culture medium after exposure to drugs. The MICs of drugs against *S. aureus* CECT 976 are summarized in Table 1.

Table 1 – MIC and MBC of ibuprofen and piroxicam against S. aureus CECT 976.

	Ibuprofen		Piroxicam	
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
S. aureus strain CECT 976	500	1400	> 2000	> 2000

The MICs of ibuprofen and piroxicam were 500 μg/mL and > 2000 μg/mL, respectively. Whereas, the MBC of ibuprofen was 1400 μg/mL and for piroxicam was > 2000 μg/mL.

The MIC obtained for ibuprofen is in agreement with the results presented in a previous work, although in the present study the pH was not measured. Elvers & Wright (1995) verified that the MIC value for *S. aureus* exceeded 600 μg/mL at pH 7, while at pH 6 the value was 350 μg/mL.

As piroxicam did not display antibacterial activity, only ibuprofen was chosen for further tests in order to understand in more detail its mode of antibacterial action.

3.3.2 Effect of different doses of ibuprofen on *S. aureus* growth

Dose response curves within 1 h standard exposure time were performed to assess the relationship between the effects of ibuprofen on culturability of *S. aureus* as a function of the drug concentration (Figure 3). It was also of interest to evaluate the antimicrobial action of ibuprofen when *S. aureus* cells were maintained both in MH broth and in NaCl solution to infer about their effect on drug activity.

The dose response behaviour for ibuprofen in MH broth and in NaCl solution was statistically different ($p < 0.05$). The bacterial culturability after exposure to ibuprofen in MH broth remained constant until the concentration of 950 µg/mL, whereas at 1400 µg/mL ibuprofen promoted total loss of *S. aureus* capacity to proliferate in solid culture medium. These results are in agreement with the MBC of ibuprofen that was presented above. In NaCl solution, ibuprofen induced a sharp decrease in culturability, where at 125 µg/mL ibuprofen elicited a 2.5-log reduction and a total loss of bacterial culturability was verified for concentrations higher than 250 µg/mL. The distinct effect of ibuprofen in MH broth and in NaCl solution can be related with a possible interaction between the drug and the culture medium. From the pharmacokinetic data, it is known that ibuprofen is extensively (>98%) bound to whole human plasma and purified albumin (Davies 1998). As MH media contains beef extract and acid hydrolysate of casein, in some way the proteins and amino acids that are present in the culture medium can neutralize the action of ibuprofen against the bacterial cells.

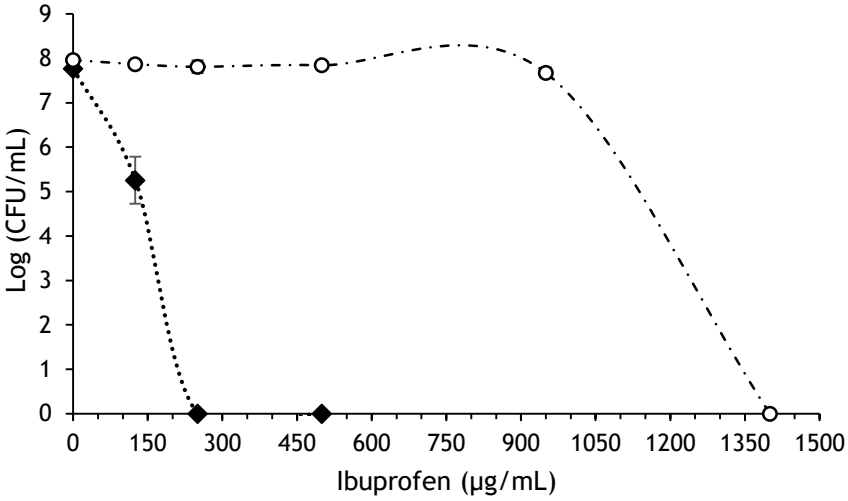

Figure 3 - Log (CFU/mL) of S. aureus as a function of ibuprofen concentration (0, 125, 250, 500, 950 and 1400 µg/mL) after 1 h of exposure in NaCl solution (····◆····) and in MH broth (- · ○ · -). Mean values ± SD for at least two independent experiments are illustrated.

3.3.3 Effect of ibuprofen on bacterial cytoplasmic membrane integrity

In order to assess the effect of ibuprofen on bacterial cytoplasmic membrane integrity, it was determined the PI uptake after 1 h of exposure to ibuprofen in NaCl solution and in MH broth (Table 2 and Figure 4). PI is commonly used as an indicator of cytoplasmic membrane integrity, as it is a nucleic acid stain to which cell membrane is usually impermeable (Simões et al. 2007; Borges et al. 2013).

The results suggest that ibuprofen compromised the integrity of the cytoplasmic membrane. The percentage of cells with damaged cytoplasmic membrane increased considerably with the contact to ibuprofen, either in NaCl solution or MH broth ($p < 0.05$). The PI uptake was not dependent of ibuprofen concentration, because it was not found statistically

significant differences ($p > 0.005$) between the concentrations used, either in NaCl solution or MH broth. Nevertheless, the PI uptake was slightly less for bacterial cells treated with ibuprofen at 500 µg/mL.

Table 2 – Permeability of S. aureus to PI after 1 h of exposure to ibuprofen in NaCl solution and in MH broth. Mean values ± SD for at least two independent experiments are illustrated.

	PI stained cells (%)	
	NaCl	MH broth
Control (cells)	2.30 ± 5.8	7.40 ± 5.4
Control (cells + DMSO)	10.6 ± 14.7	14.5 ± 4.6
Ibuprofen (µg/mL)		
125	99.1 ± 2.1	97.4 ± 4.4
250	99.8 ± 0.5	99.6 ± 1.1
500	97.9 ± 3.9	73.2 ± 18.0

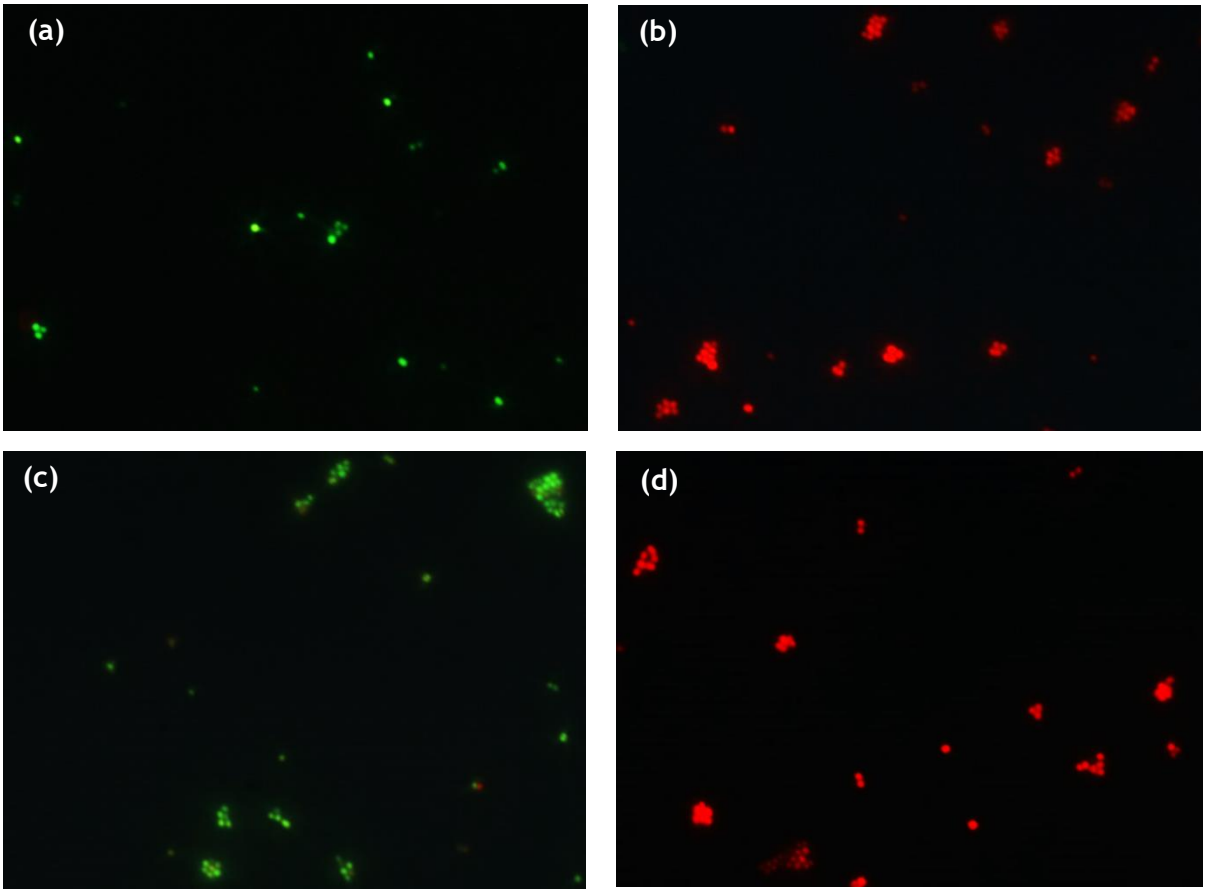

Figure 4 – PI uptake images (magnification 1000×). (a) S. aureus in NaCl solution; (b) S. aureus in NaCl solution incubated with ibuprofen at 250 µg/mL for 1 h; (c) S. aureus in MH broth and (d) S. aureus in MH broth incubated with ibuprofen at 250 µg/mL for 1 h.

Further analysis of PI uptake and dose response curves showed a contradictory behaviour between the results. The permeability of *S. aureus* to PI revealed that even for sub-inhibitory concentrations the percentage of cells with damaged cytoplasmic membrane was higher than 95%, even though most of the bacteria could form colonies on conventional medium at these concentrations. This fact led to the assumption that a possible interference of ibuprofen with the dyes' signal can explain the discrepancy between the results. Actually, there are critical aspects of this staining method for membrane integrity assessment, such as bleaching effects of SYTO9, different binding affinities of the dyes to cells and background fluorescence that can result in an under- or overestimation of the number of viable cells (Stiefel et al. 2015).

Considering that cytoplasmic membrane acts as a barrier between the cytoplasm and the extracellular medium, the specific ionic composition of the cytosol usually differs greatly from the extracellular medium. Because the internal ionic environment is generally rich in K^+, its fluxes has been used to monitor membrane damage in microorganisms (Lambert & Hammond 1973). Therefore, the results of PI uptake prompted to the additional evaluation of bacterial cytoplasmic membrane integrity by quantification of K^+ efflux.

The results of intracellular K^+ release after exposure to ibuprofen at 125, 250, 500 and 1400 μg/mL for 1 h are illustrated in Table 3. The results indicated alteration in the cytoplasmic membrane permeability. Intracellular contents were released due to the action of ibuprofen, when compared to the control samples ($p < 0.05$). However, it was not found statistically significant differences between the four concentrations tested ($p > 0.005$).

Table 3 – Concentration of K^+ (μg/mL) in solution after contact of S. aureus with ibuprofen for 1h. Mean values ± SD for two independent experiments are illustrated.

	Concentration of K^+ in solution (μg/mL)
Control (cells)	0.00 ± 0.00
Control (cells + DMSO)	0.00 ± 0.00
Ibuprofen (μg/mL)	
125	0.51 ± 0.08
250	0.51 ± 0.08
500	0.49 ± 0.08
1400	0.54 ± 0.00

Previous studies reported that microbial cytoplasmic membrane is directly affected by some non-antibiotics. Silva et al. (1979) studied the membrane effects of local anaesthetics on Gram-positive bacteria and they found that chlorpromazine, nupercain and tetracain produced characteristic micro-morphological alterations and membrane fractures. Moreover, a quick and extensive K^+ efflux induced on bacterial cells further indicated that not only the membranes were directly affected by the anaesthetics, but the membrane permeability was also primarily

disturbed. Furthermore, Pina-vaz et al. (2000) demonstrated that the antifungal activity of ibuprofen against *Candida* species was due to direct damage of the cytoplasmic membrane, confirmed by a rapid an extensive leakage of intracellular K^+, permeation to PI and severe membrane ultrastructural alterations.

3.3.4 Effect of ibuprofen on physicochemical bacterial surface properties

The physicochemical cell surface properties were determined according the van Oss approach, which allowed the quantitative assessment of the cell surface hydrophobicity expressed as the free energy of interaction between apolar or slightly polar cells immersed in an aqueous phase. The polar and apolar components of surface tension of *S. aureus*, with and without ibuprofen treatment were also determined (Table 4).

The bacterial surfaces of *S. aureus* had hydrophilic character (ΔG_{sws} > 0 mJ/m^2), nevertheless, the application of ibuprofen elicited changes on the physicochemical surface properties (p < 0.05). Bacterial surface (34.1 mJ/m^2) became less hydrophilic with the exposure to ibuprofen, particularly at 125 µg/mL (27.3 mJ/m^2) and 250 µg/mL (27.9 mJ/m^2) (p < 0.05). The polar surface tension component (γ^{AB}) of *S. aureus* was not statistically significant affected by the contact with the ibuprofen for all the concentrations used (p > 0.05). However, the electron acceptor component (γ^+) increased with ibuprofen at 250 and 500 µg/mL (p < 0.05) from 0.63 mJ/m^2 to 1.08 and 1.67 mJ/m^2, respectively. Moreover, statistically significant differences (p < 0.05) between the controls and cells treated with ibuprofen were observed in the electron donor component (γ^-), which influenced the hydrophilic character of the bacterial surface.

Table 4 – Hydrophobicity (ΔG_{sws}), apolar (γ^{LW}) and polar (γ^{AB}) surface tension components of untreated and ibuprofen treated S. aureus. Mean values ± SDs for at least two independent experiments are illustrated.

	Surface tension parameters (mJ/m^2)				
	γ^{LW}	γ^{AB}	γ^+	γ^-	ΔG_{sws} (mJ/m^2)
Control (cells)	39.7 ± 0.2	10.8 ± 1.5	0.63 ± 0.1	54.2 ± 0.8	34.1 ± 1.7
Control (cells + DMSO)	40.5 ± 0.0	10.5 ± 1.3	0.52 ± 0.1	55.0 ± 0.9	34.8 ± 1.3
Ibuprofen (µg/mL)					
125	39.5 ± 0.3	11.7 ± 0.1	0.70 ± 0.0	48.8 ± 0.1	27.3 ± 0.0
250	37.3 ± 1.1	13.8 ± 2.0	1.08 ± 0.2	49.7 ± 0.3	27.9 ± 0.0
500	38.0 ± 0.7	12.5 ± 0.5	1.67 ± 0.4	50.0 ± 0.9	30.1 ± 2.1

The alteration of physicochemical cell surface properties by non-antibiotics has been reported in previous studies. Jones et al. (1991) described the ability of three non-antibiotics

to alter the surface hydrophobicity of the clinical strains *E. coli*, *S. saprophyticus*, *S. epidermidis* and *C. albicans*. Other work indicated that incubation of *E. coli* with sub-inhibitory concentrations of ibuprofen isobuthanolammonium resulted in alterations of surface hydrophobicity in four out of seven strains (Drago et al. 2002).

3.3.5 Combinatorial activity of ibuprofen with antibiotics

Combinations of two or more antimicrobial drugs are commonly used for the treatment of bacterial infections and may represent an effective approach to combat MDR bacteria (Brooks & Brooks 2014; Worthington & Melander 2013). Additionally, the enhancement of antibiotic activity or the reversal of antibiotic resistance by non-antibiotics has been already suggested and supported by a number of investigations (Kristiansen & Amaral 1997; Kristiansen et al. 2007; Gunics et al. 2000; Ejim et al. 2011). This positive interaction with conventional antibiotics has been demonstrated for non-antibiotics from different classes, including antipsychotic drugs, tricyclic antidepressants, anti-neoplastic and anti-inflammatory drugs, among others as previously mentioned in Chapter 2, sub-section 2.2.2.

Consequently, in this study it was also intended to test the hypothesis that ibuprofen could have synergistic interaction with conventional antibiotics. Three antibiotics belonging to different drug classes were chosen: the fluoroquinolone CIP, the macrolide ERY and the board-spectrum TET. The combinatorial activity of ibuprofen with these antibiotics was assessed against the collection strain *S. aureus* CECT 976 and the resistant strains *S. aureus* SA1199B, *S. aureus* RN4220 and *S. aureus* XU212. The SA1199B strain has harbouring resistance to fluoroquinolones through overexpression of the NorA efflux pump and is a ciprofloxacin resistant. The RN4220 strain is resistant to 14- and 15-membered macrolides including erythromycin and contains multicopies of plasmid pU5054 that carries the gene encoding the MsrA macrolide efflux protein. Finally, the XU212 strain possesses the TetK efflux pump and is also an MRSA strain (Abreu et al. 2014; Chan et al. 2011).

The MICs of the antibiotics alone and in combination with ibuprofen against the different *S. aureus* strains and the corresponding FICI values are shown in Table 5. The preliminary results revealed that the combination of ibuprofen with one of each antibiotics tested generally elicited an antagonism interaction. Only the combination ibuprofen-CIP against the resistant strains *S. aureus* SA1199B produced an indifferent interaction, even with a 32-fold reduction in the MIC value of CIP.

The antagonism interaction usually occurs between bacteriostatic and bactericidal antibiotics. It has been noted that if the bactericidal drugs are the most potent with actively dividing cells, then an overall reduction of efficacy could be observed due to the inhibition of growth induced by a bacteriostatic drug. On the other hand, the combination of a bacteriostatic drug with a bactericidal agent against bacteria that are relatively resistant to the bactericidal

antibiotic can cause an additive effect (Satoskar et al 2009; Ocampo et al. 2014; Basri et al. 2014). Considering this and the fact that ibuprofen do not have bactericidal behaviour at concentrations < 1400 μg/mL, the combinatorial activity of ibuprofen with CIP could well be due to the bacteriostatic effects of ibuprofen and the bactericidal action of CIP. However, this hypothesis do not explain the results obtained for the combination with ERY or TET, both bacteriostatic antibiotics (Ocampo et al. 2014).

Table 5 – MIC and FICI of ibuprofen and the antibiotics CIP, ERY and TET against the different S. aureus strains tested.

S. aureus strains	Agents	MIC (μg/mL)		FICI	Outcome
		Alone	Combination		
CECT 976	Ibuprofen	500	> 1000	> 4	Antagonism
	CIP	1	> 2		
	Ibuprofen	500	> 1000	> 4	Antagonism
	ERY	0.24	> 0.48		
	Ibuprofen	500	> 1000	> 4	Antagonism
	TET	0.96	> 1.92		
SA1199B	Ibuprofen	500†	1000	2	Indifferent
	CIP	128	4		
RN4220	Ibuprofen	500†	> 1000	> 4	Antagonism
	ERY	256	> 512		
XU212	Ibuprofen	500†	> 1000	> 4	Antagonism
	TET	128	> 256		

MIC of the antibiotics were obtained from the work of Abreu et al. (2014). † MIC values of ibuprofen against the resistant strains were not tested and assumed equal to the collection strain.

Therefore, the predominant antagonism observed in this work suggest that ibuprofen should not be therapeutically used in combination with standard antibiotic in order to avoid the opposing action phenomenon. Nevertheless, it is important to address that the results shown here are only indicative of the combinatorial activity of ibuprofen with conventional antibiotics. Some assumptions were made that could influence the calculation of FICIs, particularly in the case of the resistant strains where the MIC value of ibuprofen was considered the same of the collection strain.

3.4 CONCLUSIONS

The antimicrobial activity of ibuprofen against *S. aureus* was confirmed, since it was obtained a MIC value of 500 µg/mL and a MBC value of 1400 µg/mL. Furthermore, the comparison of dose response curves in NaCl solution and in MH broth allowed the statement that the action of ibuprofen is affected by the culture medium and a most effective antibacterial activity can be achieved in NaCl solution.

The assessment of bacterial cytoplasmic membrane integrity by PI uptake after exposure to ibuprofen revealed permeation to PI, and the leakage of intracellular K^+ indicated that the antibacterial action of ibuprofen was due to damage of the cytoplasmic membrane. Moreover, the physicochemical characterization of bacterial surface revealed that the *S. aureus* surface was less hydrophilic when incubated with ibuprofen.

Preliminary results of combinatorial activity of ibuprofen with conventional antibiotics suggested a predominant antagonism interaction against the collection strain and the resistant strains of *S. aureus*. However, further analyses are needed in order to have a better understanding on the activity of ibuprofen-antibiotic combinations.

Chapter 4

THE EFFECT OF IBUPROFEN ON SESSILE *Staphylococcus aureus*

4.1 INTRODUCTION

Staphylococci are frequent commensal bacteria on the human skin and mucous surfaces and are among the most likely germs to infect any medical device that penetrates those surfaces. According to Otto (2008), staphylococci are recognized as the most frequent causes of biofilm-associated infections.

The formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients (Costerton et al. 1987). Moreover, because in these sessile communities bacteria have inherent resistance to antimicrobial agents, biofilms are responsible for many persistent and chronic bacterial infections (Romling et al. 2014).

The research performed in many biofilm-forming organisms has revealed that the development of a biofilm is a sequential process involving the transport of microorganisms to surfaces, initial reversible and irreversible adhesion to a substratum, microcolony formation through cell division and extracellular matrix production, maturation of attached bacteria in a differentiated biofilm, and finally the detachment and dispersal of planktonic cells from the biofilms, which contributes to the colonization of new areas (Costerton et al. 1999; Garrett et al. 2008; Vogeleer et al. 2014). The current biofilm prevention and biofilm treatment strategies try to interfere with this developmental cycle of biofilm formation. These approaches include the use of antiadhesive surfaces with altered physical, chemical and topographical properties, application of compounds that inhibit the production of functional bacterial adhesins, physical treatment of the biofilms, photodynamic therapy, targeting of the biofilm matrix degradation, interference with biofilm regulation, among others (Römling & Balsalobre 2012).

In this chapter it was intended to evaluate the effects of ibuprofen on sessile cells of *S. aureus*. To test the hypothesis of preventive effect on initial bacterial adhesion, it was analysed its action on bacterial motility, changes in the theoretical potential of adhesion to PS and the bacterial adhesion to PS microtiter plates. It was also examined the ability of ibuprofen to prevent biofilm formation and control monolayer adhered cells and 24 h old biofilms.

4.2 MATERIAL AND METHODS

4.2.1 Bacterial strain and culture conditions

S. aureus CECT 976 was the bacterium chosen for adhesion and biofilm studies. The culture conditions were the same described in Chapter 3, sub-section 3.2.1. Finally, the cell culture was standardized in fresh MH broth to an optical density of 0.04 ± 0.02 at 620 nm.

4.2.2 Ibuprofen and other reagents

Ibuprofen was selected for adhesion and biofilm studies and the stock solutions were prepared in DMSO as described in Chapter 3, sub-section 3.2.2. The reagents needed for these methods are Alamar blue solution at 400 µM, crystal violet at 1% (v/v), 99% ethanol and 33% glacial acetic acid that were obtained from VWR (Portugal).

4.2.3 Motility assay

Motility study was performed according to Borges et al. (2012). Overnight culture grown on Luria-Bertani broth (LB; Fisher Scientific, USA) was used to characterize bacterial motility. Fifteen µL of standardized bacterial culture with an optical density of 0.1 ± 0.02 at 600 nm were applied at the center of plates containing 1% (w/v) tryptone, 0.25% (w/v) NaCl and 0.3% (w/v) agar for colony spreading motility analysis. Ibuprofen at 250 and 500 µg/mL was incorporated into the growth medium after the sterilization and cooling of the mediums. Plates were incubated at 37 ºC and the diameter (mm) of the bacterial motility halos were measured at 24, 48 and 72 h.

4.2.4 Free energy of adhesion

The free energy of adhesion (ΔG_{iwI}^{Tot}) between the bacterial cells and PS surfaces was assessed according to the procedure described by Simões et al. (2010). When studying the interaction between substances *i* and *I* that are immersed or dissolved in water (w), the total interaction energy (ΔG_{iwI}^{Tot}) can be calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by Dupré equation (Equation 4):

$$\Delta G_{iwI}^{Tot} = \gamma_{iI} - \gamma_{iw} - \gamma_{Iw} \quad (4)$$

For instance, the interfacial tension for one diphasic system of interaction (γ_{iI}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{iI} = \gamma_{iI}^{LW} + \gamma_{iI}^{AB} \quad (5)$$

$$\gamma_{iI}^{LW} = \gamma_{i}^{LW} + \gamma_{I}^{LW} - 2\sqrt{\gamma_{i}^{LW}\gamma_{I}^{LW}} \quad (6)$$

$$\gamma_{iI}^{AB} = 2\left(\sqrt{\gamma_i^+\gamma_i^-} + \sqrt{\gamma_I^+\gamma_I^-} - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+}\right) \quad (7)$$

The other interfacial tension components, γ_{iw} and γ_{Iw}, were calculated in the same way. The value of the free energy of adhesion was obtained by the application of Equations 4-7, which allowed the assessment of thermodynamic adhesion:

$$\Delta G_{iwI}^{Tot} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} +$$

$$+ 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+}\right] \quad (8)$$

Thermodynamically, if ΔG_{iwI}^{Tot} < 0 mJ/m^2 adhesion is favoured, whereas adhesion is not expected to occur if ΔG_{iwI}^{Tot} > 0 mJ/m^2.

4.2.5 Bacterial adhesion and biofilm prevention

The microtiter biofilm assay is a useful method for testing bacterial attachment (Merritt et al. 2005). Bacterial adhesion and biofilm formation was performed according to Stepanović et al. (2000) with some modifications. Briefly, sterile 96-well PS microtiter plates were filled with 180 µL of bacterial suspension and 20 µL of ibuprofen at MIC and sub-inhibitory concentrations (½ MIC and ¼ MIC). The plates were covered and incubated at 37 ºC and 150 rpm for 2 h, to allow bacterial attachment, and 24 h, for biofilm formation. Afterwards, the attached bacteria and 24 h old biofilms were analysed in terms of biomass, metabolic activity and culturability.

The ability of bacteria to form biofilms was also classified according to the scheme of Borges et al. (2012):

- Non-biofilm producer (0): OD ≤ OD_{nc};
- Weak biofilm producer (+): OD_{nc} < OD ≤ 2 × OD_{nc};
- Moderate biofilm producer (++): 2 × OD_{nc} < OD ≤ 4 × OD_{nc};
- Strong biofilm producer (+++): 4 × OD_{nc} < OD.

This classification was based on the optical density (OD) values from biomass quantification (crystal violet staining). The OD_{nc} refers to negative control.

4.2.6 Monolayer adhered cells and biofilm control

The microtiter biofilm assay was also used to assess the ability of ibuprofen to disrupt monolayer adhered cells and 24 h old biofilms. Briefly, sterile 96-well PS microtiter plates were filled with 200 µL of bacterial suspension and incubated for 2 h (monolayer bacterial adhesion) and 24 h (biofilm formation) at 37 ºC and 150 rpm. After the incubation period, the content of each well was aspirated and washed one time with 200 µL of NaCl solution. Then, 180 µL of MH broth and 20 µL of ibuprofen (at MIC, 5 × MIC and 10 × MIC) were applied in each well. After 1, 6 and 24 h of exposure at 37 ºC and 150 rpm, the adhered cells and biofilms were analysed in

terms of biomass, metabolic activity and culturability. A parallel study was performed to assess the action of ibuprofen when the cells were maintained in NaCl solution instead of MH broth. For this, after monolayer bacterial adhesion, biofilm formation and washing steps, 180 µL of NaCl solution and 20 µL of ibuprofen (at MIC, 5 × MIC and 10 × MIC) were applied, and the plates were incubated for 1 h at 37 ºC and 150 rpm. Afterwards, the cells were analysed as referred above.

4.2.7 Biofilm mass quantification by crystal violet (CV) staining

Firstly, the content of each well was removed and the wells were washed with 250 µL of NaCl solution to remove all non-adherent and weakly adherent bacteria. The remaining attached bacteria were fixed with 250 µL of 96% (v/v) ethanol and, after 15 min, the microtiter plates were emptied. Then, 200 µL of 1% CV were added to each well and the biofilms were stained for 5 min at room temperature. Afterwards, the excess of stain was gently withdraw and 200 µL of 33% (v/v) glacial acetic acid was added to solubilize the dye. Finally, the biofilm mass was quantified by measuring the optical density at 570 nm using a microplate reader (FLUOstar Omega; BMG LABTECH, Germany). The results were presented as the biofilm mass reduction (%) in relation to biofilms non-exposed to ibuprofen.

4.2.8 Biofilm metabolic activity quantification by Alamar blue assay

The content of each well was removed and the wells were washed with 250 µL of NaCl solution to remove all non-adherent and weakly adherent bacteria. For the staining procedure, 190 µL of fresh MH broth and 10 µL of Alamar blue indicator solution were added to each well. Then, the microtiter plates were incubated for 20 min in darkness and room temperature. Metabolic activity was quantified by measuring the relative fluorescence at excitation and emission wavelengths of 570 nm and 590 nm, respectively, using a microplate reader (FLUOstar Omega; BMG LABTECH, Germany). The results were presented as the biofilm inactivation (%) in relation to biofilms non-exposed to ibuprofen.

4.2.9 Quantification of adhered and biofilm culturable cells

Culturability was defined as the capacity of the bacterial cells to proliferate in solid culture medium after drug exposure. Firstly, the content of each well was removed and the wells were washed with 250 µL of NaCl solution to remove all non-adherent and weakly adherent bacteria. Then, biofilms were scraped three times for 1 minute each with the addition of 200 µL of NaCl solution to resuspend the attached sessile cells. The content of each well was transferred to independent Eppendorfs. Afterwards, seven-fold serial dilutions in NaCl solution were performed and 10 µL of each dilution was placed on MH agar plates. Finally, the plates

were incubated at 37 ºC for 24 h. The number of colony forming units (CFU) was visually counted for plates containing 10 < CFU < 300 colonies and expressed as CFU per area of microtiter plate well (CFU/cm^2).

4.2.10 Statistical analysis

Both mean and standard deviation (SD) within samples were calculated for all cases. At least two independent experiments were performed for each condition tested. One-way ANOVA with Bonferroni test was performed for data assuming a normal distribution. Other data were statistically analysed by the non-parametric Wilcoxon test. Statistical analysis was based on a confidence level ≥95% ($p < 0.05$ was considered statistically significant).

4.3 Results and Discussion

4.3.1 Effect of ibuprofen on motility

Motility mechanisms are often employed by bacterial pathogens for host colonization and play an important role in the initial adhesion to a surface and consequent biofilm formation (Pratt & Kolter 1998). *S. aureus* is historically defined as a non-motile organism (Shaw et al. 1951), however, it has been previously demonstrated that colonies of *S. aureus* can passively expand across the surface of soft agar plates. Henrichsen (1972) proposed that the expansion forces of dividing *S. aureus* cells cause the motility phenomenon that was named darting motility. More recently, a different form of *S. aureus* motility was defined as colony spreading (Kaito & Sekimizu 2007; Tsompanidou et al. 2011). Therefore, the possible ability of ibuprofen to interfere with the colony spreading of *S. aureus* was investigated. Table 6 shows the motility results of bacteria with and without exposure to ibuprofen at 250 and 500 µg/mL.

Table 6 – Colony spreading (mm) of S. aureus in the absence and presence of ibuprofen. Mean values ± SD for at least two independent experiments are illustrated.

	Colony spreading (mm)		
	24 h	48 h	72 h
Control	7.2 ± 0.3	7.9 ± 0.2	8.6 ± 0.4
Ibuprofen (µg/mL)			
250	7.6 ± 0.4	8.4 ± 0.4	8.9 ± 0.3
500	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

It was not possible to verify the colony spreading ability of *S. aureus*. The bacterial cells in the absence and presence of ibuprofen at 250 µg/mL formed colonies with diameter similar to the size of the spotted area before incubation, even with 72 h of incubation. In the plates with ibuprofen at 500 µg/mL it was not observed bacterial growth, which can be related to the

fact that this concentration was the MIC value determined in the previous experiments (Chapter 3, sub-section 3.3.1). These results are not consistent with the findings of other investigations, where the colonies of *S. aureus* in the control plates had diameters between 20-85 mm (Borges et al. 2012; 2014; Abreu et al. 2014). Moreover, Kaito & Sekimizu (2007) demonstrated the ability of *S. aureus* to spread on soft agar surfaces at a speed of 100 μm/min. However, in their work it was addressed that the colony spreading is enhanced when the period of plate drying before incubation is shortened, whereas colony spreading is inhibited when the period is prolonged. Actually, in the present work it was verified that the absorption of the bacterial suspension by the medium was slow and it took more than the 15 min recommended by Kaito & Sekimizu (2007).

In contrast to the inconclusive results of the present work, it is known that some non-antibiotics inhibit bacterial motility. Imperi et al. (2013b) demonstrated that the anthelmintic drug niclosamide exerts a dramatic inhibitory effect on swarming mobility of *P. aeruginosa*, but swimming and twitching motilities are less affected. It was also reported that loperamide, an opioid-receptor agonist used to decrease the frequency of diarrhoea, decreased bacterial motility of *P. aeruginosa*, *Salmonella enterica* and *E. coli* at sub-inhibitory concentrations (Ejim et al. 2011). Additionally, the swarming motility of *P. aeruginosa* is also decreased by simvastatin, lovastatin and mevastatin (Hennessy et al. 2013).

4.3.2 Effect of ibuprofen on bacterial adhesion and biofilm prevention

Bacterial adhesion to surfaces is one of the initial steps leading to biofilm formation and is affected by many factors, including some characteristics of the bacteria itself, the target material surface, and the environmental factors, such as the presence of antimicrobial substances (An & Friedman 1997). In this work, the effect of ibuprofen on bacterial adhesion was evaluated by two different methods, the thermodynamic prediction of adhesion potential by quantification of the free energy of adhesion and by microtiter plate assays. Like previous studies (Simões et al. 2010; Borges et al. 2012; 2014; Abreu et al. 2014), PS was used as a model surface for adhesion and biofilm formation.

The characterization of the free energy of interaction (ΔG_{iwI}^{Tot}) between *S. aureus* and PS surface, when immersed in water in absence and presence of ibuprofen, is presented in Table 7. Ibuprofen was tested at MIC and at sub-inhibitory concentrations, i.e. ½ MIC and ¼ MIC.

Based on the thermodynamic prediction, *S. aureus* had no potential for adhesion to PS ($\Delta G_{iwI}^{Tot} > 0$ mJ/m^2), in the absence or with exposure to ibuprofen at 250 μg/mL. When exposed to ibuprofen at 125 and 500 μg/mL, the free energy of adhesion was < 0 mJ/m^2, which suggested that at these concentrations the adhesion is favoured.

Table 7 – Free energy of adhesion (ΔG_{iwI}^{Tot}) of untreated and ibuprofen treated S. aureus to PS when immersed in water. Mean values ± SD for at least two independent experiments are illustrated.

	Control (cells)	Control (cells + DMSO)	Ibuprofen (μg/mL)		
			125	250	500
ΔG_{iwI}^{Tot} (mJ/m^2)	2.0 ± 0.2	1.7 ± 0.7	-1.7 ± 0.0	1.0 ± 0.8	-1.8 ± 1.4

The ability of microorganisms to adhere to materials can also be predicted by direct analysis of the physicochemical surface properties (Simões et al. 2007; Simões et al. 2010; Ener & Douglas 1992; Chavant et al. 2002). Depending on the hydrophobicity of both bacteria and material surfaces, bacteria have different adhesion abilities. Generally, bacteria with hydrophobic properties prefer hydrophobic material surfaces, whereas the ones with hydrophilic characteristics prefer hydrophilic surfaces (An & Friedman 1997).

In Chapter 3, the physicochemical characterization of bacterial surface by the van Oss approach indicated that all the bacterial surfaces had hydrophilic character (ΔG_{sws} > 0 mJ/m^2), however, with the application of ibuprofen, the bacterial surfaces became less hydrophilic. As it had been demonstrated that PS surface is hydrophobic (ΔG_{sws} = -44 mJ/m^2) (Simões et al. 2010), the analysis of the physicochemical properties of both bacteria and PS surfaces also suggested that *S. aureus* is not expected to adhere on PS and with the exposure to ibuprofen the adhesion could be favoured.

In order to confirm the effect of ibuprofen on *S. aureus* adhesion, a comparison between the theoretical thermodynamic adhesion evaluation and the adhesion to PS microtiter plates was performed. The action of ibuprofen on *S. aureus* adhesion to microtiter plates within 2 h of exposure was evaluated based on its effects on biomass, metabolic activity and culturability. Ibuprofen was applied at sub-inhibitory concentrations, i.e. ½ MIC and ¼ MIC, to prevent growth inhibition and at MIC. Figures 5 presents the percentage of biomass reduction and metabolic inactivation. Figure 6 presents the Log (CFU/cm^2) that were able to adhere on PS when grown in the presence of ibuprofen for 2 h.

According to the results, the bacterial cells were able to adhere on PS microtiter plates. Moreover, the results of metabolic activity (Figure 5b) and culturability (Figure 6) revealed that ibuprofen had no significant effect on *S. aureus* adhesion (p > 0.05). However, the data of biomass reduction (Figure 5a) indicated that ibuprofen at the tested concentrations diminished cell adhesion and thus biofilm formation (p < 0.05). It was also observed that ibuprofen at lower concentrations led to a greater biomass reduction, as could be verified by the statistically significant difference (p < 0.05) between ibuprofen at 125 and 500 μg/mL. The differences between the results of biomass reduction, metabolic activity or culturability suggest that the

action of ibuprofen within the 2 h of exposure can possible be through slight reduction of the accumulation of organic or inorganic particles.

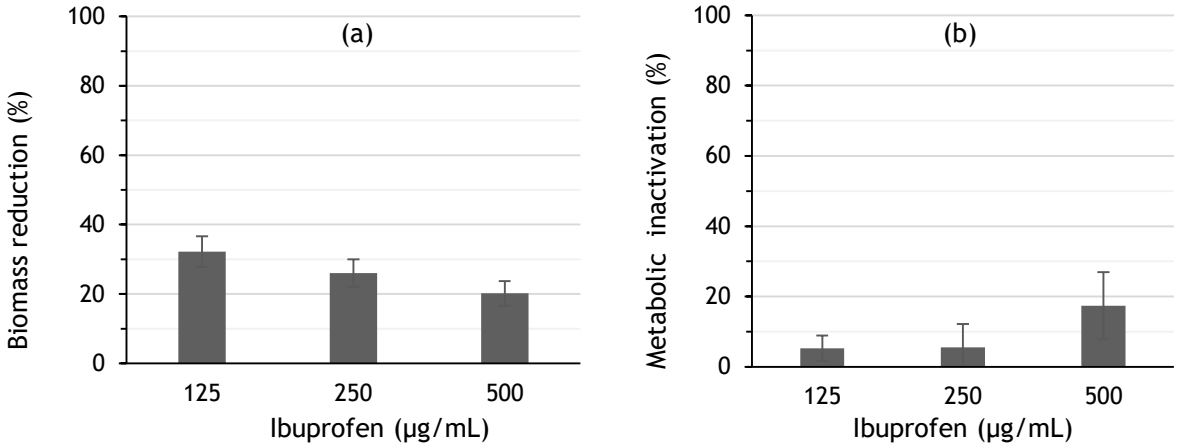

Figure 5 – Effect of ibuprofen on S. aureus adhesion to PS microtiter plates within 2 h of exposure. Percentage of biomass reduction (a) and metabolic inactivation (b). Mean values ± SD for at least two independent experiments are presented.

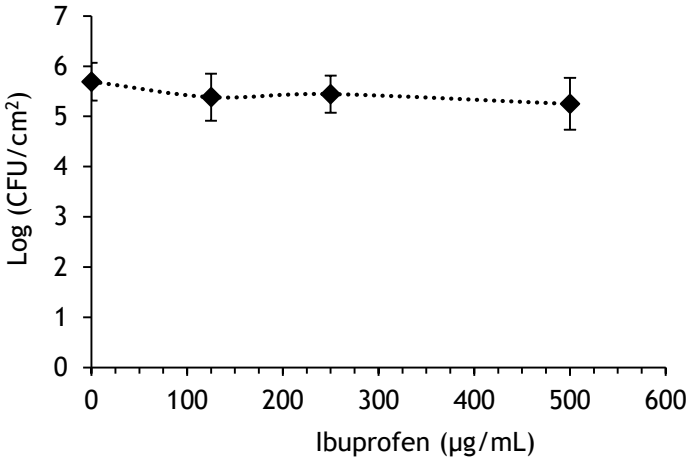

Figure 6 - Log (CFU/cm^2) of S. aureus cells that were able to adhere to PS microtiter plates within 2 h of exposure to ibuprofen at 125, 250 and 500 µg/mL. Mean values ± SD for at least two independent experiments are illustrated.

The lack of agreement between the theoretical thermodynamic adhesion evaluation and the adhesion assay suggest that *S. aureus* adhesion on PS is not only influenced by the surface physicochemical properties. Actually, a positive correlation between cell surface hydrophobicity and bacterial adhesion has been found by some authors (Pompilio et al. 2008; Chavant et al. 2002; Ener & Douglas 1992), but there are other works where the thermodynamic approach demonstrated to be ineffective as predictor for microbial adhesion (Silva-Dias et al. 2015; Borges et al. 2014; Abreu et al. 2014; Simões et al. 2010). This fact can be due to the multiplicity of parameters involved in the adhesion process, such as molecular and cellular interactions with expression of extracellular structures, in addition to the physicochemical ones (An & Friedman 1997; Garrett et al. 2008).

To ascertain the ability of ibuprofen to prevent biofilm formation, planktonic *S. aureus* was grown in the absence and presence of ibuprofen within 24 h of exposure to form biofilms on PS microtiter plates. Ibuprofen was applied at MIC and sub-inhibitory concentrations, i.e. ½ MIC and ¼ MIC. The biofilm prevention was evaluated based on the effects of ibuprofen on biomass production, metabolic activity and culturability (Figure 7 and 8).

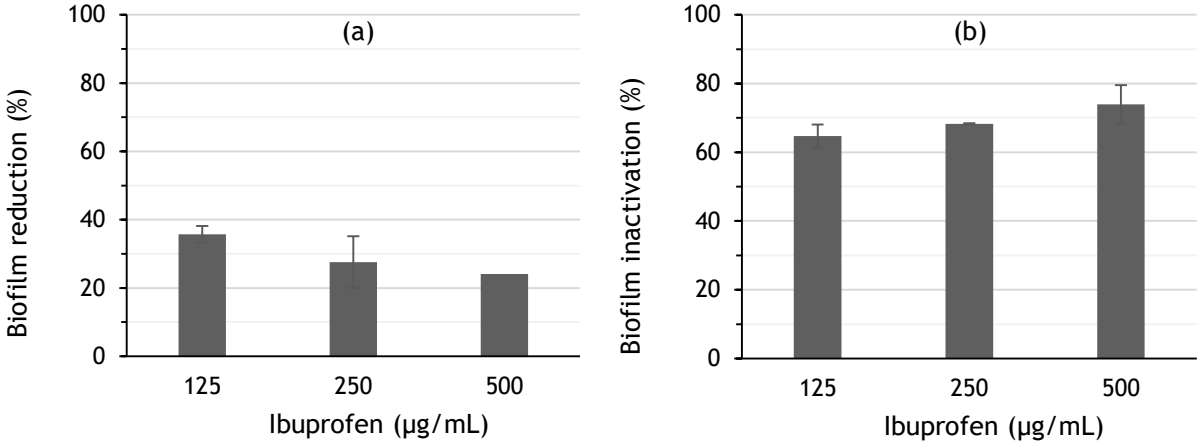

Figure 7 – Effect of ibuprofen on biofilm formation within 24 h of exposure. Percentage of biofilm reduction (a) and inactivation (b). Mean values ± SD for at least two independent experiments are illustrated.

A rank of biofilm formation was also performed according to the scheme of Borges et al. (2012), which classifies bacteria as non-biofilm producers, weak biofilm producers, moderate biofilm producers, or strong biofilm producers. *S. aureus* revealed to be a weak biofilm producer in PS substratum for all of the conditions tested, with and without ibuprofen exposure. The same result for the control sample was verified by Borges et al. (2012; 2014). Nevertheless, according to the obtained results, ibuprofen had preventive effects on biofilm formation ($p < 0.05$), either in terms of biomass, metabolic activity or culturability. The results of biofilm reduction (Figure 7a) assessed by the CV staining had a similar behaviour to the adhesion assay (Figure 5a), where biofilm reduction of ibuprofen at 125 µg/mL was statistically significant higher ($p < 0.05$) than ibuprofen at 500 µg/mL. However, it is important to note that the percentages of biofilm reduction presented above never exceeded 38%.

On the other hand, the presence of ibuprofen elicited a metabolic inactivation between 60-80% (Figure 7b). Moreover, while ibuprofen at lower concentrations led to a greater biofilm reduction, the opposite was observed in the Alamar blue assay. Ibuprofen at 500 µg/mL caused a higher biofilm inactivation than at 125 and 250 µg/mL ($p < 0.05$).

In terms of culturability (Figure 8), only ibuprofen at 500 µg/mL produced changes on the ability of bacterial cells to proliferate on solid culture medium, with total Log (CFU/cm^2) reduction (7-log (CFU/cm^2) reduction. For ibuprofen at 125 and 250 µg/mL, the Log (CFU/cm^2) value remained in the same magnitude of the control.

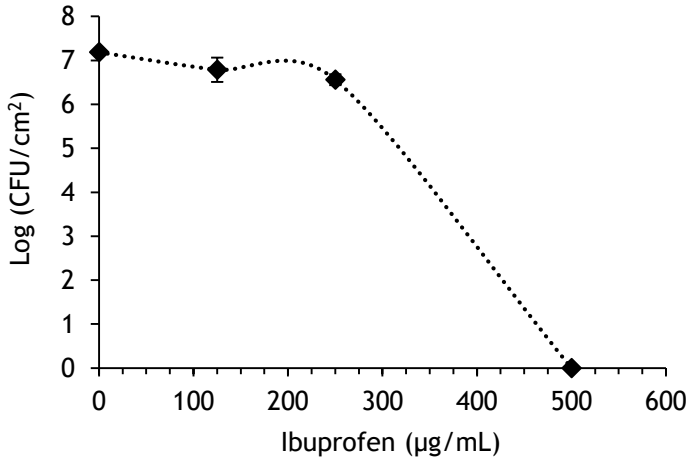

Figure 8 - Log (CFU/cm²) of S. aureus sessile cells after 24 h growth in the presence of ibuprofen at 125, 250 and 500 µg/mL. Mean values ± SD for at least two independent experiments are illustrated.

The comparison between the results obtained for biofilm reduction, biofilm inactivation and culturability (Figure 7 and 8) shows that the preventive effect of ibuprofen on biofilm formation is through different mechanisms and reinforces the idea that these three assays allow the observation of distinct phenomena. The incubation of planktonic *S. aureus* with ibuprofen at 125 and 250 µg/mL within the 24 h prompted a percentage of biofilm inactivation between 65-68%, however, the bacterial cells had the same ability to proliferate on solid culture medium than the control. This suggests that ibuprofen at these concentrations only interact with biofilms inducting the cells to enter in a dormant state, but when the drug is removed and these cells are placed on solid culture medium they can return to the metabolic active state and proliferate.

4.3.3 Effect on monolayer adhered cells and biofilm control

The effect of ibuprofen on monolayer adhered cells formed in PS microtiter plates within 1, 6 and 24 h of exposure was evaluated based on its effects on biomass, metabolic activity and culturability (Figures 9 and 10). Concerning the difficult process of microbial control when microorganisms are in sessile state, ibuprofen was applied at MIC, 5 × MIC and 10 × MIC, since the biofilm-associated cells are usually 10 to 1000× more resistant than in the planktonic state (Donlan & Costerton 2002). In Chapter 3 it was concluded that the antibacterial action of ibuprofen is enhanced in NaCl solution, so it was also of interest the comparison between the effect of ibuprofen on sessile bacteria when maintained in MH broth or in NaCl solution.

The results of biomass reduction (Figure 9a) showed that, for each exposure time tested, the ability of ibuprofen to remove the monolayer adhered cells in MH broth was more significant for ibuprofen at 500 and 2500 µg/mL than at 5000 µg/mL ($p < 0.05$). Curiously, the same behaviour was already observed in the study of the preventive effect on bacterial adhesion and biofilm formation. Comparing the biomass reduction after 6 h of exposure to ibuprofen and the

other exposure times, the results suggested that ibuprofen is more effective on the detachment of adhered cells after 1 or 24 h of incubation. However, for all the tested conditions, the percentage of biomass reduction of the monolayer adhered cells after incubation with ibuprofen was always less than 40%.

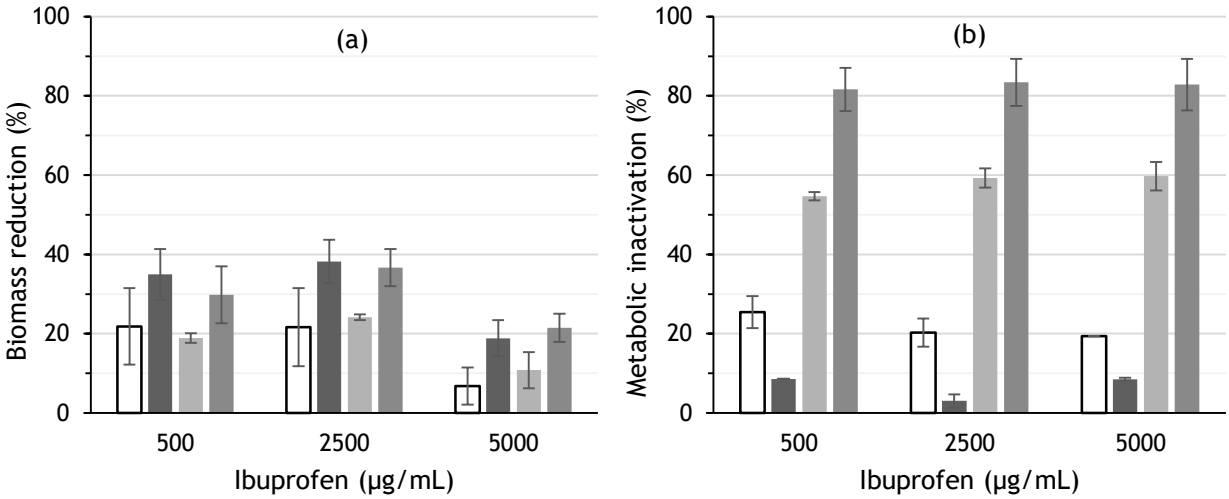

Figure 9 – Percentage of biomass reduction (a) and metabolic inactivation (b) of monolayer adhered S. aureus after exposure to ibuprofen for 1 ( ), 6 ( ) and 24 h ( ) in MH broth and for 1 h ( ) in NaCl solution. Mean values ± SD for at least two independent experiments are illustrated.

In terms of metabolic activity (Figure 9b), it was not found statistically significant differences between the ibuprofen concentrations ($p > 0.05$), for each exposure time tested. On the other hand, the comparison of the results between the different exposure times revealed that the metabolic inactivation was enhanced when the period of incubation with ibuprofen was prolonged. After 24 and 6 h of incubation, the metabolic activity of the monolayer adhered cells was reduced to approximately 80% and 60%, respectively. Nevertheless, in the treatments of 1 h, it was found that the percentage of metabolic inactivation was higher with ibuprofen in NaCl solution than in MH broth.

Once again, the analysis of the results presented in Figure 10 showed that the action of ibuprofen is improved in NaCl solution rather than in MH broth. Actually, the Log (CFU/cm^2) value of the monolayer adhered cells after exposure to ibuprofen at 500 µg/mL in NaCl solution was only comparable with the action of ibuprofen at the same concentration in MH broth within 24 h of exposure, where a 3-log (CFU/cm^2) reduction was observed in both cases. In contrast to the results of biomass reduction and metabolic inactivation, a complete growth inhibition on solid culture medium after the treatment with ibuprofen at 2500 and 5000 µg/mL was verified for all of the conditions tested (Figure 10).

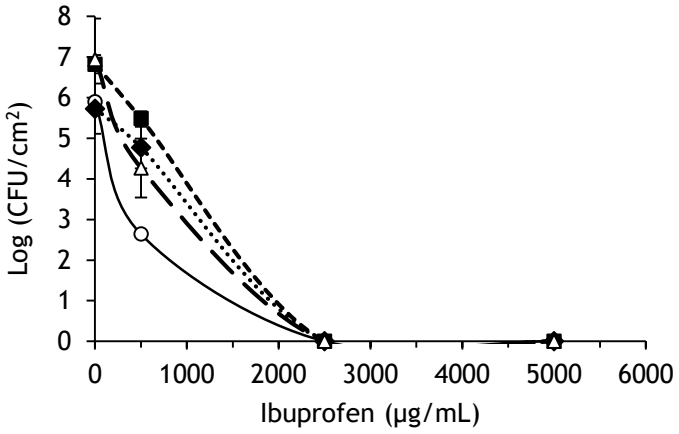

Figure 10 - Log (CFU/cm²) of monolayer adhered bacteria after exposure to ibuprofen at 500, 2500 and 5000 µg/mL for 1 (···◆···), 6 (- -■- -) and 24 h (—△—) in MH broth and for 1 h (—○—) in NaCl solution. Mean values ± SD for at least two independent experiments are illustrated.

A similar study was conducted to assess the ability of ibuprofen to control 24 h old biofilms of *S. aureus*. The biofilms were incubated for 1, 6 and 24 h with ibuprofen at MIC, 5 × MIC and 10 × MIC. Additionally, a treatment with ibuprofen in NaCl solution for 1 h was also performed. In Figure 11 is presented the percentage of biofilm reduction and inactivation. The value of Log (CFU/cm²) of the biofilm cells after incubation with ibuprofen is shown in Figure 12.

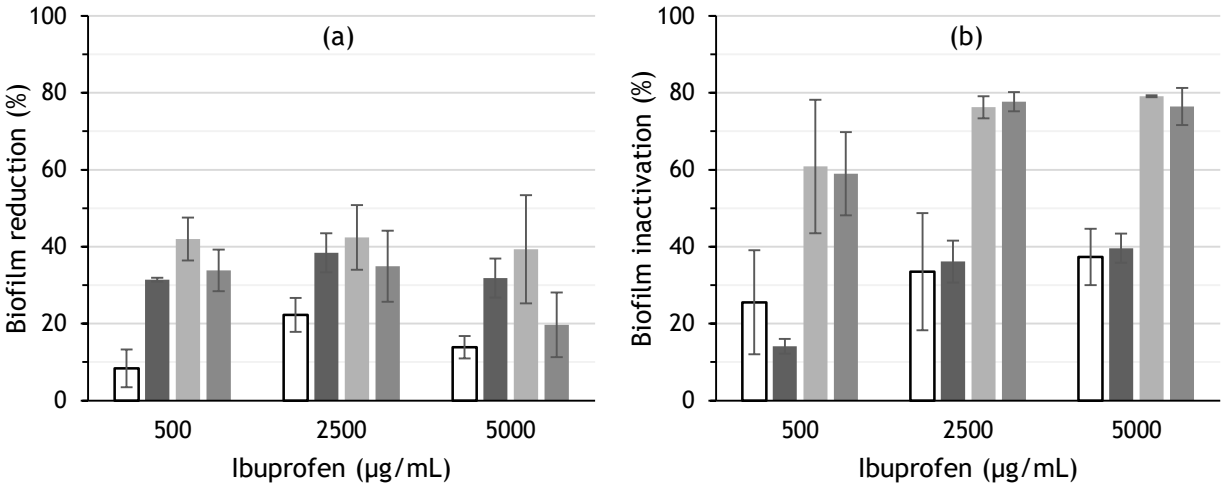

Figure 11 - Percentage of 24 h old biofilm mass reduction (a) and inactivation (b) after exposure to ibuprofen for 1 (■), 6 (■) and 24 h (■) in MH broth and for 1 h (□) in NaCl solution. Mean values ± SD for at least two independent experiments are illustrated.

Concerning the effect of ibuprofen on biofilm mass reduction (Figure 11a), the incubation for 1 h either in MH broth or in NaCl solution presented better results for ibuprofen at 2500 µg/mL ($p < 0.05$). However, with 6 h of exposure, no statistically significant differences between the three concentrations were observed ($p > 0.05$). The comparison of the results for different exposure times indicated that the biofilm reduction was improved when ibuprofen

was allowed to contact with the biofilm for 6 h. Nevertheless, the percentages of biofilm reduction were always ≤ 40%.

In terms of metabolic activity (Figure 11b), it was not found statistically significant differences between ibuprofen at 2500 and 5000 µg/mL ($p > 0.05$), for all of the exposure times. Additionally, for each incubation period, these concentrations allowed higher biofilm inactivation than ibuprofen at 500 µg/mL ($p < 0.05$). On the other hand, the treatment with ibuprofen for 6 and 24 h promoted similar biofilm inactivation.

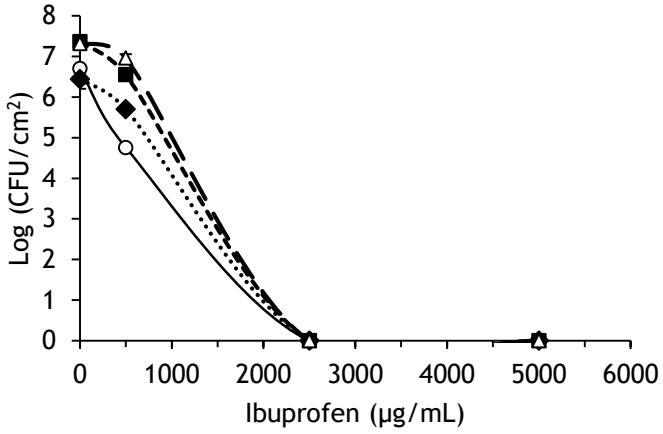

Figure 12 - Log (CFU/cm²) of 24 h old biofilm cells after exposure to ibuprofen at 500, 2500 and 5000 µg/mL for 1 (···◆···), 6 (- -■- -) and 24 h (—△—) in MH broth and for 1 h (—○—) in NaCl solution. Mean values ± SD for at least two independent experiments are illustrated.

The results of the effects of ibuprofen on biofilm culturability (Figure 12) showed a similar behaviour than those of the effect on monolayer adhered cells. Although, the analysis of metabolic activity indicated a percentage of active cells between 60-20% after the treatment with ibuprofen at 2500 and 5000 µg/mL, a complete growth inhibition on solid culture medium was verified for all of the conditions tested. Generally, the underestimation of viable cells by the plate count method can be due to the presence of injured cells or potentially viable but non-culturable cells, the inadequate culture conditions and the aggregation of bacteria that can lead to the formation of one colony from more than one cell (Banning et al. 2002; Simões et al. 2005; Gomes et al. 2016).

Finally, the comparison between biofilm reduction and inactivation allowed to conclude that ibuprofen have modest ability to remove biofilms, but can kill or induce a dormant state on the bacterial cells present in the biofilm. Additionally, the positive control effects for the incubation period of 6 h are curiously connected with the pharmacokinetics of the drug, since it is recommended the administration of ibuprofen every 6 h to maintain plasma concentrations within the therapeutic range (Davies 1998).

4.4 CONCLUSIONS

In this chapter, the effect of ibuprofen on sessile cells of *S. aureus* was demonstrated by the analysis of initial bacterial adhesion and biofilm formation, as well by evaluation of its ability to control monolayer adhered cells and 24 h old biofilms.

The action on the colony spreading motility of *S. aureus* was inconclusive and ibuprofen was not able to prevent initial bacterial adhesion within the standard period of 2 h. Additionally, it was verified that the thermodynamic approach was inappropriate for the prediction of *S. aureus* adhesion to PS, either in the absence or presence of ibuprofen. The effect on biofilm formation within 24 h was mainly through metabolic inactivation of the bacterial cells in the biofilm, rather than effective preventive action of biofilm accumulation, since the percentages of biofilm reduction never exceeded 38%. It was also confirmed that biofilm reduction, biofilm inactivation and biofilm culturability represent different phenomena, as it was verified by the distinct results obtained after exposure to ibuprofen.

The analysis of the effect on monolayer adhered cells and biofilm control demonstrated that the incubation with ibuprofen produces percentages of biomass reduction ≤ 40%, offering a moderate ability to remove attached cells and biofilms. However, the drug revealed to be more effective on metabolic activity with biofilm inactivation up to 80%. The comparison of the treatment of monolayer adhered cells and 24 h old biofilms with different concentrations of ibuprofen suggested that at 2500 μg/mL it is possible to achieve the best commitment between the amount of drug and efficacy. In terms of exposure time, the incubation period of 6 h showed relevant effects in biofilm control.

Chapter 5

CONCLUSIONS AND PERSPECTIVES FOR FURTHER RESEARCH

5.1 GENERAL CONCLUSIONS

In order to overcome the issues of MDR bacteria that are often related with pathogenic biofilms, there is an urgent need to discover new antimicrobials and anti-biofilm agents. Drug repurposing is now considered a promising and faster approach to face this alarming situation (Ribeiro et al. 2016). In this work, the ability of ibuprofen to control *S. aureus* growth in planktonic and sessile states was evaluated. The determination of different bacterial physiological indices enabled the assessment of the antimicrobial activity and the understanding of the mode of action against *S. aureus* cells. The study with sessile *S. aureus* disclosed the principal effects of ibuprofen on biofilm formation and biofilm control.

The present work confirmed that ibuprofen has antimicrobial activity against *S. aureus*, with a MIC of 500 µg/mL and a MBC of 1400 µg/mL. Moreover, the antibacterial activity against planktonic cells was enhanced when ibuprofen was allowed to contact with bacteria in NaCl solution, promoting loss of culturability even at sub-inhibitory concentrations. The exposure of *S. aureus* to ibuprofen elicited cell permeation to PI, release of intracellular K^+ and changes in the physicochemical properties of bacterial surface, supporting the statement that the antibacterial action is through cytoplasmic membrane destabilization. After contact with ibuprofen, *S. aureus* cell surface became less hydrophilic. The preliminary study of the ibuprofen-antibiotic interaction indicated that careful management in the combination of ibuprofen with conventional antibiotics is needed due to the predominant antagonistic effect.

Ibuprofen also displayed action on sessile *S. aureus*. Although it was not possible to conclude about the effect on bacterial motility and it was not detected the prevention of initial bacterial adhesion, ibuprofen interfered with biofilm formation mainly by metabolic inactivation. The reduction of biomass accumulation was at a lesser extent. Additionally, the thermodynamic approach was inappropriate for the prediction of *S. aureus* adhesion to PS, either in the absence or presence of ibuprofen, due to the contrasting results with the bacterial adhesion assay. This fact reinforces the idea that bacterial adhesion to solid surfaces is not only influenced by the surface physicochemical properties. In terms of the control of sessile bacteria, ibuprofen displayed moderate ability to remove attached cells and biofilms. However, the drug elicited metabolic inactivation up to 80%, which demonstrated that ibuprofen could be adsorbed by the monolayer adhered cells and 24 h old biofilms. The biofilm control

experiments revealed that the treatment with ibuprofen at 2500 µg/mL for 6 h allowed to achieve the best commitment between the amount of drug, period of exposure and efficacy. Despite this period of time is curiously related with the pharmacokinetic properties for anti-inflammatory purposes, the amount of 2500 µg/mL is above the plasma concentration that is frequently detected after administration of the drug. Therefore, taking all together, ibuprofen may be a good drug repurposing candidate for application as an antimicrobial and anti-biofilm agent.

5.2 PERSPECTIVES FOR FURTHER RESEARCH

Although this study revealed some of the effects of ibuprofen on *S. aureus* growth, it was possible to recognize that further research is still needed in order to achieve an insightful understanding of its action on planktonic and sessile bacteria.

Further investigation about the mechanisms of antibacterial action of ibuprofen may include the monitoring of the inhibition of key pathways, such DNA replication, RNA synthesis (transcription), protein synthesis (translation), cell wall (peptidoglycan) synthesis, and fatty acid (lipid) biosynthesis. This can be accomplished by macromolecular synthesis assay where radioactively labelled precursors are accumulated by bacterial cells and incorporated into specific macromolecules (Cotsonas King & Wu 2009; Thangamani et al. 2015b). Additionally, the cytoplasmic membrane destabilization can also be confirmed by measurement of membrane potential depolarization, transmembrane pH gradient and Zeta potential (Cotsonas King & Wu 2009; Kaatz et al. 2000; Ejim et al. 2011; Halder et al. 2015; Borges et al. 2013). Since, in the present work, it was only performed a preliminary study of the combinatorial activity of ibuprofen with conventional antibiotics, it would be of interest to perform a rational development of stable mixtures based on analytical techniques (DSC, FTIR and NMR spectroscopy) in order to analyse the stability and structural changes of the mixtures. Moreover, the investigation of the drug interaction through the use of bioinformatics tools, such as molecular docking, could help to predict the biological activity of the dual combinations.

In the study of the effects on sessile bacteria, there are some aspects that need further investigation as well. It has been recognized that QS is an important regulatory mechanism that might influence biofilm formation and differentiation (Irie & Parsek 2008; Borges et al. 2017). So, the evaluation of the potential of ibuprofen to inhibit QS could bring new findings about its importance on the prevention and control of biofilms. Additionally, it would be of interest to test the combinatorial anti-biofilm activity with N-acetyl-L-cysteine, a mucolytic agent that has been shown effects on extracellular polysaccharide production and biofilm formation (Marchese et al. 2003; Olofsson et al. 2003). It would be also important to evaluate the *in vivo* antibacterial and anti-biofilm efficacy of ibuprofen. Finally, with other experimental conditions

not explored in the present work, it could be possible to conclude about the efficacy of ibuprofen in the control of biofilms formed by cells previously exposed to the drug and its impacts on biofilm regrowth.

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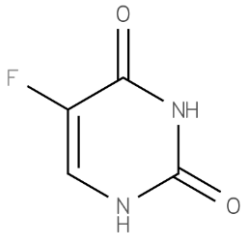
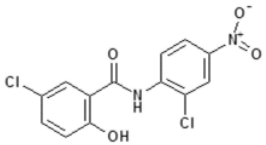
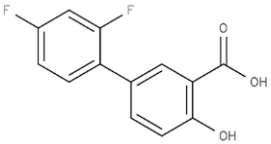
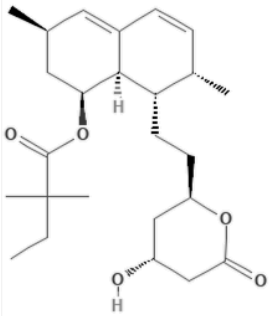
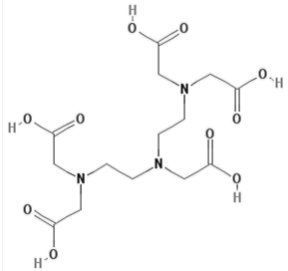
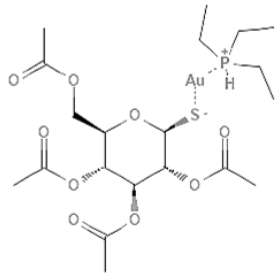
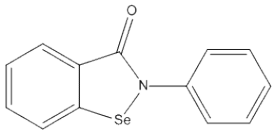
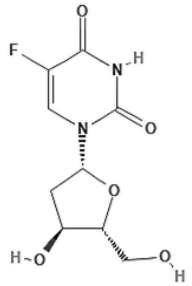
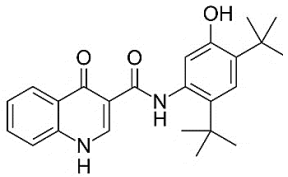
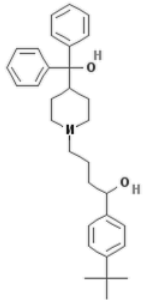
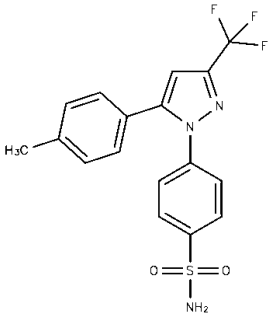
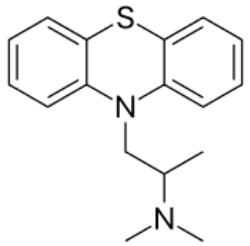
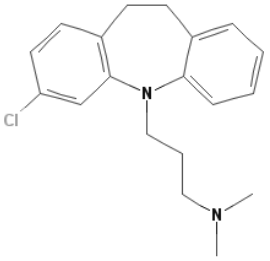
ANNEXES

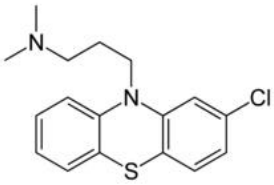
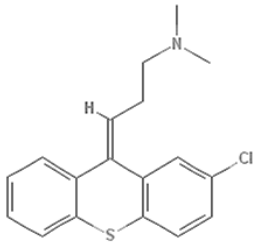
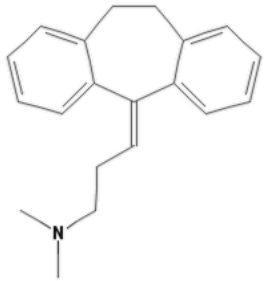
Table A. 1 – Non-antibiotics with antibacterial and anti-biofilm activity, and/or positive interaction with conventional antibiotics.

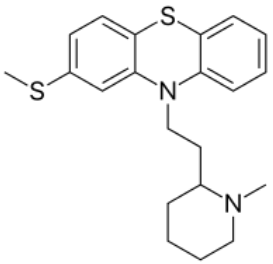
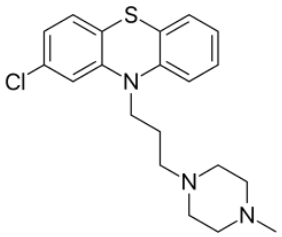
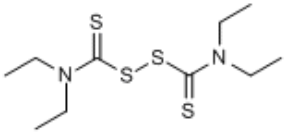
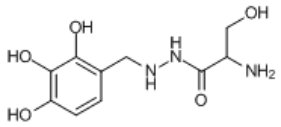
Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Gallium compounds	Treatment of hypercalcemia of malignancy	*Pseudomonas aeruginosa*	-Low concentrations inhibited growth -High levels killed planktonic bacteria	-Low concentrations prevented biofilm formation -High levels eradicated established biofilms	(Kaneko et al. 2007) (Antunes et al. 2012)
		Acinetobacter baumannii	-Inhibited the growth in both chemically defined medium and human serum, at 2 μM to 80 μM and from 4 to 64 μM, respectively - **Strong synergism with colistin**		
5-Fluorouracil 	Anticancer drug; also used for the treatment of actinic keratosis and Bowen's disease	*Staphylococcus aureus*	-Inhibited growth with MIC 50 ≤ 0.8 μg/mL -**Synergistic with tobramycin**		(Gieringer et al. 1986) (Hussain et al. 1992) (Ueda et al. 2009) (Attila et al. 2009)
		Staphylococcus epidermidis	-Inhibited growth with MIC 50 ≤ 0.8 μg/mL	-At levels below MIC greatly diminished biofilm formation	
		Pseudomonas aeruginosa		-Repressed biofilm formation	
		Escherichia coli		-Decreased biofilm formation by around 5-fold with 25 μM	
Niclosamide 	Anthelmintic drug	*Pseudomonas aeruginosa*	- inhibited QS with IC 50 = 10 μM and suppressed production of secreted virulence factors	- suppressed surface motility and reduced biofilm formation	(Imperi et al. 2013b)
Diflunisal 	Nonsteroidal anti-inflammatory drug	Methicillin-resistant *Staphylococcus aureus* (MRSA)	- inhibited the production of toxins α-hemolysin and phenol-soluble modulin α in a dose-dependent manner without inhibiting bacterial growth		(Khodaverdian et al. 2013)

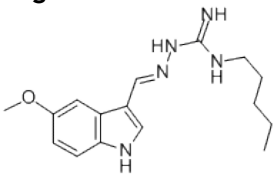
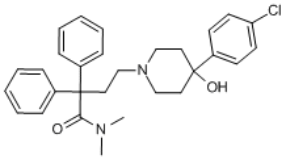
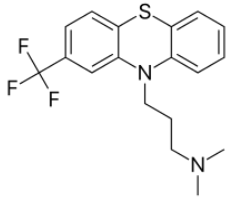
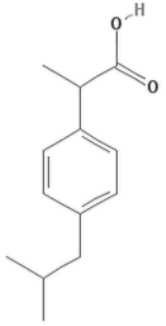
Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Statins  (Simvastatin)	Lower cholesterol levels and have anti-inflammatory functions	*Pseudomonas aeruginosa*	-Without significant inhibitory effect on bacterial growth	-Swarming motility was decreased by 100 µM of SIM, LOV and MEV - SIM at 100 µM attenuated the attachment of cells	(Hennessy et al. 2013) (Graziano et al. 2015)
		Staphylococcus aureus	-SIM's MIC = 15.65 µg/mL for *S. aureus* 29213 and 31.25 µg/mL for other strains of *S. aureus*	-SIM at 1/16xMIC to 4xMIC inhibited adhesion and biofilm formation -SIM was also able to act against mature biofilms, reducing cell viability and extra-polysaccharide production	
DTPA, pentetic acid 	Chelating agent used for preparing radiopharmaceuticals	*Pseudomonas aeruginosa*	-Suppressed the production of exoprotease elastase at 20 µM -Transcription of the elastase-encoding *lasB* gene and levels of the *Pseudomonas* quinolone signal (a molecule that mediates QS) were significantly downregulated	- Decreased biofilm formation	(Gi et al. 2014)
Auranofin 	Therapy of rheumatoid arthritis	*Staphylococcus* sp. including MRSA, VISA and VRSA	-Had bactericidal effects with MIC values ranging from 0.0625 µg/mL to 0.5 µg/mL	- Disrupted established *in vitro* biofilms of *S. aureus* and *S. epidermidis*	(Cassetta et al. 2014) (Thangamani et al. 2016)

Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Ebselen 	Anti-inflammatory, anti-oxidant, cytoprotective and neuroprotective agent	*Staphylococcus* including MRSA, VRSA and VISA	-Antimicrobial activity with MIC_{90}= 0.25 mg/L -Acts through inhibition of protein synthesis and subsequently inhibited toxin production in MRSA	-Reduced established biofilms	(Younis et al. 2015) (Thangamani et al. 2015b)
Floxuridine 	Antineoplastic	*Staphylococcus* including MRSA, VRSA and VISA	-Bactericidal activity, MIC_{90}= 0.0039 mg/L		(Younis et al. 2015)
Ivacaftor 	Used for the treatment of cystic fibrosis by stimulating chloride ion influx through cell membrane channels	*Staphylococcus aureus and Streptococcus pneumoniae*	-Inhibited the bacterial growth and exhibited positive interactions with antibiotics (**ceftriaxone, vancomycin, trimethoprim-sulfamethoxazole, moxifloxacin and linezolid**)		(Reznikov et al. 2014)
Terfenadine 	Antihistamine	*Staphylococcus aureus*	-Antimicrobial activity with MIC_{50}= 16 µg/mL and terfenadine analogs MIC_{50}= 1 µg/mL	-Active against established biofilms at 10x MIC	(Jacobs et al. 2013) (Perlmutter et al. 2014)
		Acinetobacter baumannii	-At MIC = 64 µg/mL the microorganism was susceptible		

Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Celecoxib 	Nonsteroidal anti-inflammatory drug	MRSA, VRSA and *Streptococcus pneumoniae*	- MICs ranging from 16 to 128 µg/mL - At 6.25 and 12.5 µM increased the sensitivity of *S. aureus* and MRSA to antibiotics **ampicillin, kanamycin, ciprofloxacin and chloramphenicol**		(Thangamani et al. 2015a) (Kalle & Rizvi 2011)
		Pseudomonas aeruginosa, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*	-MICs ranging from 8 to 32 µg/mL in combination with sub-inhibitory concentration of **colistin**		
Promethazine 	H1-antagonist with anticholinergic, sedative, antiemetic effects and some local anesthetic properties	*Escherichia coli*	- Limited inhibitory activity (MIC = 128 mg/L) but had synergistic activity with **ampicillin, tetracycline or erythromycin**		(Gunics et al. 2000)
		Staphylococcus epidermidis	- Limited inhibitory activity (MIC = 64 mg/L) but had synergistic activity with **erythromycin or tetracycline**		
Clomipramine 	Tricyclic antidepressant	*Staphylococcus epidermidis*	- Limited inhibitory activity (MIC = 64 mg/L) but had synergistic activity with **erythromycin or tetracycline**		(Gunics et al. 2000)

Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Chlorpromazine 	Psychotropic agent indicated for the treatment of schizophrenia; sedative and antiemetic agent	*Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes*	- At one-half its MIC (12.5 to 600 µg/mL) exhibited positive interactions with **penicillin, cefuroxime, methicillin, tobramycin, oxacillin and erythromycin**		(Kristiansen et al. 2010) (Kristiansen et al. 2003) (Hendricks 2006)
Trans-chlorprothixene 	Antipsychotic drug	*Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*	- At one-half its MIC (12.5 to >200 µg/mL) exhibited positive interactions with **penicillin, cefuroxime, methicillin and tobramycin**		(Kristiansen et al. 2010)
Amitriptyline 	Used for the treatment of depression, chronic pain, irritable bowel syndrome, sleep disorders, diabetic neuropathy, agitation and insomnia, and migraine prophylaxis	*Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*	- At one-half its MIC (100 to >200 µg/mL) reduced the MIC of **penicillin, cefuroxime, tobramycin and methicillin**		(Kristiansen et al. 2010)

Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Thioridazine 	Treatment of schizophrenia and generalized anxiety disorder	Methicillin susceptible *Staphylococcus aureus* (MSSA), MRSA and erythromycin-resistant *Streptococcus pyogenes*	-Inhibited the growth with MIC from 16 to 32 mg/L - Combination at 8-12 mg/L reduced the **oxacillin or erythromycin** resistance of all strains significantly.		(Kristiansen et al. 2003) (Kristiansen et al. 2007) (Hendricks 2006)
Prochlorperazine 	Antiemetic, antipsychotic, antihistaminic, and anticholinergic agent	methicillin-resistant (MRSA) *Staphylococcus aureus*	-Limited inhibitory activity (MIC = 32-64 mg/L) but the combination at 8-12 mg/L with **oxacillin** exhibited positive interaction		(Kristiansen et al. 2007) (Hendricks 2006)
Disulfiram 	Treatment and management of chronic alcoholism	*Staphylococcus aureus*	-Weak inhibitory activity (MIC = 32 to 256 µg/mL) but had synergistic activity with **minocycline**		(Ejim et al. 2011)
Benserazide 	Dopamine agent used in the treatment of Parkinson's disease	*Pseudomonas aeruginosa*	-Weak antibacterial activity (MIC = 128 - 256 µg/mL) but had synergistic interaction with **minocycline**		(Ejim et al. 2011)

Drug	Pharmacology	Microorganism	Effect on planktonic bacteria	Anti-biofilm activity	References
Tegaserod 	Serotonin 5-HT receptor antagonist for irritable bowel syndrome	*Pseudomonas aeruginosa*	-No antibacterial activity (MIC = 512 µg/mL) but had synergistic interaction with **minocycline**		(Ejim et al. 2011)
Loperamide 	Opioid receptor agonist used to treat diarrhea	*Pseudomonas aeruginosa*	-No antibacterial activity (MIC = 2048 µg/mL) but had synergistic interaction with **minocycline and other different tetracycline antibiotics**	-At 32 mg/mL decreased swimming motility	(Ejim et al. 2011)
		A. baumannii, *E. coli* and *K. pneumoniae*	-No antibacterial activity (MIC = 2048 µg/mL) but had synergistic interaction with **minocycline**		
Triflupromazine 	Management of psychoses and used to control nausea and vomiting	MSSA and MRSA	-Antimicrobial activity with MIC = 16 mg/L		(Hendricks et al. 2003)
		Pseudomonas spp. 25879	-Antimicrobial activity with MIC = 10 mg/L		
Ibuprofen 	Analgesic, anti-inflammatory and antipyretic agent	*Staphylococcus aureus*	- At pH 7, MIC > 600 µg/mL - At pH 6, MIC = 350 µg/mL		(Elvers & Wright 1995) (Naves et al. 2010)
		Escherichia coli		-Reduced biofilm development by 37.2% to 44.8% -Minimum biofilm inhibitory concentration (MBIC) from 2 to 125 mg/L	