

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO



Repurposing non-antibiotic drugs for antimicrobial purposes

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Mestrado Integrado em Bioengenharia

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Acknowledgments

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Resumo

As infecções persistentes têm sido uma grande preocupação para as comunidades médicas e científicas, já que microrganismos, geralmente associados a biofilmes, desenvolveram ao longo do tempo mecanismos de resistência a múltiplas drogas, situação que tem piorado com o passar dos anos. Os fármacos convencionais tornaram-se cada vez mais ineficazes e o desenvolvimento de novos fármacos é dispendioso e moroso. Assim, uma estratégia de reaproveitamento de medicamentos poderá ser uma excelente alternativa, pois permite reduzir os custos, o tempo e o risco associados ao desenvolvimento de novos fármacos. O objetivo do presente trabalho consistiu em avaliar a ação de quatro compostos anti-inflamatórios não esteróides (AINEs) [diclofenaco sódico (DCF), piroxicam (PXC), ácido acetilsalicílico (ASA) e naproxeno sódico (NPX)] no crescimento em estado planctônico e sésil de *Escherichia coli* (CECT 434) e *Staphylococcus aureus* (CECT 976).

A atividade antibacteriana e o modo de ação dos AINEs selecionados foram estudados usando diferentes índices fisiológicos bacterianos: concentração mínima inibitória (CMI), concentração mínima bactericida (CMB), captação de iodeto de propídio (PI) e liberação de potássio intracelular (K^+). As CMIs de todos os NSAIDs selecionados variaram entre 800 $\mu\text{g/mL}$ e >2000 $\mu\text{g/mL}$. Para o PXC as CMIs determinadas foram 800 $\mu\text{g/mL}$ e superior a 2000 $\mu\text{g/mL}$ (concentração máxima testada) para *E. coli* e *S. aureus*, respetivamente. Para o ASA, os valores de CMI foram 1750 $\mu\text{g/mL}$ e 2000 $\mu\text{g/mL}$ para *E. coli* e *S. aureus*, respetivamente. O DCF apenas foi eficaz contra *S. aureus* (2000 $\mu\text{g/mL}$) na concentração mais alta testada, uma vez que a CMI para a *E. coli* é superior a 2000 $\mu\text{g/mL}$. Da mesma forma, a CMI para o NPX é superior 2000 $\mu\text{g/mL}$, para ambas as estirpes bacterianas testadas. Os valores de CMB para todos os AINEs selecionados foi superior a 2000 $\mu\text{g/mL}$.

A integridade da membrana citoplasmática de *E. coli* e *S. aureus* foi avaliada após exposição aos AINEs selecionados através da avaliação da captação de PI e da liberação de K^+ intracelular. PXC e ASA causaram alterações na membrana citoplasmática de *E. coli*, aumentando a sua permeabilidade ao iodeto de propídio e a liberação de K^+ intracelular. Uma vez que os valores das CMIs foram maiores que 2000 $\mu\text{g/mL}$ para o PXC com *S. aureus*, para o DCF com *E. coli* e para o NPX com ambas as bactérias, nenhum ensaio de controlo de biofilmes ou testes de integridade membranar foram realizados para esses casos.

O potencial dos AINEs selecionados para erradicar biofilmes de *E. coli* e *S. aureus* (24 h) foi avaliado através de um ensaio em placa de microtitulação e caracterizado em termos de redução de massa de biofilme (coloração com violeta de cristal)/atividade metabólica (coloração com alamar azul) e culturabilidade (método de contagem em placa). PXC, DCF e ASA promoveram um elevado efeito na inativação metabólica com valores próximos a 80%, porém, apenas o PXC foi capaz de remover a biomassa com eficácia moderada para *E. coli* (28,6%). Para o ASA a 5×CIM e 10×CIM, em ambas as estirpes bacterianas, e para o DCF a 10×CIM, em *S. aureus*, verificou-se uma redução total da culturabilidade celular. No entanto, o mesmo não ocorreu para o PXC com *E. coli*, que obteve uma redução máxima de 3,8 log UFC (unidades formadoras de

colónias) por cm^2 a $10 \times CIM$.

O efeito dos AINEs selecionados quando combinados com os antibióticos tetraciclina (TET) e canamicina (KAN) em biofilmes de 24 h também foi avaliado. Apenas algumas combinações promoveram melhorias estatisticamente significativas em relação a um dos controlos (células com AINEs ou células com TET/KAN). De fato, para alguns casos, não há qualquer vantagem em combinar AINEs com ambos os antibióticos, pois o uso de um AINE ou antibiótico por si só promove um efeito igual ou melhor sobre a remoção de biomassa, inativação metabólica ou redução da culturabilidade.

Em geral, os AINEs selecionados parecem afetar a membrana citoplasmática das bactérias planctónicas e reduzir substancialmente a atividade metabólica e a capacidade de cultivo das células do biofilme. Portanto, este trabalho realça que os NSADs selecionados podem ser uma boa alternativa como agente antimicrobiano e anti-biofilme. No entanto, são necessárias novas pesquisas para melhor compreender o seu potencial contra bactérias patogénicas.

Abstract

Persistent infections have been a major concern for the medical and scientific communities since microorganisms, usually associated with biofilms, have developed mechanisms of resistance to multiple drugs over time and this situation has worsened over the years. Conventional drugs have become increasingly ineffective and the development of new drugs is costly and time-consuming. Thus, 'Drug repurposing' can be an excellent alternative, since it allows to reduce the costs, time and risk that the development of new drugs implies. The objective of the present work was to evaluate the action of four selected non-steroidal anti-inflammatory drugs (NSAIDs) [Diclofenac sodium (DCF), piroxicam (PXC), acetylsalicylic acid (ASA) and naproxen (NPX)] against *Escherichia coli* (CECT 434) and *Staphylococcus aureus* (CECT 976) growth in planktonic and sessile states.

The antibacterial activity and mode of action of the selected NSAIDs were studied using different bacterial physiological indices: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), propidium iodide (PI) uptake and intracellular potassium (K^+) release. The MIC of all selected NSAIDs ranged from 800 $\mu\text{g/mL}$ to >2000 $\mu\text{g/mL}$. For PXC, MIC values were 800 $\mu\text{g/mL}$ and higher than 2000 $\mu\text{g/mL}$ (maximum concentration tested) against *E. coli* and *S. aureus*, respectively. For ASA, the MIC values were 1750 $\mu\text{g/mL}$ and 2000 $\mu\text{g/mL}$ for *E. coli* and *S. aureus*, respectively. DCF was only effective against *S. aureus* (2000 $\mu\text{g/mL}$) at the highest concentration tested, since the MIC for *E. coli* is above 2000 $\mu\text{g/mL}$. Similarly, the MIC of NPX was greater than 2000 $\mu\text{g/mL}$, for both bacterial strains tested. The MBC for all selected NSAIDs were higher than 2000 $\mu\text{g/mL}$.

The integrity of *E. coli* and *S. aureus* cytoplasmic membrane was evaluated after exposure to the selected NSAIDs by PI uptake and leakage of K^+ . PXC and ASA caused changes in the cytoplasmic membrane of *E. coli*, increasing its permeability to propidium iodide and intracellular K^+ release. Once the MICs were greater than 2000 $\mu\text{g/mL}$ for PXC with *S. aureus*, DCF with *E. coli* and NPX with both bacteria, no biofilm control or membrane integrity assays were performed for these cases.

The potential of selected NSAIDs to eradicate pre-formed *E. coli* and *S. aureus* biofilms (24 h old) was performed using a microtiter plate assay and characterized in terms of biofilm mass (crystal violet staining)/metabolic activity (alamar blue staining) reductions and culturability (plate count agar method). PXC, DCF and ASA promoted high effect on metabolic inactivation with values close to 80%, however, only PXC was able to remove biomass with moderate efficacy for *E. coli* (28.6%). Total loss of the culturability of the biofilm cells was observed for ASA at 5×MIC and 10×MIC for both bacterial strains and for DCF at 10×MIC for *S. aureus*. However, the same did not occur for PXC with *E. coli*, with the maximum reduction of 3,8 log CFU (colony-forming units) per cm^2 at 10×MIC.

The effect of the selected NSAIDs combined with the tetracycline (TET) and kanamycin (KAN) antibiotics on 24 h biofilms was also evaluated. Only a few combinations promoted statistically significant improvements over one of the controls (cells with NSAIDs or TET/KAN cells).

In fact, for some cases there is no advantage in combine NSAIDs with both antibiotics, since the use of a NSAID or antibiotic alone promotes an equal or even better effect on biofilm cell inactivation, biomass removal and reduction of the culturability.

In general, the selected NSAIDs appear to affect the cytoplasmic membrane of planktonic bacteria and substantially reduce the metabolic activity and culturability of biofilm cells. Therefore, this work emphasizes that selected NSADs may be a good candidates for repurposing as an antimicrobial and anti-biofilm agent. Nevertheless, further research is still needed to understand its full potential against pathogenic bacteria.

Declaration

I hereby declare, on my word of honor, that this work is original and that all non-original contributions were properly referenced with source identification.

Date: Wednesday, June 26, 2019

Cláudia Leão

“Create the highest, grandest vision possible for your life, because you become what you believe”

Ed Lester

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Abbreviations

ADME	Absorption, distribution, metabolism and excretion
AI	Autoinducer
ASA	Acetylsalicylic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CECT	'Colección Española de Cultivos Tipo'
CFU	Colony forming units
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COX	Cyclooxygenase
DCF	Diclofenac sodium
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric matrix
ExPEC	Extraintestinal pathogene <i>E. coli</i>
InPEC	Intestinal pathogene <i>E. coli</i>
IZD	Inhibition Zone Diameter
KAN	Kanamycin
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistance
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant <i>S. aureus</i>
NPX	Naproxen sodium
NSAID	Nonsteroidal anti-inflammatory drug
OD	Optical Density
PCA	Plate count agar
PG	Prostaglandin
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2 α	Prostaglandin F2 α
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostacyclin
PI	Propidium iodide
PXC	Piroxicam
QS	Quorum sensing
STR	Streptomycin
TET	Tetracycline
tNSAID	Traditional Nonsteroidal Anti-inflammatory Drug
TXA2	Prothrombotic thromboxane A2

Chapter 1

Introduction

1.1 Background and Project Presentation

Over the past 60 years, the early recognition and treatment of infectious diseases have reduced the morbidity and mortality associated with these infections [1]. In addition, the better understanding of biological mechanisms allied to the fast development of efficacious and safe antimicrobial drugs allowed the treatment of infectious diseases [1, 2]. These antimicrobial drugs are known as antibiotics and they are the most successful chemotherapeutic drugs known to prevent and treat bacterial infections. The first systemic antibiotics created were antibiotics from the groups of sulfonamides and penicillins [2].

Initially, antibiotics were scarce and expensive, so they were available only for military use during World War II. Later, with the development of new antibiotics and optimization of the production processes, they became accessible to the entire population [2, 3]. However, the high availability and easy access to antibiotics led to them being used abusively, either in prophylactic use, or to treat any type of infections, even the most common of them. Moreover, the inappropriate prescription and the overuse of these antibiotics in the treatment of animal diseases and in agriculture contributed to the crisis of resistance to antibiotics [4, 5]. As early as in 1945, Alexander Fleming reported to 'The New York Times' that the high availability and abuse of antibiotics would lead to an evolution of the resistance of microorganisms to these drugs, causing more serious and more difficult to treat infections [6]. As expected, in less than one year of use, the number of strains resistant to penicillin increased and, a few years later, about 50% of the strains become non-susceptible to these antibiotics [2]. Today, the situation is even worse because, bacteria that were susceptible to antimicrobial drugs in the past, have developed resistance to different antibiotic classes, even to the most recently developed [7]. Thus, bacteria that only caused severe nosocomial infections in hospitalized patients, are now installed in the community, causing serious infections in patients who were considered non-vulnerable [2, 8]. By the way, studies shown 80% of human infections are caused by sessile microbial communities named biofilms, which are less susceptible to host defenses and highly resistant to antimicrobial agents when compared to planktonic cells [9, 10].

1.2 Objectives

In this work it was also intended to study the antimicrobial activity and the mode of action of nonsteroidal anti-inflammatory drugs (NSAIDs), acetylsalicylic acid (ASA), diclofenac sodium (DCF), piroxicam (PXC) and naproxen sodium (NPX) against selected strains, *E. coli* and *S. aureus*, taking into account the variables of concentration and time.

Another objective of this work was determine whether selected NSAIDs are able to control *E. coli* and *S. aureus* biofilms and their ability to improve the activity of common broad-spectrum antibiotics belonging to different classes, ciprofloxacin (CIP), tetracycline (TET) and streptomycin (STR) and kanamycin (KAN). Of the four antibiotics referred, it was intended to select the two antibiotics with the lowest effect on the studied strains, *E. coli* and *S. aureus*, in order to later evaluate the antimicrobial activity of the NSAIDs as antibiotic adjuvants.

At the end, it was pretended to evaluate if NSAIDs are good alternatives to fight the rising of biofilm resistance to antimicrobial drugs and if 'Drug Repurposing' seems to be a good strategy to combat this serious problem, or whether efforts should be made to create new antimicrobial drugs.

1.3 Structure of the Dissertation

Aside from the introductory chapter, this dissertation is still composed of four more chapters.

In chapter 2, all the state of art and the information collected in works already carried out on biofilms are described, including the resistance to the antimicrobial agents and on tests already carried out with the selected NSAIDs and also with other NSAIDs.

Chapter 3 focuses on the antibacterial activity and mode of action of selected NSAIDs against *E. coli* and *S. aureus*. So, all the planktonic tests are presented, where it was discussed the susceptibility of the *E. coli* and *S. aureus* bacterial strains to the selected NSAIDs (ASA, PXC, DCF and NPX) and antibiotics (CIP, TET, KAN and STR). Moreover, drug dose-time influence on the bacterial growth was discussed in this chapter. This chapter also includes studies of the interaction of the selected NSAIDs with the cytoplasmic membrane of both *E. coli* and *S. aureus* bacteria. The study of the physical and chemical properties of these drugs was also performed.

The chapter 4 provides the study of the ability of NSAIDs to eradicate pre-established biofilms, including their ability to remove biofilms, inactivate the metabolic biofilm cells and reduce the culturability of biofilm cells. The same assays were also performed for a mixture of NSAIDs with antibiotics, in order to evaluate their combined effect and their interaction.

In the chapter 5 are included the main conclusions about this study and the future perspectives regarding the use of NSAIDs as antimicrobial drugs and also the strategy of drug repurposing.

Chapter 2

Literature review

2.1 Microbial sessil communities

Biofilms are structured communities of sessile bacterial cells enclosed in a self-produced polymer matrix of polysaccharides, proteins and DNA [11, 12].

The formation of a biofilm occurs when planktonic bacteria adhere reversibly to a surface and undergo multiple changes until the formation of a mature biofilm, as can be seen in Figure 1.

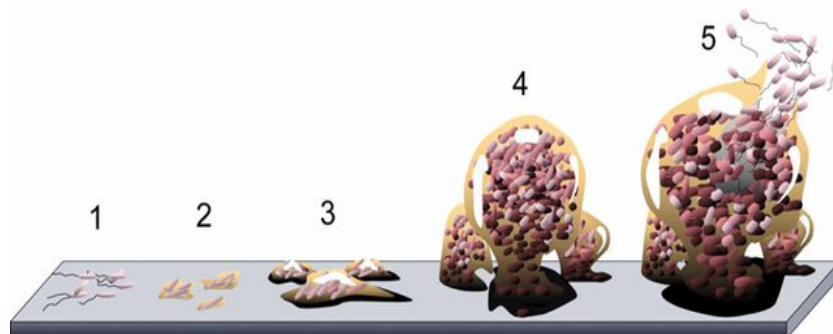


Figure 1: The five stages of biofilm formation. 1 - Planktonic cells adherence to the surface. 2 - Cells aggregates, form microcolonies and excrete extracellular polymeric substances (EPS). Irreversible attachment. 3 - Biofilm is formed and has multilayered clusters. 4 - A mature three-dimensional biofilm is formed. 5 - The critical mass of the biofilm is reached and it releases planktonic cells to colonize other surfaces. Figure by Dr. Erik Unosson from "Antibacterial Strategies for Titanium Biomaterials" [13].

At the first stage, although adhered, the bacteria are still susceptible to antibiotics. Then, cells aggregate and binds irreversibly to the surface to start the cell multiplication process, forming microcolonies. In a stage of biofilm maturation, microcolonies are responsible to produce a polymeric matrix of exopolysaccharides, proteins and DNA [14]. Furthermore, bacteria are able to transmit chemical signals to communicate with other bacterial cells, through signaling molecules called autoinducers (AIs). In this process named quorum sensing (QS), autoinducers are responsible to induce the expression of target genes, which consequently change the behavior of the

bacteria, in order to synchronize its activity with the activity of the other bacteria present in the cellular community [15]. Thus, communities move from a set of cells that act individually to a set of cells that work together to control a process, such as the control of many physiological functions, the regulation of the formation/differentiation of biofilm and expression of genes related to survival, virulence and pathogenicity [16]. In a mature stage, the biofilm exhibits the highest resistance to the antibiotics. Subsequently, a stage of cell detachment occurs where the planktonic bacteria moves to another place to initiate a new process of biofilm formation [11, 17, 18].

2.1.1 Mechanisms of antibiotic resistance in biofilms

As previously mentioned, biofilms are communities of microorganisms adhered to a surface and now it's known that these microorganisms express different characteristics from their equivalent planktonic cells, namely the greater resistance to antimicrobial agents [19]. There are several factors that confer resistance to microbial cells, among of the presence of extracellular polymeric matrix (EPS) that reduces diffusion and penetration of antimicrobial agents and protects biofilm microorganisms against various environmental stresses like starvation and dehydration [20]. In fact, studies shown that this matrix is not a impenetrable barrier to the antimicrobial agents, however it causes a delay in the diffusion of these drugs [19]. Nevertheless, the matrix prevents a sufficient intake of nutrients and oxygen in the inner regions of biofilms, inducing a vegetative state in some bacteria [21]. In this stationary phase these bacteria lose the ability to produce enzymes that could degrade the drugs and they're less susceptible to antimicrobial agents since the antimicrobial drugs do not act on metabolically inactive cells [22]. Furthermore, due to the heterogeneity of nutrients in microbial population, the subpopulation of metabolic inactive cells can exhibit multidrug resistance [23]. These cells are known as persisters and it's during the stationary phase that most of the persisters are formed [22].

Biofilms cells also develops adaptive responses like express induced resistance genes that lead to the appearance of enzymes that neutralize or degrade antibiotics [11] and the ability to overexpress efflux pumps, allowing the extrusion of the antibiotic out of the cell and prevents the antibiotic from acting efficiently against bacteria. Moreover, the ability of the cells to program apoptosis allow the community to reestablish itself with the nutrients released by the lysed cells [24]. Nevertheless, none of these mechanisms would be developed without cellular communication through quorum sensing system. This mechanism explained earlier in this chapter in section 2.1 is important to regulate the heterogeneous activity of biofilms, allowing the cells to act unequally in order to achieve the same end which is the resistance to antimicrobial agents [22].

Studies show that each mechanism imposes a barrier to the success of antimicrobial drugs but only with a combination of all these mechanisms is possible to achieve high antimicrobial resistance, once all of these mechanisms together allows biofilm communities to be 100 to 1000 times less susceptible to antimicrobial agents than equivalent planktonic bacteria [19, 25].

2.1.2 *Escherichia coli* and *Staphylococcus aureus* biofilms

E. coli is a rod-shaped Gram-negative bacterium. This non-sporulating and facultative anaerobic bacterium is the most abundant commensal microorganism in the gastrointestinal tract of warm-blooded living beings [26]. However, some pathogenic strains of *E. coli* causes a lot of common bacterial infections which are the major cause of morbidity and mortality. There are two types of *E. coli*, intestinal (InPEC) and extraintestinal (ExPEC). The first one includes toxin-producing strains related to Crohn's disease, enteric syndromes and hemorrhages. The second one includes infections of the urinary tract, neonatal sepsis and meningitis [27]. This bacterium is also associated with infections in medical devices such as prostheses and joints, shunts, urethral and intravascular catheters [28]. Among other factors, *E. coli* has extracellular appendages that facilitate its adhesion to surfaces and also allows their motility [29].

In the past, *E. coli* was treated with ampicillin in many industries for years and their resistance, whereby it's highly resistant to this antibiotic. However, in last decades, *E. coli* has developed resistance to other antibiotics, including the most recently developed from fluoroquinolones and cephalosporins classes [30].

S. aureus is a spherically shaped Gram-positive bacterium. It's also a non-sporulating and a facultative anaerobic bacterium and are commonly found on the skin and mucous membranes of humans, such as the armpits, anterior nostrils and perineum. However, they can also be found in the oropharynx, mouth, vagina, intestinal tract and mammary glands [31,32]. Similarly to *E. coli*, *S. aureus* are associated with infections in medical devices [33].

They are opportunistic bacteria that can cause simple localized infections in the skin, as well as serious infections such as meningitis, pneumonia, renal carbuncle and endocarditis [34]. *S. aureus* appears, predominantly, in the anterior nostrils and the nasal passages allow this microorganism to migrate to other areas of the body and, through epithelial rupture, to spread in the circulatory system. Besides, *S. aureus* produce an amorphous polysaccharide, located on bacterial capsule, which allows their binding to smooth surfaces, resulting in biofilm formation. They also produce extracellular toxins and enzymes that destroy the cells and tissues of the host, causing a serious and dangerous immune response [35]. Moreover, *S. aureus* has the ability to export drugs to the outside environment through several types of efflux pumps [36].

Initially, strains of *S. aureus* were treated with sulfonamides and penicillins and these infections were reduced dramatically. However, these strains have developed a resistance to these antibiotics. Later, was introduced semisynthetic penicillins, like methicillin and oxacillin, that brought under control these infections again, until the arrival of strains of *S. aureus* who developed resistance to all these antimicrobial drugs, the multidrug-resistant or methicillin-resistant *S. aureus* (MRSA) [33,37].

E. coli and *S. aureus* are commensal microorganisms, which are usually associated with infections in the community and hospitals and quickly form antibiotic resistant gene clusters through resistance plasmids and other genetic elements, who confer resistance to several antibiotics at once. Besides, antibiotic resistance quickly emerge and progress through bacterial populations,

apart from that adapts quickly to the existent treatment options [38]. Moreover, these pathogens are biofilm-producing bacteria involved in some of the most common bacterial infections worldwide. *S. aureus* belong to a group of pathogens termed ESKAPE that includes bacteria such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp* [39]. These bacteria are the major cause of nosocomial infections worldwide and many of them are resistant to multiple drugs which is one of the biggest threats to global public health, a problem who deserves some concern [40,41].

As a ESKAPE bacterium, *S. aureus* has a large amount of resistance mechanisms including biofilm formation, inactivation or alteration of drug properties, alteration of drug binding sites, and changes in cellular permeability, preventing intracellular accumulation of the drug, through efflux pumps and loss of porins. While efflux pumps allow removal of drugs from the inside of the cell, loss of porins, channels that allow the passage of hydrophilic drugs prevents drug uptake because hydrophilic drugs are not able to enter the cell through the cytoplasmic membrane [41]. It is known that one of the most critical mechanisms in resistance to antimicrobial drugs in *S. aureus* biofilms is the quorum sensing system regulated by the gene (*agr*) responsible for biofilm attachment/detachment [42]. Biofilm detachment plays an important role in biofilm infections, since it allows free cells to be lodged elsewhere in the body through the bloodstream, initiating a new infection at these sites [43]. Moreover, without a quorum sensing system, bacteria are not able to form highly differentiated biofilms making them more susceptible to antimicrobial drugs [42].

E. coli biofilms, similar to other biofilms, are a barrier to the treatment of bacterial infections with conventional treatments. The matrix of exopolysaccharides and cell-to-cell communication are characteristic of *E. coli* biofilms that provide protection against the entry of antimicrobial agents and against the conditions of the external environment and allow the cells to live cooperatively within this matrix [29]. In addition, *E. coli* has fimbriae that facilitate its adhesion to surfaces and self-aggregating factors that facilitate the aggregation of bacteria and the consequent formation of microcolonies. These characteristics provides sufficient conditions for the biofilm formation. *E. coli* also has motility which allow it to move through the biofilm in search of areas with greater availability of nutrients [44].

Thus, a better understanding of these pathogens regarding their virulence, resistance, transmission and pathogenicity can help to the development of new antimicrobial agents and treatment options [41].

2.2 Conventional therapies: Antibiotics

Until the 20th century, infectious diseases were one of the major causes of morbidity and mortality due to either the lack of knowledge of the causes of infectious diseases or the lack of knowledge of effective ways of treating them. Nevertheless, from the end of the 19th century the first studies on bacteria and on the antimicrobial chemicals by the hand of Paul Ehrlich begin to appear until the accidental discovery of one of the most important antibiotics by Alexander Fleming, the penicillin [45]. These events marked the beginning of the 'antibiotics era', however

the period from 1950 to 1970 was marked as the 'golden era' at which the great majority of the classes of antibiotics were developed [7].

Antibiotics are cytotoxic and cytostatic agents for bacteria, helping the host's immune system to eliminate them [7]. They are classified in several groups according to their mode of action (Table 1) so, they can act on the bacteria of different ways as for example, β -lactams and glycopeptide agents act at the level of bacterial cell-wall synthesis, as well as macrolides and tetracyclines are involved in the inhibition of protein synthesis, such as DNA gyrase, RNA polymerase and some other enzymes. Fluoroquinolones and rifamycins interfere with the nucleic acid synthesis and the sulfonamides usually inhibit metabolic pathways [8, 46].

The 'antibiotic era' began with the discovery of the first chemotherapeutic drug by Paul Ehrlich in 1910. This antimicrobial drug, arsphenamine was very important in the treatment of syphilis and is therefore one of the most commonly used drugs until the arrival of penicillin in 1928. After the discovery of arsphenamine, Ehrlich began a systematic screening approach and, at the beginning of his research on antibiotics, he discovered sulfa drugs, which were grouped into a class named sulfonamides. Currently, sulfonamides are used in combination with pirymethoxazole in the treatment of two opportunistic infections, pneumonia and toxoplasmosis. Penicillin was then discovered by Alexander Fleming in 1928, however, it was only used in 1942 to treat a streptococcal septicemia. Its application was only possible due to the studies of Howard Florey and Ernst Chain who studied the mode of action of this antimicrobial drug. Penicillin belongs to the class of β -lactams and rapidly the microorganisms developed the ability to cleave these β -lactam rings through the production of penicillinases, thus resulting in resistance to penicillin. For this reason, in 1970, penicillinases inhibitor compounds, such as clavulanic acid, sulbactam and tazobactam, were combined with penicillin to avoid this phenomenon. Due to the rapid development of resistance mechanisms, semi-synthetic and second-generation penicillins, ampicillin and amoxicillin were created from 1961. A third generation of penicillins was marked by the creation of carbenicillin, ticarcillin and piperacillin [6, 47].

The first antibiotic from aminoglycosides class to be isolated was streptomycin in 1943 and was the first solution for the treatment of tuberculosis. This antibiotic revolutionized medicine since tuberculosis was the greatest cause of death in the world, since the 19th century. Moreover, a second class of β -lactam antibiotics was discovered in 1945, the first generation cephalosporins such as cephalothin, cefazolin and cephalexin that were able to treat the typhoid fever crisis that plagued Italy. A second generation was later synthesized with the ability to cross the blood-brain barrier, revolutionizing the treatment of meningitis. There are also third and fourth generation cephalosporins and the effort for the synthesis of new generations of cephalosporins continues to this day [6, 47].

These antibiotics have revolutionized medicine in the treatment of diseases that were completely deadly prior to their discovery, however many other antibiotics of various classes were isolated or synthesized in order to combat resistance to these early antibiotics. However, as already mentioned, since its inception antibiotics have quickly become available and accessible to everyone, including in the industry. The 'wonder drug' was consumed in an abusive and irre-

Table 1: Classes and modes of action of the antibiotics and their resistance mechanisms. Taken from "A Review on Antibiotic Resistance: Alarm Bells are Ringing.", by Zaman et al. [7].

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β -Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem) monobactams (aztreonam)	Peptidoglycan biosynthesis; Hydrolysis efflux, altered target	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Translation	Hydrolysis, glycosylation, efflux, phosphorylation, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicol	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Efflux, altered target

sponsible way contributing to the appearance of the resistance of some microorganisms to these drugs.

Resistance has increased over the years and has been a major concern for the scientific community, as solutions are increasingly scarce and in the 21st century, the population is still not sufficiently aware of the danger of irresponsible taking of these drugs. Moreover, despite the existing awareness, some industries such as livestock production and feed production continue to abuse in the use of these antibiotics, increasing the antimicrobial resistance in these animals and

consequently in the people who feed on them [7,48].

2.3 New Strategies to fight Biofilms

The mechanisms of resistance developed by bacteria have led to the need to search of new new compounds with antimicrobial and antibiofilm properties that can replace the use of antibiotics or improve their activity, suppressing bacterial resistance mechanisms [38]. Knowledge of antimicrobial resistance factors to antibiotics including in the sessile mode of growth may be an important strategy for the search of new antimicrobial agents. Regarding biofilms, compounds that affect the ability of bacteria to adhere, prevent their communication (QS), affect their formation and inhibit expression of efflux pumps can be an interesting alternative [49]. Other strategy consists of select compounds that already exist on the market, used in the treatment of certain diseases, and use them for different treatment purposes [50]. This strategy is known as 'Drug Repurposing' and it's a promising strategy since it allows to reduce cost, time and risk of new drug development [51].

2.3.1 Non-antibiotic drug approach: nonsteroidal anti-inflammatory drugs

The NSAIDs are the most prescribed non-antibiotic drugs and are highly used to treat pain and inflammation [52]. Their mode of action consists in inhibit pro-inflammatory enzyme cyclooxygenase (COX), who blocks prostaglandins (PGs) production, as observed in Figure 2 [52–54]. Prostaglandins are important in the generation of the inflammatory response and are responsible for the development of cardinal signs (rubor (redness), calor (heat), tumor (swelling) and dolor (pain)) [55].

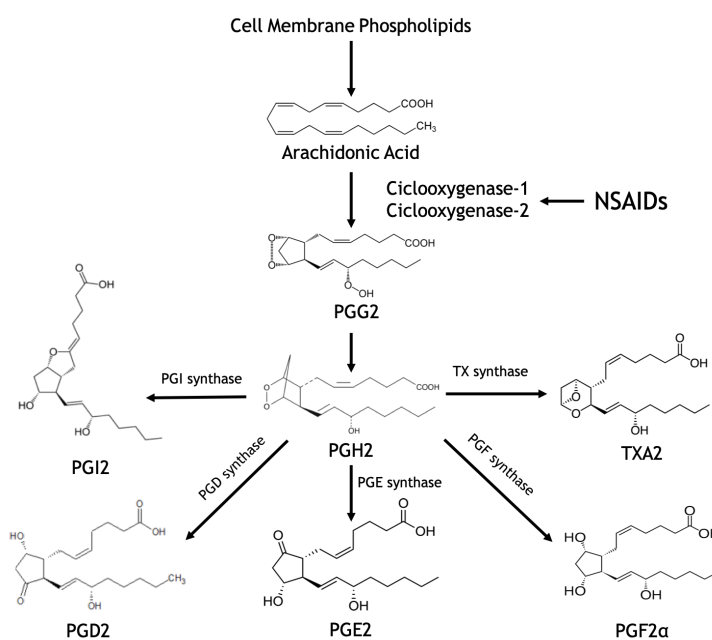


Figure 2: Prostaglandin biosynthetic pathway and NSAIDs action site. Adapted from [52].

While COX-2 is related to pain mediated by prostaglandin synthesis, COX-1 protects the gastric mucosa and maintains platelet homeostasis [56]. COX-1 is expressed at high levels in cells and tissues such as endothelium, platelets, monocytes, seminal vesicles, and renal collecting tubules. On the other hand, COX-2 is usually expressed in cells and tissues such as vascular endothelium, monocytes, macrophages, osteoclasts and synovial joint and has an important role in reproduction, renal physiology, bone resorption and neurotransmission [53].

NSAIDs include two classes, the traditional non-selective NSAIDs (tNSAID) that nonspecifically inhibits COX-1 and COX-2, and selective COX-2 inhibitors [57]. Selective COX-2 inhibitors have been developed with the aim of producing best anti-inflammatory and analgesic agents with reduced adverse effects compared to traditional NSAIDs [53, 56]. This new class of NSAIDs are safer than other for ulceration's of the gastrointestinal mucosa. However, there is evidence that selective COX-2 inhibitors lead to an increased risk of cardiovascular events, including an increased chance of heart attack and stroke in some patients [58]. NSAIDs are currently associated with gastrointestinal, renal, cardiovascular and cancer problems.

Currently there are about twenty NSAIDs and those are grouped into six families according to their chemical structure. In addition, those six groups are grouped into two larger groups, the acetylated and non-acetylated NSAIDs. NSAIDs are weak organic acids with hydrophobic properties which facilitates their binding to COX which is a membrane protein whose active site is located at the end of a hydrophobic channel [59].

Among the existing NSAIDs, in this work, four of them from different classes will be studied, diclofenac sodium (DCF), piroxicam (PXC), acetylsalicylic acid (ASA) and naproxen sodium (NPX) (Figure 3). ASA (also known as Aspirin®) (Figure 3a) belongs to the salicylates family (acetylated) acts by inhibiting PG synthesis, NF-Kappa B and iNOS and promoting L-selectin shedding [52, 59], however it's a NSAID that works slightly differently than others, since it's the only NSAID belonging to the group of acetylates. All the other NSAIDs are non-acetylated [60]. ASA is used in the relief of all grades of pain and inflammation related with rheumatoid arthritis osteoarthritis, ankylosing spondylitis and systemic lupus erythematosus [61]. Besides, ASA is used as antiplatelet agent in cardiovascular diseases. It also used in a rare syndrome that manifests in young children, Kawasaki syndrome [62].

PXC (Figure 3b) belongs to the oxicams family, derived from the enolic acids class, besides the function that is common to all NSAIDs which is the inhibition of PG synthesis, it also inhibits β_2 integrin activation [52, 59]. PXC is a medicine used in the treatment of osteoarthritis and rheumatoid arthritis, reducing inflammation, stiffness and joint pain caused by those diseases [61, 63].

DCF (Figure 3c), which belong to the acetic acids family is indicated in the relief of all grades of pain and inflammation related with a wide range of conditions, including musculoskeletal disorders, arthritic conditions and other painful conditions caused by trauma [61, 64]. DFC, besides inhibiting PG synthesis, also inhibits VLA-4 activation and allow L-selectin shedding [52, 59].

NPX (Figure 3d) is a NSAID belonging to the propionic acids family, which inhibits PG synthesis and Akt-dependent chemotactic pathways [52, 59]. Moreover, naproxen sodium is a

medicine used for pain relief and inflammation related to arthritic conditions, musculoskeletal disorders and trauma events. It's also used for dysmenorrhea and ankylosing spondylitis [61, 65]. All the selected NSAIDs are classified as non-selective NSAIDs [66].

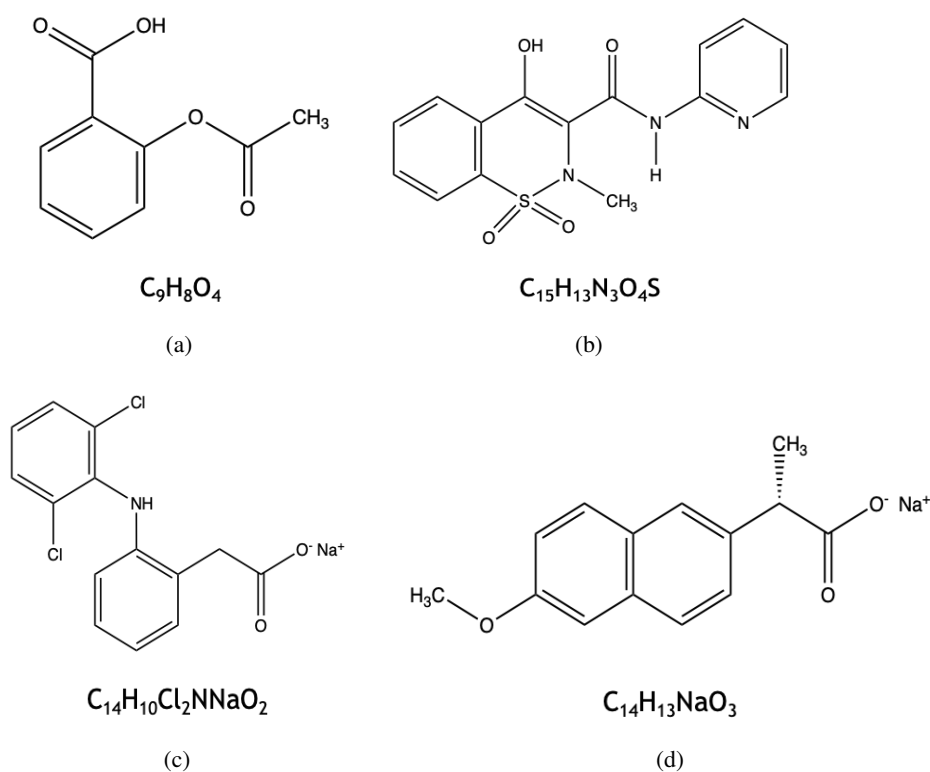


Figure 3: Chemical structures and molecular formula of some examples of NSAIDs: (a) acetylsalicylic acid, (b) piroxicam, (c) diclofenac sodium and (d) naproxen sodium.

2.3.2 NSAIDs ADME properties

The absorption, distribution, metabolism and excretion (ADME) properties are important properties for predicting the pharmacokinetic properties of a drug [67]. The discovery of new drugs often fails during clinical trials due to their poor ADME properties, implying a high waste of time and money [68]. Predicting the pharmacokinetic properties of drugs will substantially reduce the cost of drug development and allows it to focus only on high quality drugs with the potential to be absorbed, distributed, metabolized and excreted by the organism [69].

Absorption:

After oral administration, NSAIDs absorption are usually fast and complete and will be similar for any NSAID since all of them are extremely hydrophobic substances and are expected to share similar absorption characteristics [70].

Distribution:

The distribution of NSAIDs is facilitated by their high affinity with plasma proteins, essentially albumin. Given the acid and hydrophobic nature of NSAIDs and the basic and also hydrophobic character of albumin, they bind strongly through ionic and hydrophobic interactions. Given this strong interaction between NSAIDs and albumin, when NSAIDs are ingested simultaneously with anticoagulants, thyroid hormones, oral antidiabetic agents and the anticancer agent methotrexate, they cause saturation of plasma proteins, increasing the levels of free drug (unbound), leading to a phenomenon called plasma protein displacement [70]. Knowing that the unbound drug is the one that exhibit pharmacological effects, an increased level of unbound drug can lead to an increased activity and consequent toxicity [71]. A well-known example of plasma protein displacement is the simultaneous ingestion of Aspirin® and warfarin [70].

Metabolism:

Typically phase I of NSAID metabolism produces polar metabolites that are not sufficiently hydrophobic to compete with arachidonic acid for binding to COX. Thus, most NSAIDs are initially metabolized into inactive products which are subsequently converted into highly hydrophobic groups, transforming them into a non-polar metabolite. These metabolites are then active products of the reaction that compete with arachidonic acid, inhibiting COX [70].

Excretion:

NSAIDs can be excreted in three different ways: mostly as phase-II glucuronides; sometimes as sulfate conjugates; and in a small percentage of the times can be excreted in the urine without any chemical transformation [70].

2.4 State-of-the-art on the antibacterial and biofilm activity of NSAIDs

Due to the mechanisms of resistance developed by bacteria against antibiotics, many studies are done to find new solutions to this serious problem that society faces. Several studies have shown that NSAIDs may even be a solution as co-adjuvants of antibiotics or even as substitutes. Through an analysis of the used NSAIDs in the studies, the predominant NSAIDs were ASA (aspirin), ibuprofen, DFC, indomethacin and mefenamic acid.

Recent studies have shown that ibuprofen, DCF and aspirin exhibited antibacterial activity against Gram-positive bacteria, such as *Bacillus cereus* and *S. aureus*. However, only aspirin showed a broth spectrum activity and has exhibited antibacterial activity on Gram-negative bacteria, such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Salmonella choleraesuis*. Only mefenamic acid had no bacterial activity against the bacteria tested on this study [72–74].

Another study showed that mefenamic acid and PXC are the less effective NSAIDs tested and, although it had no effect on Gram-positive bacteria, they demonstrated a lower effect on Gram-negative bacteria [74].

The ability of DCF and indomethacin to decrease bacterial clearance from kidneys of mice infected with *E. coli* was also reported [75]. In another study with two different NSAIDs, sulindac sulfide and COX-2 selective NS-398, it was demonstrated substantial inhibitory and bactericidal activity. However, for this bacterium, sulindac sulfoxide and aspirin showed minimum inhibitory concentrations higher than therapeutic serum concentrations [76].

Many studies highlight the ability of anti-inflammatory drugs to inhibit and prevent the formation of biofilms. However, there are not many studies on their ability to eradicate them. Although not abundant, studies have shown that some NSAIDs such as aspirin and ibuprofen are able to control pre-established biofilms [77, 78].

It is clear that the use of NSAIDs as an antimicrobial agent depends on their minimum inhibitory concentration and their therapeutic serum concentration. If minimum inhibitory concentration were bigger than therapeutic serum concentration, the drug is clinically irrelevant [72, 76].

Chapter 3

Antibacterial activity and mode of action of selected NSAIDs

3.1 Introduction

Resistance to antibiotics has grown steeply over the years and has become a public health problem. Previously easy-to-treat infections begin to resist to conventional treatments. Therefore, strategies are currently being sought to rapidly obtain effective drugs in the treatment of these resistant bacteria [79]. Relying on existing drugs and understanding their mode of action against pathogenic bacteria is a good strategy to avoid the long process and cost of developing a new drug, since the propensity of this new drug to fail at some level of clinical testing is very high [51, 80].

In this project NSAIDs from different classes, PXC, DCF, ASA and NPX against *E. coli* CECT 434 and *S. aureus* CECT 976 were repurposed. The mode of action and susceptibility to the selected NSAIDs were evaluated in this Chapter.

3.2 Materials and Methods

3.2.1 Microorganisms and Culture Conditions

The model microorganisms used in this study was the collection strains *S. aureus* CECT 976 and *E. coli* CECT 434. The strains stored at -80°C were transferred to a Mueller–Hinton (MH, Sigma-Aldrich, Portugal) agar plate and grown overnight at 37°C . Then, they were inoculated in MH broth at 37°C , under agitation (150 rpm).

3.2.2 NSAIDs

The nonsteroidal anti-inflammatory drugs ASA, DCF and NPX were obtained from Sigma-Aldrich and PXC from Alfa aesar. The solutions were prepared in dimethyl sulfoxide (DMSO). The stock solutions of each NSAID were stored at 4°C until use (no more than 24 hours).

3.2.3 Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

The MIC values of NSAIDs against bacterial strains *E. coli* and *S. aureus* were determined by broth microdilution method in sterile 96-well microtiter plates (Orange Scientific, Belgium) according to Oliveira et al. [78]. Overnight cell cultures were adjusted with fresh MH broth to an OD_{600nm} of $0,132 \pm 0,02$ and added to the microtiter plates at a volume of 180 μ L. Then, 20 μ L of each NSAID were added at different concentrations in a range between 6,25 and 2000 μ g/mL for both bacteria. Cell suspensions without NSAIDs and cell suspensions with DMSO (10% v/v) was included as negative controls. The absorbance was measured using a microplate reader (SPECTROstar Nano; BMG LABTECH, Germany) before ($t=0$ h) and after ($t=24$ h) the incubation period. Plates were incubated for 24h at 37°C under agitation (150 rpm). MIC values corresponds to the concentration in which the final OD was equal or below to the initial OD. All tests were performed with six replicates with a minimum of two repeats.

For MBC determination, 10 μ L of each concentration equal and above the MIC value was added to a plate count agar (PCA; Oxoid, England) plates. The plates were incubated for 24h at 37°C and then analyzed for bacterial growth. MBC values corresponds to the concentration that inhibited totally the growth of bacteria [81]. All tests were performed in duplicate with a minimum of two repeats.

3.2.4 Time Kill Curves

In order to evaluate the bacterial growth/death over time, as a function of a range of NSAID concentration (considering the MIC and MBC previously determined), a time kill curves assay was executed. Overnight bacterial cultures were standardized in fresh MH broth at a OD_{600nm} of $0,132 \pm 0,02$. Then, 20 μ L of each NSAID at different concentrations ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and MBC) and 180 μ L of bacterial suspension were added to the wells of 96-well polystyrene microtiter plates. Cell suspensions without NSAIDs and with DMSO (10%, v/v) were used as negative controls. Afterwards, absorbance (600nm) was measured hourly for 24 hours using a micoplate reader (SPECTROstar Nano; BMG LABTECH, Germany) at 37°C. The results were expressed in absorbance (600nm) per hour.

3.2.5 Dose Response Curves

Dose response curves were determined trough the quantification of culturable cells, according Borges et al. [10] with some modifications. Overnight bacterial cultures were adjusted to an OD_{600nm} of $0,132 \pm 0,02$ were added to a microcentrifuge tubes (Eppendorf, Germany). Then, were added to the microcentrifuge tubes in a total volume of 1 mL. The cell suspensions in the presence of NSAIDs were incubated for 1, 2, 3, 4, 5 and 6h at 37°C, under agitation (150 rpm). Cell suspensions without NSAIDs and with DMSO (10%, v/v) were used as negative controls. After exposure, 1:10 serial dilutions in saline solution (0,85%, NaCl) was performed and 10 μ L of each dilution was plated on plate count agar (PCA) plates and were incubated at 37°C during 24h.

Colony forming units (CFU) were counted after the incubation period and the results expressed in log CFU/mL. At least two independent experiments were performed for each condition.

3.2.6 Propidium Iodide (PI) Uptake

In order to assess the the membrane integrity of *S. aureus* CECT 976 and *E. coli* CECT 434 it was determined the PI uptake after 6 h of exposure to each NSAID, using a Live/Dead BacLight™ kit (Invitrogen, USA), according to Oliveira et al. [78], with some modifications. This kit own two different nucleic acid stains, SYTO 9™ (Green) and propidium iodide (PI) (Red), in order to differentiate live bacteria with unbroken membranes from dead bacteria with compromised membranes, respectively, through a selective stain exclusion method [82]. Bacterial suspensions were grown overnight and were centrifuged at 4000 rpm (Eppendorf centrifuge 5810R; Eppendorf AG, Germany), during 10 min. Bacterial suspensions were washed once with saline solution (0,85%, NaCl) and were resuspended in MH broth at a DO_{600nm} of $0,132 \pm 0,02$. Then, 900 μ L of cell suspension were incubated with 100 μ L of different NSAIDs at MIC at 37°C, during 6 h, under agitation (150 rpm). Bacterial suspension containing only cells and with DMSO (10%, v/v) were used as negative controls. Afterwards, one 1:10 serial dilution with saline solution (0,85% NaCl) was performed. 300 μ L of each sample were filtrated using a Nucleopore® (Whatman, UK) black polycarbonate membrane (pore size 0,22 μ m) and is stained for 7 min with 250 μ L of SYTO 9™ and 50 μ L of PI, in the dark. Then, the bacterial suspensions were filtered one more time. Then, the polycarbonate membranes were mounted in a microscope slide with BacLight™ mounting oil, and covered with cover glass (24 x 24 mm, Thermo Fisher Scientific™, USA), as described by manufacturer [82]. At the end, samples were analysed and observed on a LEICA DMLB2 epifluorescence microscope (LEICA Microsystems, Germany) coupled with a LEICA DFC300 FX camera and a 100 $\times$ oil immersion fluorescence objective. Images of stained samples were obtained trough a LEICA IM50 Image Manager, Image processing and Archiving software. Total cells and PI-stained cells were counted from ten different view fields. Two independent experiments were performed for all conditions. The number of live and dead cells, green and red, respectively, were determined through Equation 3.1, according Baptista et al. [25]:

$$N = \frac{n \times A_M}{A_{MF} \times V} \times D \quad (3.1)$$

where N is the number of cells per milliliter (mL) in the sample, n is the number of cells per microscopic field, A_M is the membrane area (cm^2), A_{MF} is the microscope field area (cm^2) and V is the volume of the sample (mL).

The results are shown as a % of PI stained cells as a ratio of the number of PI stained cells/mL and the total number of cells/mL.

3.2.7 Potassium (K^+) Release

The amount of K^+ ion in cell suspensions treated with NSAIDs were assessed by atomic absorption spectroscopy, according Oliveira et al. [78], with some modifications. Overnight cell

cultures were firstly centrifuged 4000 rpm for 10 min. Cells were washed once with ultrapure water and OD_{600nm} was adjusted to $0,132 \pm 0,02$. Afterwards, samples were incubated with each NSAID at four different final concentrations ($1/2 \times MIC$, $1/4 \times MIC$, MIC, and MBC) at $37^\circ C$ for 6 h, under agitation (150rpm). Cell suspensions containing only cells and with 10% (v/v) DMSO were used as negative controls. After NSAIDs exposure, samples were filtrated with Fisherbrand (USA) syringe filter pore size $0,22 \mu m$ and were analyzed in an atomic absorption spectrometer GBC 932 plus device using GBC Avante 1.33 software.

3.2.8 "Drug-likeness" prediction

"Drug-like" properties of NSAIDs were evaluated according Lipinski's rule of five that predicts the absorption, distribution, metabolism and excretion (ADME) [83]. Chemical structures and molecular weight of NSAIDs were consulted on the PubChem platform and the other parameters were determined by Molinspiration calculation software.

3.2.9 Statistical analysis

Mean and deviation values were determined for all performed tests. All the tests were performed at least two times and with several replicates. The data were analyzed using t-student test from Microsoft Excel 2018. Statistical analysis was determined assuming a confidence level $\geq 95\%$. Thus, $p < 0,05$ were considered statistically significant.

3.3 Results and Discussion

3.3.1 Minimum Inhibitory and Bactericidal Concentrations of selected NSAIDs

The MIC and MBC values of selected NSAIDs, ASA, DCF, PXC and NPX against *E. coli* CECT 434 and *S. aureus* CECT 976 were determined and the results are shown in Table 2. Only ASA shows to be effective to both bacteria, however PXC and DCF shows to be effective to *E. coli* and *S. aureus*, at the range of concentrations evaluated. This could mean that PXC has more ability to inhibit Gram-negative and DCF have more ability to inhibit Gram-positive bacteria. MIC values range between 800 and 2000 $\mu g/mL$ and the lowest MIC value was obtained for PXC against *E. coli* (800 $\mu g/mL$). Moreover *E. coli* shows to be more susceptible to ASA (1750 $\mu g/mL$) than *S. aureus* (2000 $\mu g/mL$). MIC value for DCF was also 2000 $\mu g/mL$. No inhibitory effect was found to NPX at the range of concentrations evaluated for any bacteria. Once the minimum inhibitory concentrations were greater than 2000 $\mu g/mL$ for PXC with *S. aureus*, DCF with *E. coli* and NPX with both bacteria, no dose response curves, biofilm control or membrane integrity assays were performed for these cases.

No MBC values were found for any anti-inflammatory, at the range of concentrations evaluated, which means that all MBC values were higher than 2000 $\mu g/mL$. Can also conclude that all MBC values were higher than MIC values to the same anti-inflammatory and bacterium. The summary of the MIC and MBC values are shown in the Table 2.

MIC and MBC values of PXC was determined in two different situations. Initially, *E. coli* was grown in MHB in contact with PXC however, PXC precipitates due to its hydrophobic properties, which results in a MIC value of 2000 $\mu\text{g/mL}$. In order to avoid the formation of the precipitate it was added to the medium lactose. This sugar is an proper excipient commonly used to stabilize the solution of PXC in culture medium, such as MHB [84]. Lactose is also used as a filler-binder or as a diluent in pharmaceutical formulations in oral capsule, tablet formulations and intravenous injections, due to their availability and cost effectiveness [85,86]. Besides characteristics such as a good compatibility with active ingredients and other excipients, low sweetness and hygroscopicity, a reasonable water solubility and high chemical and physical stability lead to the use of lactose in 70% of pharmaceutical formulations [87].

Different solutions of PXC at 2500 $\mu\text{g/mL}$ in MHB with different concentrations of lactose (0,5%, 1%, 1,5%, 2%, 5%, 10%, 15% and 20% (w/v)) were incubated for 6h, in order to select the concentration that provides a better result. Lactose concentrations above 10% (w/v) were discarded, once lactose becomes insoluble and precipitates. Lactose is one of the less soluble sugars, being even less soluble than its monosaccharide components. Solubility of lactose at 20 °C is 19,5% (w/v) [61], however lactose wasn't soluble at this concentration in MHB.

After 6h exposure, the samples were visually analyzed and it was possible to verify that concentrations of 1%, 1,5%, 2%, 5% (w/v) promotes the stabilization of the solution, preventing the precipitation of PXC. Once these four concentrations promotes a similar result, it was chosen the lower one, 1% (w/v). Studies shown that excipients like lactose are used in pharmaceutical formulations at a very low concentration (0,5% and 2% (w/w)), approximately, in order to avoid interference on drug action [88–90]. Besides, the threshold level for lactose-free formulations was established in 10 % (w/v), which means that PXC formulation with lactose does not presuppose a danger to the lactose intolerant people [86]. Afterwards, a MIC value of 800 $\mu\text{g/mL}$ was determined in MHB with lactose (1% (w/v)). However, MBC value remained above 2000 $\mu\text{g/mL}$.

The values obtained for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were close to those found in other studies [91]. A recent study evaluated the effect ASA and DCF for a variety of Gram-positive and Gram-negative bacteria and verified that both *E. coli* and *S. aureus* strains were susceptible to ASA. However, the same did not occur for DCF, which only has effect on *S. aureus* strain. These authors found MIC values for DCF of 1250 $\mu\text{g/mL}$ against *S. aureus* and a concentration above 5000 $\mu\text{g/mL}$ for *E. coli*, which corroborates the results obtained in this study that *S. aureus* is more susceptible to this NSAID than *E. coli*. Moreover, this study shows MIC values of 2500 $\mu\text{g/mL}$ and 5000 $\mu\text{g/mL}$ for ASA against *S. aureus* and *E. coli*, respectively. Despite the MIC values for *S. aureus* was similar to those obtained in the present work, the MIC value for *E. coli* was much higher, it's important to note that the differences observed can be related with the fact that the bacterial strains used are not the same. Other aspect that must be taken in to account is the methodology applied. By the way, values of MBC were much higher than MIC values, confirming the obtained results [72].

In another study performed by Laudy et al. [92], the antimicrobial effect of PXC against *E. coli* ATCC 25922 was evaluated and it was verified that MIC value for *E. coli* was 625 $\mu\text{g/mL}$ [93].

A study about NPX against *E. coli* ATCC 25922 reports MIC values around 3200 $\mu\text{g/mL}$. This corroborates our results, since only concentrations up to 2000 $\mu\text{g/mL}$ were obtained.

Table 2: MIC and MBC values of PXC, DCF, ASA and NPX against *E. coli* CECT 343 and *S. aureus* CECT 976

		MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
PXC	<i>E. coli</i>	800	>2000
	<i>S. aureus</i>	>2000	>2000
DCF	<i>E. coli</i>	>2000	>2000
	<i>S. aureus</i>	2000	>2000
ASA	<i>E. coli</i>	1750	>2000
	<i>S. aureus</i>	2000	>2000
NPX	<i>E. coli</i>	>2000	>2000
	<i>S. aureus</i>	>2000	>2000

Time kill curves were used to monitor the bacterial growth and death of *E. coli* and *S. aureus* after exposure to selected NSAIDs, as well as, to corroborate the results of antimicrobial activity. This analysis was done for PXC, DCF and ASA at $1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and at a concentration above MIC, as it was not possible to determine MBC values for all selected anti-inflammatory drugs, up to the maximum tested concentration of 2000 $\mu\text{g/mL}$. Since it was not possible to determine MIC neither MBC values for NPX up to the maximum tested concentration of 2000 $\mu\text{g/mL}$, it was chosen four random concentrations (500, 1000, 2000 and 2500 $\mu\text{g/mL}$).

First of all, time kill curves for *E. coli* and PXC were determined for two different conditions: the first assay was performed with MHB containing PXC at concentrations of 500, 1000, 2000 and 2500 $\mu\text{g/mL}$ (Figure A.1.1) and the second one was performed in MHB with 1% (w/v) lactose containing PXC at concentrations of 200, 400, 800 and 1500 $\mu\text{g/mL}$ (Figure A.1.2).

It's possible to verify that the effect of PXC without a stabilizer is affected because, even at concentrations 2,5 times higher than the lactose assay, it did not inhibit *E. coli* growth. Analyzing Figure A.1.2 it was possible to verify that the control with DMSO and the PXC concentrations of 200 and 400 $\mu\text{g/mL}$ promotes a significant reduction ($p < 0,05$) on bacterial growth, although the effect is similar for the various concentrations ($p > 0,05$), with specific growth rates between 0,132 and 0,163 h^{-1} (Table A.1.1, Appendix A).

Despite DMSO has a specific growth rate lower than PXC at 200 $\mu\text{g/mL}$, in the end of the assay, it affects bacterial growth more than all sub-inhibitory concentrations (200 and 400 $\mu\text{g/mL}$). As expected, PXC at concentrations of 800 and 1500 $\mu\text{g/mL}$ (MIC and >MIC) completely inhibited bacterial growth, representing a significant effect when compared to sub-inhibitory concentrations ($p < 0,05$).

Comparing Figures A.1.1 and A.1.2, it can be noted that the growth curve of *E. coli* with the addition of lactose behaves differently from its growth curve under normal conditions. Despite

E. coli grows faster in the medium with lactose, with a specific growth rate of $0,47\ h^{-1}$ against $0,23\ h^{-1}$, it does not reach a cell concentration as high as *E. coli* growing under normal conditions (Table A.1.1, Appendix A). This difference can rely on the *E. coli* metabolism which produces about two times more lactate in the fermentation of glucose than lactose's [94].

Lactate accumulates when the production of pyruvate is higher than its consumption, through glycolysis in order to produce energy [95,96]. Once the production of lactate is higher for glucose fermentation than lactose's, it is possible to conclude that the production of energy in first case is also higher. In other words, *E. coli* grown up more consuming glucose than lactose.

Time kill curves for *E. coli* and *S. aureus* strains exposed to ASA were also tested, at a similar range of concentrations (437,5, 875, 1750 and 2500 $\mu\text{g/mL}$ for *E. coli* and 500, 1000, 2000 and 2500 $\mu\text{g/mL}$ for *S. aureus*) (Figures A.1.3 and A.1.4). Despite the differences of behaviour between *E. coli* and *S. aureus*, the effect of ASA was similar for both strains. *E. coli* grown to an OD_{600nm} of 1,1 until 12h and remains practically unchanged until 24 h (final OD_{600nm} of 1,2). In contrast, *S. aureus* grown at a higher rate in the first 8 hours of the assay, reaching an OD_{600nm} of 0,8 and then grown more slowly in the following hours until an OD_{600nm} of 1,1. In other words, the two strains have different behaviours of growth, although the final concentration of cells is similar after 24h. Besides, the specific growth rates of *E. coli* and *S. aureus* were also similar with values of $0,23\ h^{-1}$ and $0,26\ h^{-1}$, respectively (Table A.1.1, Appendix A).

In terms of bacterial activity of ASA, the behaviour was very similar for both strains. As it was observed for PXC, the control with DMSO and with the sub-inhibitory concentrations promoted a significant decrease of the bacterial growth ($p<0,05$), although there isn't a significant difference between the different concentrations ($p>0,05$). The specific growth rates vary between $0,17$ and $0,20\ h^{-1}$ for *E. coli* and between $0,21$ and $0,22\ h^{-1}$ for *S. aureus* (Table A.1.1, Appendix A).

As expected and in similarity to time kill curves for PXC, at concentrations of 1750 and 2500 $\mu\text{g/mL}$ for *E. coli* and 2000 and 2500 $\mu\text{g/mL}$ for *S. aureus* (MIC and >MIC), the bacterial growth was completely inhibited, without no significant differences between both concentrations ($p>0,05$).

The bactericidal/bacteriostatic activity of DCF was also tested in terms of time kill curves at concentrations of 500, 1000, 2000 and 2500 $\mu\text{g/mL}$ (Figure A.1.5). Similar to previous results, *S. aureus* had a specific growth rate of $0,26\ h^{-1}$ (Table A.1.1, Appendix A). DCF promoted a significant inhibition on bacterial growth for all tested concentrations and the effect of different sub-inhibitory concentrations and the control with DMSO were different ($p>0,05$), with specific growth rates of $0,23\ h^{-1}$, $0,14\ h^{-1}$ and $0,08\ h^{-1}$ for control with DMSO, 500 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$, respectively (Table A.1.1, Appendix A).

Although DCF at 500 $\mu\text{g/mL}$ delays the bacterial growth more than DMSO, this last one had a higher effect on the inhibition of bacterial growth. This can happen because with DCF at 500 and 1000 $\mu\text{g/mL}$, *S. aureus* grows slowly until the end of the assay. However, DMSO allows a faster grow of the bacteria at the beginning and prevents its growth from 12 h. DCF at 2000 and 2500 $\mu\text{g/mL}$ (MIC and >MIC) completely inhibited the bacterial growth ($p<0,05$).

Finally, time-kill assay was also performed for *E. coli* and *S. aureus* with NPX was performed with at concentrations of 500, 1000, 2000 and 2500 $\mu\text{g/mL}$ (Figures A.1.6 and A.1.7). It's clear that NPX had a bigger effect on *E. coli* than *S. aureus*. For *S. aureus*, there is no effect on the inhibition of the bacterial growth for any concentration, when compared with the control. Regarding *E. coli*, NPX at 500 $\mu\text{g/mL}$ didn't inhibit the bacterial growth ($p>0,05$), however, at concentrations of 1000, 2000 and 2500 $\mu\text{g/mL}$ there was a significant inhibition in relation to control (DMSO) ($p<0,05$). Despite this, there was no difference between the inhibition for concentrations of 2000 and 2500 $\mu\text{g/mL}$ ($p>0,05$). Specific growth rates for *E. coli* range between $0,18\text{ h}^{-1}$ and $0,23\text{ h}^{-1}$ and between $0,21\text{ h}^{-1}$ and $0,26\text{ h}^{-1}$ for *S. aureus* (Table A.1.1, Appendix A).

3.3.2 Effect of different doses of the selected NSAIDs on *E. coli* and *S. aureus* planktonic cells culturability

Dose response curves within 6 h exposure time were performed to assess the relationship between the effects of selected NSAIDs on culturability (log CFU/mL) of *E. coli* and *S. aureus* as a function of the NSAID concentration. Figure 4 shows the viable *E. coli* cells (log CFU/mL) after exposure to PXC at different concentrations (400, 800 and 1500 $\mu\text{g/mL}$). PXC had a maximum reduction of 1,3 log CFU/mL corresponding to the concentration of 1500 $\mu\text{g/mL}$, however it is not concentration dependent, since there were no statistically significant differences between the three concentrations ($p>0,05$) (Table A.2.1, Appendix A).

In contrast, it is clear that ASA was concentration dependent since, the reduction for the concentration of 875 $\mu\text{g/mL}$ (0,2 log CFU/mL) was lower than 1750 and 2500 $\mu\text{g/mL}$ (0,8 and 0,9log, respectively) ($p<0,05$) (Figure 5, Table A.2.1 in Appendix A). However, it is still possible to notice that there were no statistically significant differences between concentrations 2000 and 2500 $\mu\text{g/mL}$ ($p>0,05$).

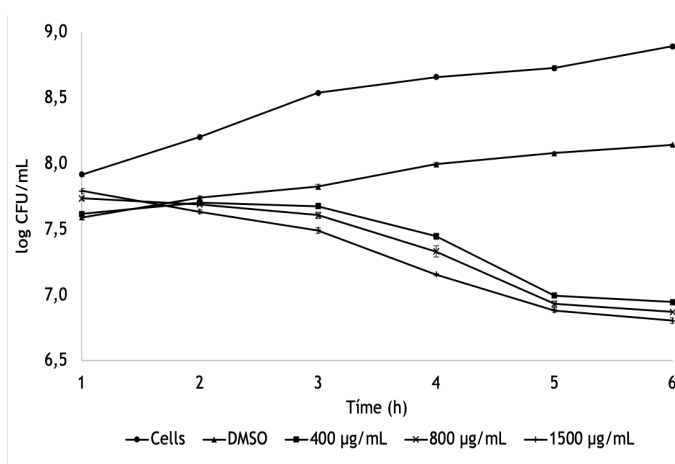


Figure 4: Dose response curves. Log CFU/ml of *E. coli* planktonic cells (grown in broth culture medium with lactose) as a function of PXC concentration ($1/2\times\text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NSAIDs and cells with DMSO were used as negative controls. Data points are from a single experiment.

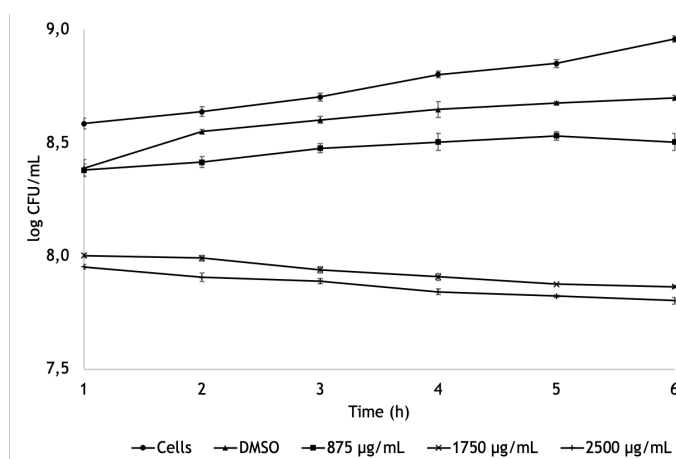


Figure 5: Dose response curves. Log CFU/ml of *E. coli* planktonic cells as a function of ASA concentration ($1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NSAIDs and cells with DMSO were used as negative controls. Data points are from a single experiment.

Now evaluating the effect of DCF and ASA on *S. aureus*, it was verified that DCF showed to be concentration dependent with reductions of 2,5, 3,9 and 5,7 log CFU/mL for concentrations of 1000, 2000 and 2500 µg/mL, respectively ($p < 0,05$) (Figure 6, Table A.2.1 in Appendix A).

With regard to ASA, it reduced approximately 1,0 log CFU/mL at 2000 µg/mL and 2500 µg/mL (7), however there were no statistically significant differences between the two concentrations ($p > 0,05$) Table A.2.1 in Appendix A. Moreover, there was no reduction for the sub-inhibitory concentration (1000 µg/mL).

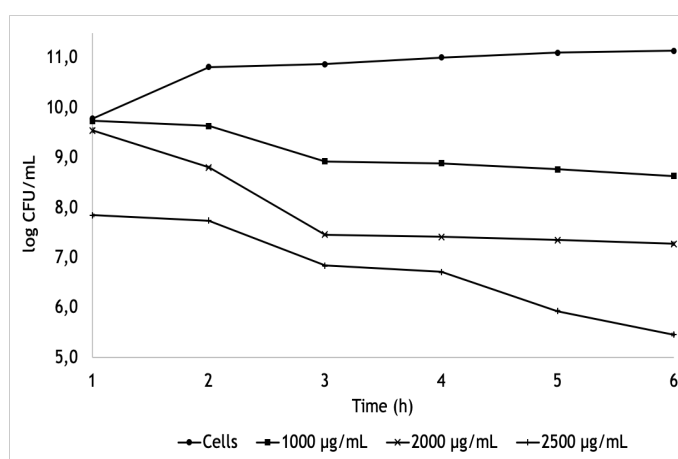


Figure 6: Dose response curves. Log CFU/ml of *S. aureus* planktonic cells as a function of DCF concentration ($1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NSAIDs and cells with DMSO were used as negative controls. Data points are from a single experiment.

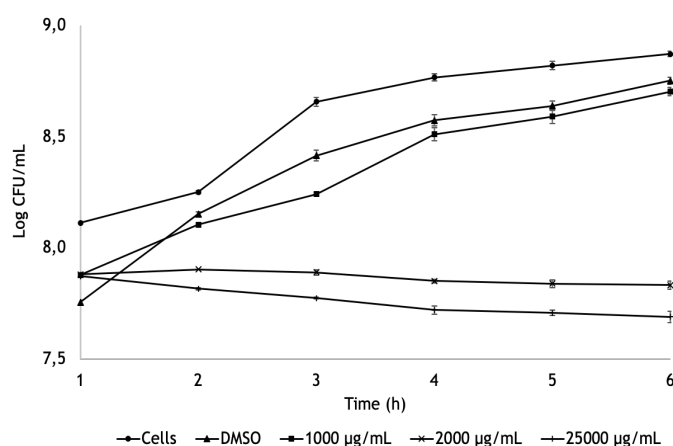


Figure 7: Dose response curves. Log CFU/ml of *S. aureus* planktonic cells as a function of ASA concentration ($1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NSAIDs and cells with DMSO were used as negative controls. Data points are from a single experiment.

3.3.3 Effect of selected NSAIDs on bacterial cytoplasmic membrane integrity

The membrane integrity of both *E. coli* and *S. aureus* strains was evaluated through PI uptake and K^+ release, after 6h exposure to PXC, ASA and DCF at different concentrations.

In a mixture of the SYTO 9 and PI stains, the ability of PI to penetrate in the cytoplasmic membrane and bind to DNA of cell with damaged membranes makes it is an indicator of membrane integrity, since damaged membranes stain fluorescent red, while intact cell membranes stain fluorescent green [82, 97].

Cells maintains homeostasis through the cytoplasmic membrane. K^+ is the most abundant intracellular ion and plays a key role in cell functioning, so it is necessary to maintain K^+ levels in an adequate proportion [98]. The electrogenic transmembrane proteins Na^+-K^+ -ATPase pumps, are responsible for maintaining a potential difference between Na^+ and K^+ across the membrane, where the concentration of K^+ inside the cell should be higher than its concentration in the outside [99].

As mentioned above, K^+ is the most abundant cation in human being organism, in which 98% are located in intracellular fluid and only 2% are located in extracellular fluid [100]. Therefore, due to this delicate balance, large amounts of K^+ in extracellular fluid imply the existence of K^+ leakage, which is a sign of membrane damage [101].

PI uptake results suggest that after 6h of exposure to PXC and ASA at MIC the integrity of the cytoplasmic membrane of *E. coli* was compromised ($p < 0,05$) (Figure 8). The percentage of cells stained with PI was 24,0% for PXC and 17,2% for ASA.

Futhermore, the ability of DCF and ASA to compromise the membrane integrity of *S. aureus* cells after 6 h of exposure was also assessed and it was verified that the percentage of PI stained cells was 27,1% and 21,1%, respectively (Figure 9). It's possible to verify that both bacteria were

more resistant to ASA than the other NSAIDs, since it showed lower percentages of PI stained cells than PXC and DCF. Percentages of PI stained cells are summarized in Table A.3.1, in Appendix A.

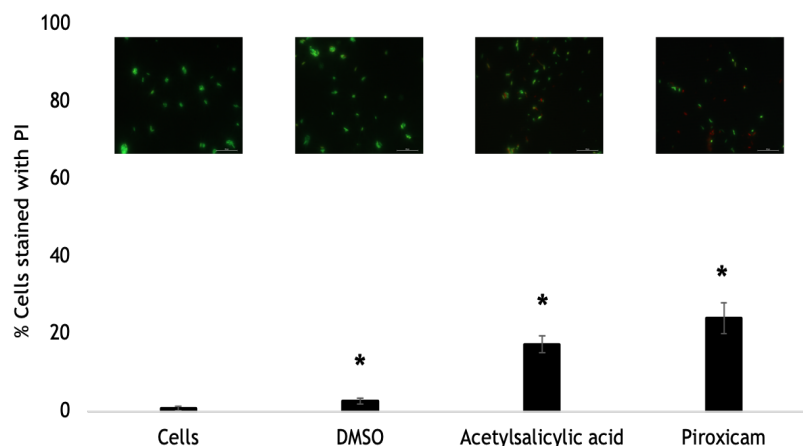


Figure 8: Permeability of *E. coli* to PI after exposure to PXC and ASA at MIC for 6 h. The mean $\pm$ SD for two replicates are illustrated. Bars with * are statistically significant from the control.

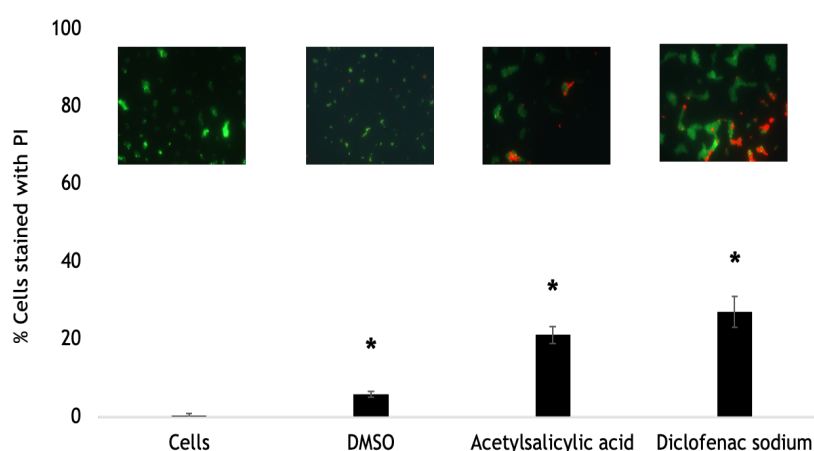


Figure 9: Permeability of *S. aureus* to PI after exposure to DCF and ASA at MIC for 6 h. The mean $\pm$ SD for two replicates are illustrated. Bars with * are statistically significant from the control.

Regarding K^+ release, it is possible to see that intracellular contents were released due to action of tested NSAIDs (Figure 10). For *E. coli*, PXC and ASA, at concentration higher than MIC, induced a maximum K^+ release of 0,48 $\mu\text{g/mL}$ (concentration of 1500 $\mu\text{g/mL}$) and 0,43 $\mu\text{g/mL}$ (concentration of 2500 mg/mL), respectively (Figure 10a)(Table A.3.2, Appendix A). The effect of PXC is concentration-dependent ($p < 0,05$), however, there are no statistically significant differences between the concentrations $1/2 \times \text{MIC}$ and MIC ($p > 0,05$). The effect of ASA was also

concentration-dependent ($p < 0,05$), however, there are no statistically difference in the K^+ release for the two intermediate concentrations ($1/2 \times \text{MIC}$ and MIC) ($p > 0,05$).

For *S. aureus*, DCF and ASA also were concentration-dependent. For ASA the concentration of K^+ released for the highest concentration was statistically significant higher than lowest concentrations ($p < 0,05$). Besides, there were no differences between the two lowest concentrations ($p > 0,05$). DCF is also concentration-dependent, however there were no differences between the two intermediate concentrations ($1/2 \times \text{MIC}$ and MIC) ($p > 0,05$). The alteration in the *S. aureus* cytoplasmic membrane caused by DCF and ASA induced a maximum release of $0,51 \mu\text{g/mL}$ and $0,29 \mu\text{g/mL}$ of K^+ , respectively, for the highest concentration tested ($>\text{MIC}$) (Figure 10b) (Table A.3.2, Appendix A).

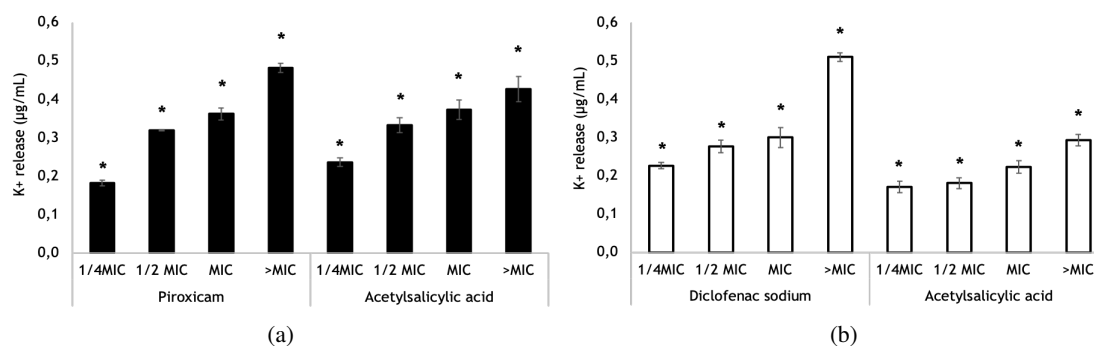


Figure 10: Permeability of *E. coli* (a) and *S. aureus* (b) to PI after exposure to PXC, ASA and DCF at MIC , $5 \times \text{MIC}$, $10 \times \text{MIC}$ and a concentration above MIC ($>\text{MIC}$), for 6h. The mean $\pm$ SD for two replicates are illustrated. Bars with * are statistically significant from the control.

Summing up, the results from both assays suggests that the three NSAIDs are able to cause damage in cytoplasmic membrane and promote the release of cellular content, simultaneously. Moreover, it possible to conclude that ASA had a higher effect on *E. coli* ($0,43 \mu\text{g/mL}$) than *S. aureus* ($0,51 \mu\text{g/mL}$).

In fact, their are evidences that NSAIDs act on the cytoplasmic membrane in a different ways, including changes in the fluidity and thickness of the membrane and changes in conformation of the phospholipid layer [59]. Besides, unstable membranes can affect, among others, cell division, permeability, adhesion and shape changes [78].

In this way, some study suggests that NSAIDs form pores in the cytoplasmic membranes of *E. coli* allowing the leakage of small ions such as K^+ and phosphate and later larger molecules such as DNA and RNA. In addition, they report that the mode of action of the NSAIDs can be related with membrane disruption, thus preventing bacterial growth. However, these mechanisms are still not well defined and there are evidences that DCF may also affect DNA synthesis [102, 103].

Moreover, because of their amphiphilic character, it is possible that NSAIDs act on the phospholipid layer, altering its permeability and fluidity. There are indeed evidences that NSAIDs such

as aspirin and PXC alter membrane permeability and fluidity by exerting hydrophobic and electrostatic interactions with the acyl chains of the phospholipids and polar groups of the phospholipid head [104]. However, this interaction of NSAIDs with phospholipids has not been shown to be feasible for some NSAIDs such as mefenamic acid, flufenamic acid, and celecoxib. Therefore it can be concluded that the membrane permeabilization method may vary according to the type of anti-inflammatory [105].

3.3.4 "Drug-likeness" prediction

The ADME properties (Absorption, Distribution, Metabolism, and Excretion) of selected drugs were predicted based on Lipinski's "rule of five", obeying to the following conditions: molecular weight (≤ 500 Da), $-2 \leq \log P \leq 5$, hydrogen bond donors (≤ 5) and hydrogen bond acceptors (≤ 10) [83].

The NSAIDs selected for this study satisfy all the previous conditions with molecular weight values between 180,16 and 331,35 Da, $\log P$ range from 0,66 and 2,06, hydrogen bond donors range from 0 to 2 and hydrogen bond acceptors range from 3 to 7. Besides, the ADME properties of selected antibiotics were also predicted (Table 3).

Contrary to NSAIDs, the selected antibiotics do not fulfill all the conditions since molecular weight range from 331,35 Da to 581,58 Da, $\log P$ from -5,70 to -0,24, hydrogen bond donors range from 7 to 16 and hydrogen bond acceptors range from 6 to 19. Excluding CIP, all antibiotics failed at least one of the model parameters and STR failed all parameters. All data are shown in the Table 3.

Thus, the selected NSAIDs are drug-like compounds although the same does not happen for both antibiotics. If the compound fails rule of five, the likelihood of oral activity problems is very high, however, compliance with the rule of five requirements does not ensure that these problems do not exist, because this method of classification does not take into account the specific chemistry structural properties of drugs [83]. Besides, other properties like lipophilicity and electronic distribution and characteristics like transport, reactivity, toxicity, affinity to proteins and metabolic stability on human being organism, should be into account to better predict the absorption of a drug [106]. A lot of these characteristics have already been studied and are well defined, once NSAIDs are widely used drugs in therapeutic treatments.

It's also important to note that Lipinski's rule of five does not predict the oral absorption in a quantitative way and its classification of well absorbed or poorly absorbed compound is not categorical, however it's a simple model that allows to classify the compound reasonably and quickly [106]. Besides, studies shown that 20% of all drugs administered orally fail at least one of the correlations of Lipinski's rule of five [107].

Lipinski's model is based on the thousands of drugs parameters, so it's natural that some of them are outside the limits of the model. Curiously, most of the compounds that are outside the limits of the model belong to a restricted set of therapeutic classes, among them the antibiotics, cardiac glycosides, antifungals and vitamins [108], which corroborates the collected data for KAN, TET and STR.

Table 3: Lipinski's "rule of five" parameters for the selected NSAIDs and antibiotics

Drug	Molecular weight (Da)	logP	Hydrogen bond donors	Hydrogen bond acceptors
PXC	331,35	2,06	2	7
DCF	318,13	1,85	1	3
ASA	180,16	1,43	1	4
NPX	252,24	0,66	0	3
KAN	484,50	-5,70	15	15
TET	444,44	-0,24	7	10
CIP	331,35	-0,70	2	6
STR	581,58	-5,35	16	19

3.4 Conclusions

This study highlights NSAIDs as an alternative or complementary strategy to inhibit bacterial growth of *E. coli* and *S. aureus* strains. In fact, the selected NSAIDs demonstrated that have antimicrobial effect against both tested bacteria by inhibiting their growth. Moreover, it appears that this effect may be related to the ability of NSAIDs to destabilize the cytoplasmic membrane of these bacteria, allowing the PI uptake and intracellular K^+ release, through the disrupted membrane.

However, its medicinal use should be ruled out since the recommended maximum concentrations per mL of plasma are lower than MIC values for all NSAIDs tested. For PXC, the maximum recommended concentration is only 4 $\mu\text{g/ml}$ which represents about 0.5% of the MIC [63]. In the case of ASA and DCF the maximum recommended concentrations are 800 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$, which corresponds to a 40 % and 20% percentage, respectively [62,64]. NPX has a maximum recommended concentration of 200 $\mu\text{g/ml}$, however the MIC value was greater than 2000 $\mu\text{g/ml}$, so this NSAID has not been tested in later trials [65]. In addition, all NSAIDs meet the requirements of Lipinski's rule of five and are therefore considered "drug-like" compounds.

At this point of the work, repurposing NSAIDs for antimicrobial treatments seems to be a good strategy, however, its effect on biofilm eradication will be evaluated in next chapter.

Chapter 4

Biofilm eradication activity of selected NSAIDs

4.1 Introduction

The emergence of antibiotic resistance is due in large part to biofilms. Some bacteria have developed survival mechanisms that allow them to live in community and cooperate with each other to form a matrix of exopolysaccharides that acts as a barrier to antimicrobial agents and external stresses [109]. Once formed the biofilm, these bacteria develop a series of other mechanisms that allow them to be about 1000 times more resistant than in the planktonic state [110]. Eradicating these cellular communities has been a major challenge for the medical and scientific community since they are becoming more vulgar and more resilient [111].

This chapter addresses studies of the ability of selected NSAIDs to eradicate pre-established biofilms and their ability to potentiate the effect of broad-spectrum antibiotics, KAN and TET.

4.2 Materials and methods

4.2.1 Microorganisms and Culture Conditions

The model microorganisms used in the biofilms studies were the collection strains *E. coli* CECT 434 and *S. aureus* CECT 976. The culture conditions were the same described in Chapter 3, sub-section 3.2.1.

4.2.2 Antibiotics and NSAIDs

The antibiotics CIP - 5 μ g/disc, TET - 30 μ g/disc, STR - 10 μ g/disc, and KAN - 30 μ g/disc] were obtained from Sigma-Aldrich and the solutions were prepared in water according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [91]. The nonsteroidal anti-inflammatory drugs acetylsalicylic acid (ASA), diclofenac sodium (DCF) and naproxen sodium (NPX) were obtained from Sigma-Aldrich and piroxicam (PXC) from Alfa aesar. The solutions were prepared

in dimethyl sulfoxide (DMSO). The stock solutions of each compound (antibiotics and NSAIDs) were stored at 4 °C until use (no more than 24 hours). Stock solutions were prepared in the same conditions mentioned in Chapter 3, sub-section 3.2.1.

4.2.3 Antibacterial activity assessment by disc diffusion method

Antibiotic susceptibility of *S. aureus* and *E. coli* strains was determined by disc diffusion method, according Abreu et al. [24]. Overnight cell cultures were adjusted to an OD_{600nm} of $0,132 \pm 0,02$ with fresh MH broth. Bacterial cells were seeded in MH agar plates using a sterilized cotton swab. Then a volume of 10 μ L of CIP, TET, STR and KAN prepared according to the CLSI guidelines [91], is added to a 6 mm diameter sterile blank discs which were placed on the agar plate. To facilitate the dissolution of CIP and TET, additional drops of HCl were added. Discs with water and the same amount of HCl used in antibiotics dissolution was used as negative controls. Then, the plates were incubated for 24h at 37 °C. After incubation, each inhibition zone diameter (IZD) was recorded. Two independent experiments were performed.

4.2.4 Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

The MIC values of antibiotics against bacterial strains *E. coli* and *S. aureus* were determined by broth microdilution method in sterile 96-well microtiter plates (Orange Scientific, Belgium) according to Oliveira et al. [78]. The experimental procedure was the same as described in Chapter 3, sub-section 3.2.3.

4.2.5 Biofilm Control

4.2.5.1 Biofilm formation

Biofilm formation was performed according to Stepanović et al. [112] with some modifications, in order to assess the ability of NSAIDs to control 24h old biofilms. Briefly, overnight cell cultures were adjusted to an OD_{620nm} of $0,04 \pm 0,02$ with fresh MH broth and 200 μ L of cell suspensions were added to 96-well polystyrene microtiter plates. The plates were incubated for 24 h at 37 °C under agitation (150 rpm).

4.2.5.2 Exposure to NSAIDs

After biofilm formation, the content of the well was caught up and the wells were washed with 200 μ L of saline solution (0,85% NaCl). Afterwards, 20 μ L of different NSAIDs in three different concentrations (MIC, 5 $\times$ MIC and 10 $\times$ MIC) and 180 μ L of fresh MH broth were added to the wells and the plates were incubated for 24 h at 37 °C under agitation (150 rpm). Wells only with cell suspensions and wells with DMSO (10%, v/v) were used as negative controls. Then, biofilms were analyzed in terms of biomass reduction, metabolic activity and culturability.

4.2.5.3 Mass quantification by crystal violet staining

Mass quantification was performed according to Baptista et al. [25] in order to assess the biofilm biomass reduction promoted by selected NSAIDs. After NSAIDs exposure, the content of wells was aspirated and the wells were washed with 200 μL saline solution (0,85%, NaCl) to remove all non-adherent and weakly adherent bacterial cells. Next, cells were fixed with 250 μL of 99% ethanol for 15 min and the content was discarded at the end. Then, bacteria were stained with 1% crystal violet (CV; Sigma-Aldrich, Portugal) for 5 min and the excess of stain was removed at the end. Fixed cells were resolubilized with 200 μL of 33% (v/v) glacial acetic acid and OD was measured in a microplate reader (SpectraMax M2e) at 570 nm. At least two independent experiments were performed. The results were expressed as the biofilm removal (%) regarding biofilms non-exposed to NSAIDs (Equation 4.1):

$$\%BR = \frac{OD_C - OD_W}{OD_C} \times 100 \quad (4.1)$$

where %BR is the percentage of biofilm removal, OD_C is the OD_{570nm} value of the control wells and OD_W is the OD_{570nm} value for the NSAIDs-treated wells.

4.2.5.4 Metabolic activity quantification by alamar blue assay

Metabolic activity quantification was performed according to Baptista et al. [25] in order to assess the biofilm metabolic inactivation promoted by selected NSAIDs. After NSAIDs exposure wells were aspirated and washed as mentioned in section 4.2.5.3. For the staining procedure, 10 μL of alamar blue solution (Sigma-Aldrich, Portugal) and 190 μL of fresh MH broth were added to each well. Then, the microtiter plates were incubated for 20 min in the dark at 37°C under agitation (150 rpm).

Finally, fluorescence intensity was measured at excitation and emission wavelengths of 570 nm and 590 nm, respectively, using a microplate reader (FLUOstar Omega) (BMG LABTECH, Germany). At least two independent experiments were performed. The results are expressed as the biofilm inactivation (%) regarding biofilms non-exposed to NSAIDs (Equation 4.2):

$$\%BI = \frac{FI_C - FI_W}{FI_C} \times 100 \quad (4.2)$$

where %BI is the percentage of biofilm inactivation, FI_C is the fluorescence intensity of control wells and FI_W is the fluorescence intensity for the NSAIDs-treated wells.

4.2.5.5 Biofilm culturable cells quantification by plate count method

Biofilm culturable cells quantification was performed according to Baptista et al. [25] in order to assess the culturability reduction of biofilm cells promoted by selected NSAIDs. After the treatment with NSAIDs, the content of the wells was removed and the wells were washed to remove non-adherent bacteria, as mentioned in the sub-section 4.2.5.3. Afterwards, the attached cells were scraped three times with 200 μL of saline solution (0,85%, NaCl), during 1 min each.

The content of each well (600 μL) was transferred to a microcentrifuge tubes (Eppendorf, Germany) and it was also added 400 μL of saline solution. Then, 1:10 serial dilutions were performed in saline solution and 10 μL of each dilution were plated on PCA plates. Plates were incubated at 37°C during 24h. At least two independent experiments were performed.

CFU were counted ($10 < \text{CFU} < 200$) after the incubation period and the results were expressed per square centimeter of microtiter plate well CFU/cm^2 , according to Equations 4.3 and 4.4.

$$\frac{\text{CFU}}{\text{mL}} = \frac{N}{SV \times \text{Dilution}} \quad (4.3)$$

where N is the number of CFU in the PCA plates and SV is the volume of the sample in mL.

$$\frac{\text{CFU}}{\text{cm}^2} = \frac{\frac{\text{CFU}}{\text{mL}} \times \text{WV}}{1,53} \quad (4.4)$$

where WV is the well's volume (0,2mL) and 1,53 is the area of the well in cm^2 . The final results were expressed in $\log \text{CFU}/\text{cm}^2$.

4.2.6 Combination of NSAIDs with antibiotics

In order to evaluate the combined effect of NSAIDs and antibiotics, an overnight culture was standardized to an OD_{600nm} of $0,04 \pm 0,02$ with fresh MH broth. Biofilms were formed in a 96-well microtiter plate like was previous stated in sub-section 4.2.5.1. Afterwards, 10 μL of each NSAID, 10 μL of each antibiotics and 180 μL of fresh MHB were added to the microplates, which were incubated at 37°C for 24h. Then, the biofilms were analyzed in terms of reduction of biomass, metabolic activity and culturability, as described in sub-sections 4.2.5.3, 4.2.5.4 and 4.2.5.5, respectively.

Subsequently, in order to classify the interaction between NSAIDs and antibiotics when compared to NSAIDs and antibiotics alone, the Combinatorial Index ($\sum C_I$) for each combination was calculated and scored according to the score proposed by Baptista et al. [25]. The Combinatorial Index of NSAIDs ($C_{I|N}$) was determined through Equation 4.5:

$$C_{I|N} = \frac{R_N}{R_{N|A}} \quad (4.5)$$

where R_N are the results obtained for NSAIDs alone (at MIC, 5×MIC and 10×MIC) for each method (biofilm removal, biofilm inactivation and $\log \text{CFU}/\text{cm}^2$ reduction). $R_{N|A}$ are the results obtained for the combination of NSAIDs (at MIC, 5×MIC and 10×MIC) with antibiotics (at MIC) for each method. The Combinatorial Index of antibiotics ($C_{I|A}$) was determined in a similar way. Afterwards, $\sum C_I$ was calculated by Equation 4.6:

$$\sum C_I = C_{I|N} + (C_{I|A}) \quad (4.6)$$

$\sum C_I$ values were then scored and classified according to the interactions between NSAIDs and antibiotics in *S. aureus* and *E. coli* biofilms:

- $< 0,5$ – synergistic (+++)
- $0,5$ to 2 – additive (++)
- 2 to 4 – indifferent (+)
- > 4 – antagonistic (–)

4.2.7 Biofilm control activity classification

The effects of NSAIDs on biofilm control were classified according to the following conditions proposed by Lemos et al. [113], where "I" expresses inactivation and "R" expresses removal:

- Low efficacy: I or $R < 25\%$
- Moderate efficacy: $25\% \leq I$ or $R < 60\%$
- high efficacy: $60\% \leq I$ or $R < 90\%$
- excellent efficacy: $90\% \leq I$ or $R \leq 100\%$

4.2.8 Statistical analysis

Mean and deviation values were determined for all performed tests. All the tests were performed at least two times and with several replicates. The data were analyzed using t-student test from Microsoft Excel 2018. Statistical analysis was determined assuming a confidence level $\geq 95\%$. Thus, $p < 0,05$ were considered statistically significant.

4.3 Results and Discussion

4.3.1 Susceptibility profile of *E. coli* and *S. aureus* to conventional antibiotics

Broad-spectrum antibiotics belonging to different classes were chosen in order to evaluate the profile of susceptibility of the bacterial strains selected for this study. Antibiotics from quinolones class, CIP, tetracyclines class, TET and aminoglycosides class, STR and KAN were used in this assay [3]. The classes and mode of action of these antibiotics are summarized in Table 1 (Chapter 2, section 2.2). Bacterial susceptibility of *E. coli* and *S. aureus* strains against these antibiotics was assessed following the Clinical and Laboratory Standards Institute (CLSI) guidelines [91].

Inhibition zone diameter (IZD) values range from $26,5$ to $39,7$ mm to bacterium *S. aureus* and from $28,5$ to $43,2$ mm for bacterium *E. coli*. All the data are shown in Table 4. TET and KAN had the smallest inhibition diameters for both strains being, therefore, the antibiotics to which the bacteria offer a greater resistance. For this reason, TET and KAN were selected for the biofilm control assays, since the objective is to test the effect of the selected NSAIDs on the bacterial

resistance modification. Nevertheless, through IZD values, *S. aureus* and *E. coli* strains were classified as susceptible to all antibiotics, according to CLSI guidelines [91].

In general, *S. aureus* show to be more resistant to the selected antibiotics than *E. coli*, however, kanamycin had a similar effect on both strains ($p < 0,05$). Based on IZD values, *S. aureus* and *E. coli* were classified as susceptible to all antibiotics, according to CLSI guidelines [91].

Table 4: Inhibition zone diameters (IZD (mm))(mean $\pm$ SD) promoted by the action of the selected antibiotics. Strains were classified as resistant (R), intermediate resistant (I) or susceptible (S) to each antibiotic according to CLSI guidelines [91]

Bacterial Strain	Antibiotics	IZD (mm)
<i>S. aureus</i>	<i>CIP</i> _{5μg/disc}	38,8 $\pm$ 3,1 (S)
	<i>TET</i> _{30μg/disc}	26,5 $\pm$ 1,2 (S)
CECT 976	<i>STR</i> _{10μg/disc}	39,7 $\pm$ 3,3 (S)
	<i>KAN</i> _{30μg/disc}	29,8 $\pm$ 2,1 (S)
<i>E. coli</i>	<i>CIP</i> _{5μg/disc}	43,2 $\pm$ 2,1 (S)
	<i>TET</i> _{30μg/disc}	37,3 $\pm$ 1,4 (S)
CECT 434	<i>STR</i> _{10μg/disc}	43,7 $\pm$ 0,0 (S)
	<i>KAN</i> _{30μg/disc}	28,5 $\pm$ 0,2 (S)

Consulting the literature it's verified that inhibition zones diameters of the antibiotics are slightly higher than expected. However, these data refers to strains from a different culture collection, the American Type Culture Collection (ATCC). Literature values points to maximum values of 26 mm for KAN, valid for both strains, and maximum values of 25 mm and 30 mm for TET, for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, respectively [24, 91, 114].

In CIP case, the quality control values had a maximum of 40 mm and 30 mm for *E. coli* and *S. aureus*, respectively [24, 91, 115]. As seen, the values obtained in this experiment are slightly higher than those found in the literature, so, the selected control strains might be slightly more susceptible to said antibiotics. In addition, literature corroborates that two strains are equally susceptible to KAN and that *S. aureus* strain is more resistant to CIP than *E. coli* strain [91, 115].

On the other hand, the literature has shown that *E. coli* strain is more resistant to tetracycline than to *S. aureus* strain and also that the two strains are equally susceptible to STR, which is not in agreement with the obtained results. Besides, STR is, according to the literature, the antibiotic to which the bacteria offer the greatest resistance, with inhibition zones of approximately 20mm, which denies the obtained results, since it was the antibiotic with inhibition zone diameters [91, 114, 115].

4.3.2 Minimum Inhibitory and Bactericidal Concentrations of selected antibiotics

MIC and MBC values of TET and KAN was also evaluated in this work. For *E. coli*, MIC values range from 6 μ g/mL and 24 μ g/mL for TET and KAN, respectively. For *S. aureus* values

range from 3 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$, for KAN and TET, respectively. MBC values for KAN was the same for both bacteria (24 $\mu\text{g/mL}$) and for TET were 48 $\mu\text{g/mL}$ and 64 $\mu\text{g/mL}$ for *S. aureus* and *E. coli*, respectively. Except for the effect of KAN against *E. coli*, MBC values of all the other combinations were higher than MIC values. For KAN against *E. coli*, MIC and MBC values were the same.

It can be concluded that *E. coli* was the most resistant strain to both antibiotics, however, this strain is more resistant to KAN than to TET. On the other hand, *S. aureus* showed to be more resistant to TET. Moreover, according CLSI guidelines and MIC values, *E. coli* strain was classified as intermediate resistant to both antibiotics and *S. aureus* strain was classified as susceptible also to both antibiotics [91].

By the way, according to CLSI guidelines [91], quality control ranges for KAN are between 1 and 4 $\mu\text{g/mL}$ for both strains which allows to state that the *S. aureus* strain is within the range of usual values, however the strain of *E. coli* appears to be more resistant than expected. In the case of TET, for *E. coli* the values should be between 0.12 and 1 $\mu\text{g/mL}$ and for *S. aureus* between 0.5 and 2 $\mu\text{g/mL}$.

In the case of TET, for *E. coli* the values should be between 0.12 and 1 $\mu\text{g/mL}$ and for *S. aureus* between 0.5 and 2 $\mu\text{g/mL}$. The values determined are slightly higher, however this may be due to differences in mode of action of the bacteria used, since they belong to a different collection [91]. MIC and MBC values of selected antibiotics are shown in the Table 5.

Table 5: MIC and MBC values of KAN and TET against *E. coli* CECT 343 and *S. aureus* CECT 976. Strains were classified as resistant (R), intermediate resistant (I) or susceptible (S) to each antibiotic according to CLSI guidelines [91]

		<i>E. coli</i>	<i>S. aureus</i>
KAN	MIC	24 (I)	3(S)
	MBC	24	24
TET	MIC	6 (I)	4 (S)
	MBC	64	48

4.3.3 Effect of selected NSAIDs on the control of *E. coli* and *S. aureus* biofilms

The effect of ASA, PXC and DCF on the control of *E. coli* and *S. aureus* 24h old biofilms was studied at MIC, 5 $\times$ MIC and 10 $\times$ MIC. Their effects on biomass removal, inactivation and culturability was assessed (Figures 11, 12, 13). It is important to emphasize that PXC was tested only on *E. coli* biofilms, DCF was only tested on *S. aureus* biofilms and ASA was tested for both bacteria.

Concerning biofilm mass, while DCF and ASA were not able to remove *E. coli* and *S. aureus* biofilm cells at all concentrations tested (Figures 11a and 13a), PXC showed a moderate efficacy in the removal of biofilm cells at MIC (28,6%). However, this NSAID had a low efficacy in removal at 5 $\times$ MIC (16,8%) and had no efficacy in removal at 10 $\times$ MIC (12a). For both bacteria, the effect

of DCF and ASA wasn't concentration dependent ($p>0,05$). In fact, for PXC, the percentage of biofilm mass removal was higher for MIC than $5\times$ MIC and $10\times$ MIC ($p<0,05$) (Figure 12a).

In regard to biofilm inactivation, the three NSAIDs showed a high efficacy to inactivate biofilm cells at all concentrations tested ($\geq 66,1\%$). No significant differences were observed between the three concentrations for all the NSAIDs ($p>0,05$) (Figures 11c, 12c and 13c). The classification of the efficacy of NSAIDs on biofilm reduction and biofilm inactivation is summarized in Table B.2.1 (Appendix B).

The effect of ASA on biofilm cells culturability was very similar for both bacterial strains. It promoted a reduction of 3,6 and 3,3 log CFU/cm² for *E. coli* and *S. aureus*, respectively, at MIC. Besides, at $5\times$ MIC and $10\times$ MIC ASA reduced totally the culturability of the biofilm cells and promoted a significant reduction in relation to MIC, which means that the activity of ASA was concentration dependent ($p<0,05$) (Figure 11e). On the other hand, DCF promoted a reduction of 2,2 and 3,5 log CFU/cm² for MIC and $5\times$ MIC, respectively, and reduced totally the culturability of the biofilm cells at $10\times$ MIC (Figure 13e). This effect was concentration dependent, as the reduction of the culturability of the biofilm cells decreased with the increase of the concentration ($p<0,05$). For PXC, it was observed a reduction of 3,2, 3,6 and 3,8 log CFU/cm² of *E. coli* cells for MIC, $5\times$ MIC and $10\times$ MIC, respectively. There was a statistically significant increase from MIC to $5\times$ MIC and $10\times$ MIC ($p<0,05$), however, there was no increase from $5\times$ MIC to $10\times$ MIC ($p>0,05$) (Figure 12e).

4.3.3.1 Effect of dual combination of selected NSAIDs with selected antibiotics on the control of *E. coli* and *S. aureus* biofilms

To evaluate the ability of NSAIDs as antibiotic adjuvants on the control of 24h old biofilms, the effect of antibiotics KAN and TET alone, and their effect (at MIC) when combined with NSAIDs (at MIC, $5\times$ MIC and $10\times$ MIC) was also assessed. Once again, the results were analysed in terms of biomass removal, inactivation and culturability of biofilm cells (Figures 11, 12, 13).

KAN and TET alone had low efficacy on *E. coli* biofilm mass removal, with reductions of 22,8% and 21,5%, respectively, while they had moderate efficacy on *S. aureus* biofilm mass removal, with reductions of 30,6% and 31,2%, respectively (Table B.2.1). In terms of biofilm inactivation, KAN and TET alone had high effect for *E. coli*, with reductions of 80,8% and 66,8%, respectively, and had moderate effect for *S. aureus*, with reductions of 43,5% and 26,8%, respectively. Summing up, KAN and TET had a better activity on biofilm mass removal for *S. aureus* and had a better effect on biofilm inactivation for *E. coli*.

4.3.3.2 Acetylsalicylic acid

The ability of ASA was evaluated on the biomass removal, cell inactivation and reduction of the culturability of *E. coli* and *S. aureus* biofilm cells when combined with antibiotics TET and KAN. It is important to note that in the combination assays only the NSAID concentration changes (MIC, $5\times$ MIC and $10\times$ MIC), keeping the concentration of the antibiotic constant (MIC).

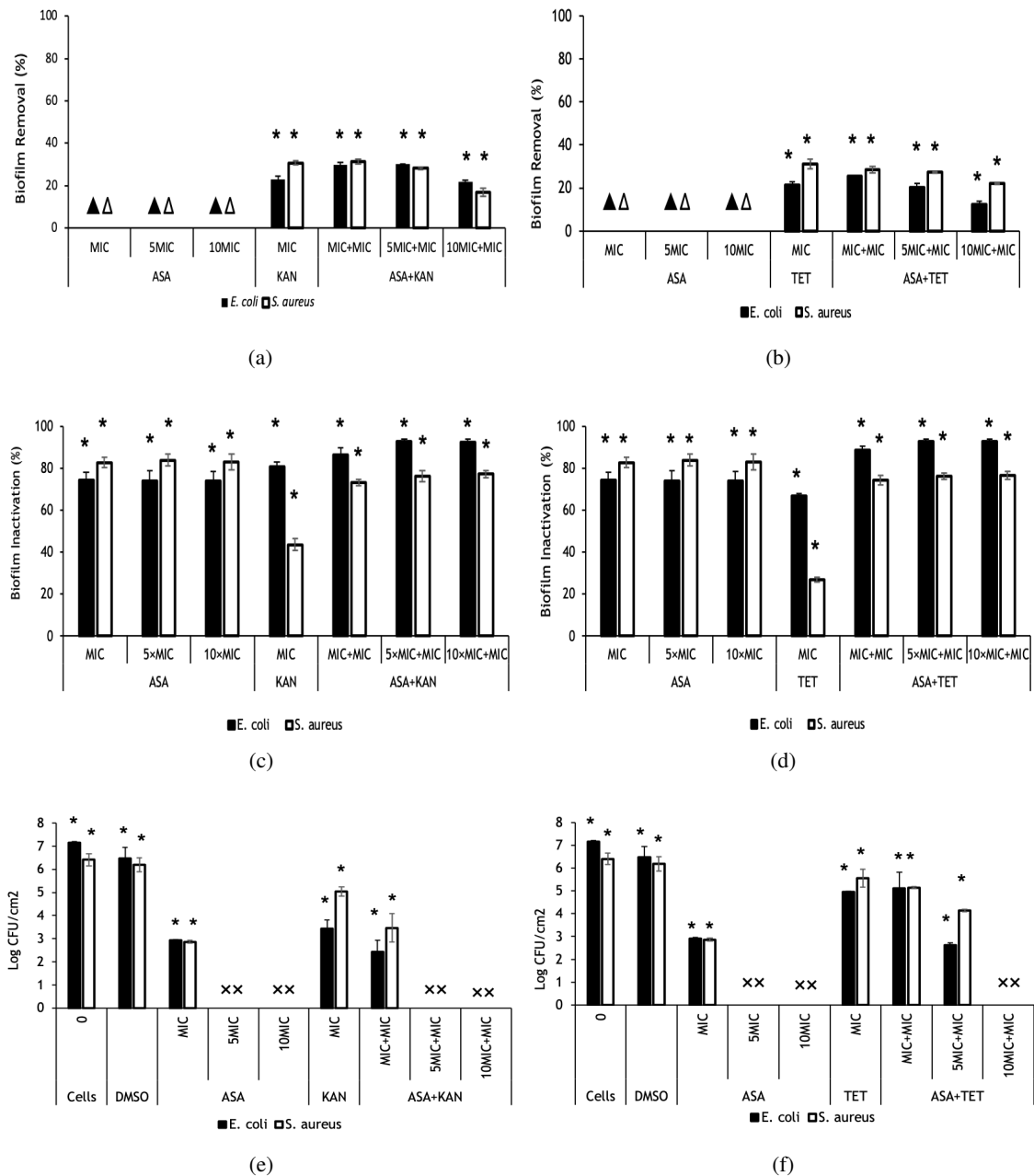


Figure 11: Effect of ASA at MIC, 5×MIC and 10×MIC alone and in combination with selected antibiotics (KAN and TET) at MIC, on 24 h old *E. coli* CECT 434 (black) and *S. aureus* CECT 976 (white) biofilms after 24 h exposure. (a)(b) Percentage of mass reduction for KAN and TET, respectively, (c)(d) biofilm removal for KAN and TET, respectively; and (e)(f) culturability – Log CFU/cm² reduction - for KAN and TET, respectively. × - no CFU/cm² detected. Δ - 0% of biofilm removal. Mean values ± SD for two or more independent experiments are illustrated. Bars marked with * are statistically different from the control ($p < 0.05$).

For *E. coli* bacterium, ASA and KAN combination led to an significant increase of the biofilm mass removal only for MIC and 5×MIC (Table 11a) ($p < 0.05$). In the case of *S. aureus* bacterium,

none of the combinations promoted improvements in the biofilm reduction. Moreover, at $10\times\text{MIC}$ there was a significant decrease of the reduction over the control of the antibiotic alone ($p<0,05$).

The interactions between ASA and KAN at all concentration tested for both bacteria were classified as additive (Table B.1.1, Appendix B). Moreover, the two lowest concentrations had a moderate effect on biofilm mass removal (Table B.2.1, Appendix B). For TET, the behaviour was very similar to the previous one. TET and ASA only had a higher effect at MIC compared to control (cells with TET) for *E. coli*, while, for *S. aureus* none of the concentration had higher effect than the control. Contrariwise, at $10\times\text{MIC}$ the biomass removal was considerably reduced compared to the control (cells with TET), for both bacterial strains ($p<0,05$) (Figure 11b). The interactions of ASA and TET was classified as additive for all concentrations (Table B.1.1, Appendix B). Moderate effect on biofilm mass removal was found for *E. coli* at MIC and for *S. aureus* at MIC and $5\times\text{MIC}$, while the other combinations had a lower effect (Table B.2.1, Appendix B).

For biofilm inactivation, combinations with KAN and TET had similar behaviours, since there were no differences between the three concentrations ($p>0,05$) (Figures 11c and 11d). *E. coli* promoted an improved cell inactivation at all concentrations, while *S. aureus* promoted the opposite, it was observed the decrease in cell inactivation at all concentrations, compared to the control. Summing up, there is no advantage in combine ASA with both antibiotics for *S. aureus*, since the use of a ASA alone promotes a better effect on biofilm cell inactivation. All combinations were rated as additive (Table B.1.1, Appendix B). Furthermore, for *E. coli* the combinatorial effects were mostly classified as excellent, while for *S. aureus* these were classified as highly effective (Table B.2.1, Appendix B).

Regarding biofilm culturability, the dual combination of ASA at MIC and KAN, promoted an increased log CFU/cm² value when compared to the control of ASA alone, for both bacteria ($p<0,05$) (Figure 11e). The same happened for the combination of ASA with TET, however this phenomenon was verified for MIC and $5\times\text{MIC}$ (Figure 11f). Besides, ASA and KAN at $5\times\text{MIC}$ and $10\times\text{MIC}$ inhibited completely the culturability of biofilm cells, as well as ASA and TET at $10\times\text{MIC}$. Considering these results, there is no advantage in combine ASA with both antibiotics and both bacterial strains, since the use of a single drug promotes an equal or lower log CFU/cm² reduction. Interactions between ASA and KAN, at MIC, were considered indifferent, while all the other interactions were considered additive, for both bacteria (Table B.1.1, Appendix B).

4.3.3.3 Piroxicam

The ability of PXC to control 24 h old *E. coli* biofilms when combined with the selected antibiotics was also evaluated. With regard to the reduction of biomass, the behaviour of the combination PXC and KAN was similar to the combination of PXC and TET, with a significant increase in the removal effect with increasing concentration ($p<0,05$) (Figures 12a and 12b). PXC behaves as a bell-shaped effect, since when PXC is used alone, its effect decreases with an increasing concentration, but when conjugated with a fixed concentration of antibiotics, its effect increases with an increasing concentration.

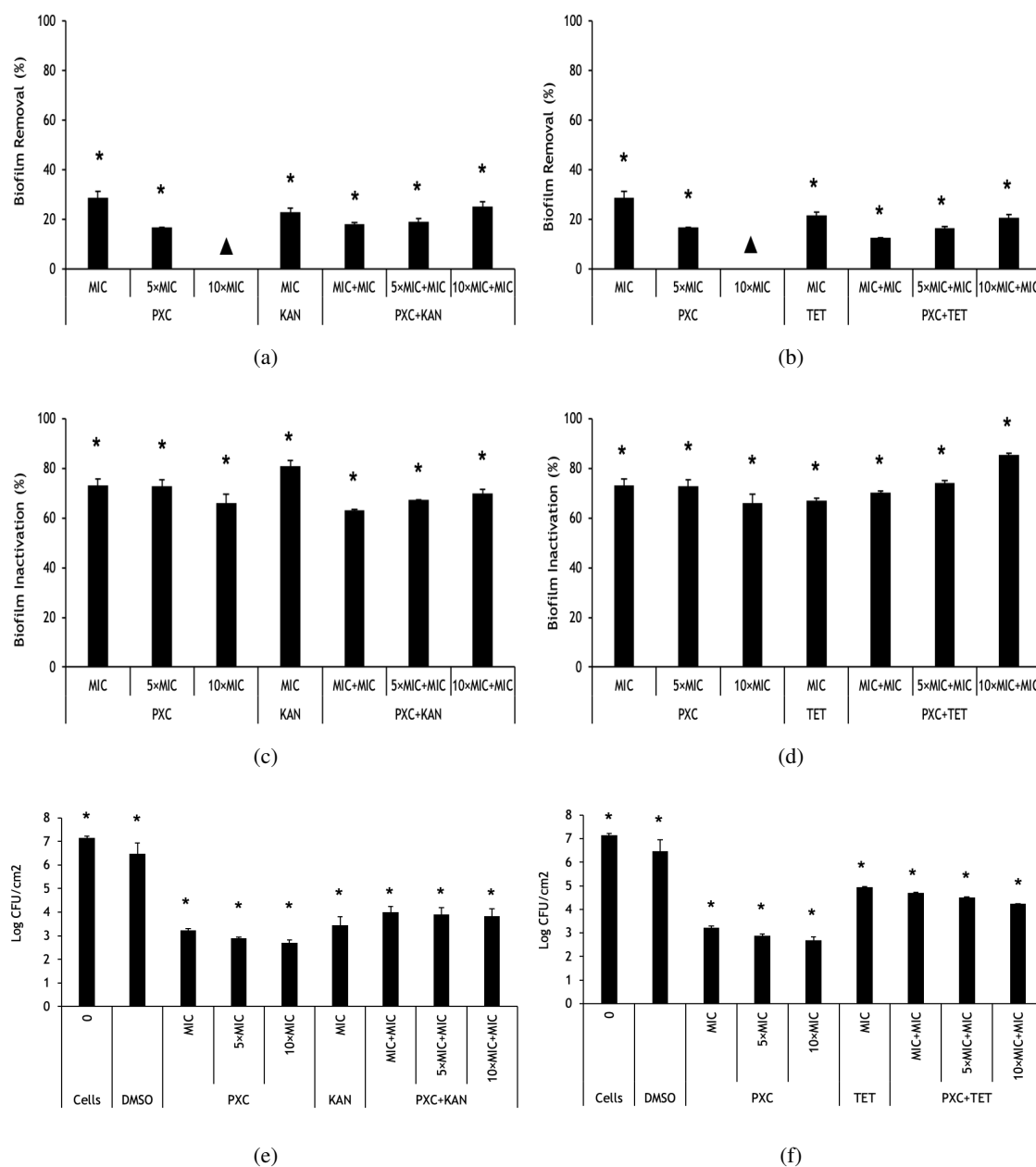


Figure 12: Effect of PXC at MIC, 5×MIC and 10×MIC alone and in combination with selected antibiotics (KAN and TET) at MIC, on 24 h old *E. coli* CECT 434 biofilms after 24 h exposure. (a)(b) Percentage of mass reduction for KAN and TET, respectively, (c)(d) biofilm removal for KAN and TET, respectively; and (e)(f) culturability – Log CFU/cm² reduction - for KAN and TET, respectively. Δ - 0% of biofilm removal. Mean values ± SD for two or more independent experiments are illustrated. Bars marked with * are statistically different from the control ($p < 0,05$).

When PXC was used alone (at MIC) it reduced more biomass than when conjugated with KAN or TET. So, there is no advantage in combine PXC with both antibiotics, since the use of a single

drug promotes a higher effect than the combination. In general, the dual combinations of PXC with tested antibiotics promoted low efficacy on biomass reduction, except for the combination of PXC and KAN at $10\times\text{MIC}$ that promoted a moderate effect (Table B.2.1, Appendix B). By the way, interactions between PXC and KAN were classified as indifferent for MIC and $5\times\text{MIC}$ and additive for $10\times\text{MIC}$. On the other side, interactions between PXC and TET were classified as antagonistic, indifferent and additive for MIC, $5\times\text{MIC}$ and $10\times\text{MIC}$, respectively (Table B.1.1, Appendix B).

Dual combinations of PXC and KAN did not improve inactivation of the biofilm cells in relation to the controls (cells with PXC and cells with KAN/TET), for any concentration (Figure 12c). Contrariwise, dual combination of PXC and TET at $10\times\text{MIC}$ improved biofilm inactivation (Figure 12d). All the combinations of PXC and KAN were classified as indifferent and all the combinations of PXC and TET were classified as additive; despite this, all these combinations with both antibiotics had high efficacy on biofilm inactivation (Tables B.1.1 and B.2.1, Appendix B).

As far as culturability is concerned, the combinations between PXC and KAN promoted log CFU/cm² reduction lower than controls and the same applies to combinations of PXC and TET (Figures 12e and 12f). Therefore it's predicted that piroxicam used alone or antibiotics alone will be more effective than the dual combination with antibiotics. Furthermore, all the combinations between PXC and both antibiotics had additive interactions (Table B.1.1, Appendix B).

4.3.3.4 Diclofenac sodium

Dual combinations of DCF with KAN and DCF with TET had similar effects in the biomass reduction of *S. aureus* biofilms (Figures 13a and 13b). In fact, combinations with DCF (both antibiotics) showed greater removal effects at higher concentrations and obtained greater removal effects than the control of cells with DCF ($p<0,05$). Simultaneously, dual combinations of DCF with KAN and TET had worse effects than the control of cell with KAN/TET ($p<0,05$).

In addition, for the combination of DCF with KAN excluding MIC, which obtained an indifferent effect between the two drugs (DCF and KAN), the two other concentrations showed additive interactions. Regarding the combination of DCF with TET, the interaction between the two drugs was additive for all concentrations (Table B.1.1, Appendix B). Almost all the combinations had low efficacy on biofilm removal, with the exception of DCF at $10\times\text{MIC}$ with TET which had a moderate effect (Table B.2.1, Appendix B).

In terms of biofilm inactivation, no differences were observed for all combinations when compared with the NSAIDs alone ($p>0,05$) but had a much higher percentage of metabolic inactivation than both antibiotics alone ($p<0,05$) (Figures 13c and 13d). However, all of these combinations were highly effective in cell inactivation, and the interaction between DCF and KAN/TET was additive (Tables B.1.1 and B.2.1, Appendix B).

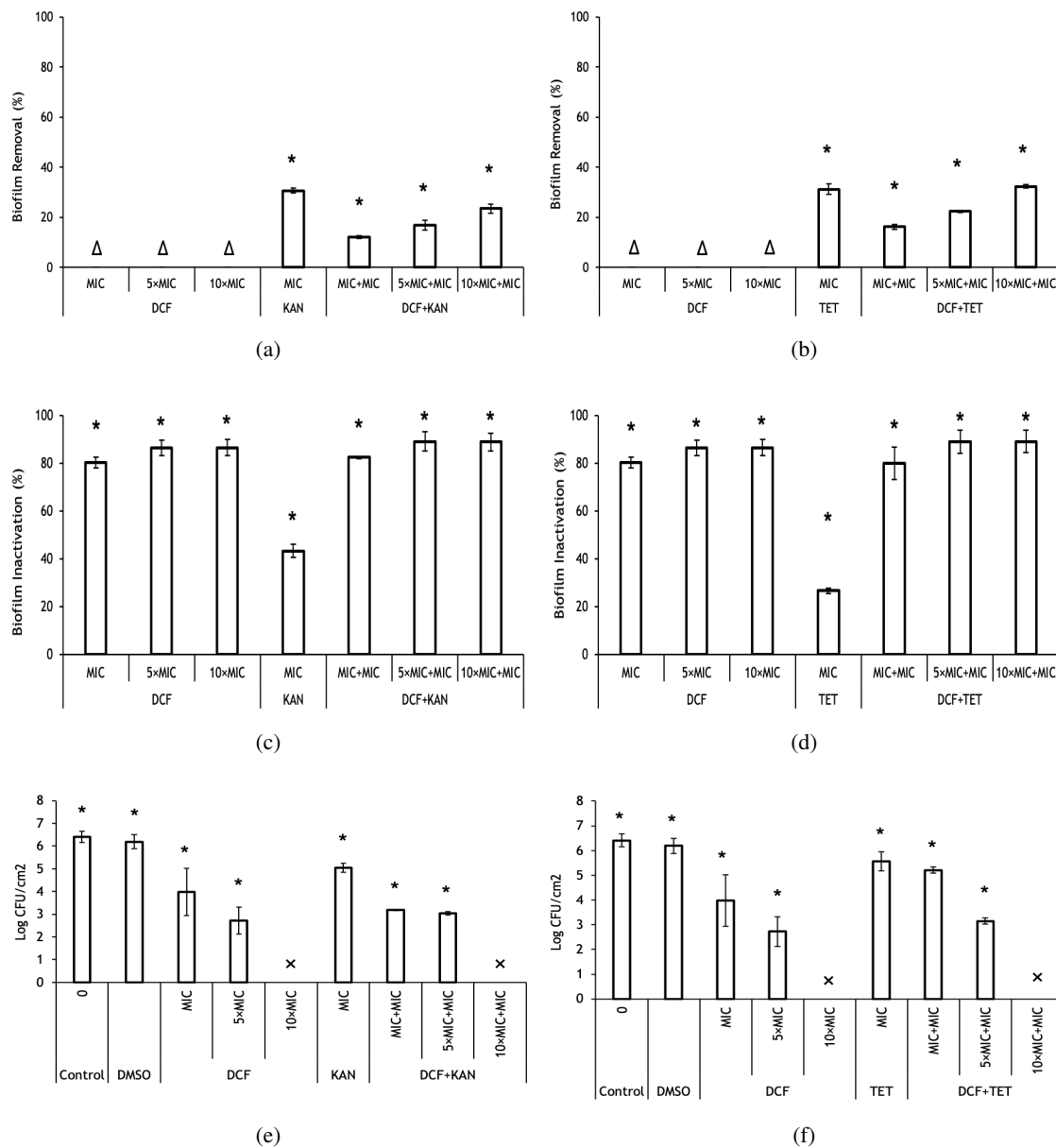


Figure 13: Effect of DCF at MIC, 5×MIC and 10×MIC alone and in combination with selected antibiotics (KAN and TET) at MIC, on 24 h old *S. aureus* CECT 976 biofilms after 24 h exposure. (a)(b) Percentage of mass reduction for KAN and TET, respectively, (c)(d) biofilm removal for KAN and TET, respectively; and (e)(f) culturability – Log CFU/cm² reduction - for KAN and TET, respectively. × - no CFU/cm² detected. Δ - 0% of biofilm removal. Mean values ± SD for two or more independent experiments are illustrated. Bars marked with * are statistically different from the control ($p < 0.05$).

In terms of culturability is concerned, combinations of DFC with KAN and TET at 10×MIC reduced completely the culturability of biofilm cells, exactly like DCF alone. So, there is no advantage in combine ASA with both antibiotics, since the use of a single drug promotes the

same effect than the combination (Figures 13e and 13f). The interaction of the combinations of DCF and KAN at MIC and 5×MIC were classified as indifferent, while at 10×MIC it was classified as additive. For the combinations between DCF and TET, an additive interaction at MIC and 10×MIC was found, while indifferent interaction was observed at for 5×MIC (Table B.1.1, Appendix B).

Some studies showed that certain drugs like NSAIDs improve the activity of certain antibiotics such as KAN and TET [116]. In addition, another study indicates that DCF and ASA are good quorum sensing inhibitors [77]. Since quorum sensing is a mechanism associated with the resistance of microorganisms to antibiotics, this may be the mode of action of these NSAIDs in the eradication of *E. coli* and *S. aureus* biofilms. Moreover, a further study demonstrated that ASA can totally reduce biofilm cells culturability, as noted above [117].

Many studies have shown that NSAIDs actually inhibit the biofilm formation, affecting their adhesion to a surface and cell growth, but there are not many studies on the action of these drugs on pre-established biofilms [118, 119].

4.4 Conclusions

This study highlights the effect of NSAIDs on biofilm eradication and their effect on antibiotic potentiation. Only piroxicam demonstrated to be able to remove the biofilm mass. However, all NSAIDs showed to be quite effective in metabolic inactivation and in the reduction of biofilm cells culturability.

For combination assays of NSAIDs with antibiotics, this study highlights that not all combinations have better effects than the individual use of NSAIDs or antibiotics. In fact, when the combined effect is similar to the effect of a single drug, there is no advantage in combining them. However, often the effect of NSAIDs is higher than the effect of antibiotics, so they could replace their use. A disadvantage can be the difference in the amount of drug required to achieve this effect. Therefore it's required a careful handling in therapeutics. Of course, another mismatch of the NSAIDs is their low ability to remove biofilm mass cells in relation to antibiotics. However, they shown to be excellent in metabolic inactivation and in the reduction of biofilm cells culturability.

Chapter 5

Conclusions and Future Work

5.1 General Conclusions

This work highlights NSAIDs as an alternative to the use of antibiotics or as co-adjuvants in order to enhance their effect or modify the resistance mechanisms of the bactérias.

Four NSAIDs were selected although only three have been shown to be effective in the cell growth inhibition. In fact, ASA, DCF and PXC were able to totally inhibit the growth of *E. coli* CECT 434 and *S. aureus* CECT 976. While PXC appears to be more effective against *E. coli* strain, DCF is more effective against *S. aureus* strain and the ASA appeared to be equally effective against both bacterial strains. Therefore, studies have been carried out only for these combinations of bacteria and NSAIDs, since the MICs were greater than 2000 µg/mL for PXC with *S. aureus*, DCF with *E. coli* and NPX with both bacteria. All MBC values were also higher than 2000 µg/mL.

Furthermore, for *S. aureus*, ASA and DCF are equally effective in the bacterial growth inhibition. For *E. coli*, PXC appeared to be more efficient than the ASA, since it required a concentration 2,2 times lower than the ASA concentration.

These NSAIDs were also evaluated for their ability to affect the integrity of the bacterial cell membranes, in order to understand if this is their mode of action. In fact, these NSAIDs showed to affect the bacterial cytoplasmic membranes, causing the release of intracellular content (K^+) to the external medium.

NSAIDs also showed to be highly effective in the reduction of metabolic activity of *E. coli* and *S. aureus* biofilms, however, only PXC showed to be able to reduce biomass, moderately. Moreover, ASA and DCF reduced totally the culturability of biofilm cells. The combinations of NSAIDs with KAN and TET in most cases showed that there is no advantage in combine NSAIDs with antibiotics. This is due to the fact that a NSAID or antibiotic alone promotes an equal or even better effect on biofilm cell inactivation, biomass removal and reduction of the culturability. However combinations of PXC with TET, ASA with KAN and ASA with TET, for *E. coli* regarding metabolic inactivation assay showed statistically significant inactivation that the controls (cells with ASA/PXC and cells with KAN/TET).

Repurposing PXC, DCF and ASA as antimicrobial drug seems to be a good strategy to overcome the problems associated with resistance to antibiotics. The selected NSAIDs are drug-like compounds and their ADME properties have already been predicted. Furthermore, anti-inflammatory drugs are commonly used and prescribed by the medical community and all the effects on the human body are well known. However, its use in medical treatment has to be ruled out since the recommended maximum concentrations per mL of plasma are lower than MIC values for all NSAIDs tested.

5.2 Future Work

Although studies on the effect of NSAIDs (ASA, PXC, DCG and NPX) against bacterial strains of *E. coli* and *S. aureus* have been conducted, further studies are needed to better understand their effects on planktonic and sessile bacterial cells.

Other mechanisms of action of these antimicrobial drugs, focusing on the inhibition of DNA replication and synthesis of RNA and proteins, requires further investigation. In addition, it is important to monitor cell wall and lipid synthesis [120–122]. All these mechanisms of action can be monitored through a synthesis assay of macromolecules by assessing the incorporation rate of the corresponding radiolabeled precursors [122, 123].

In addition, additional assays on the effect of NSAIDs on the cytoplasmic membrane of bacteria should be performed, including zeta potential assays, characterization of bacterial surfaces and analysis of hydrophobicity changes.

In addition, it's known that the quorum sensing is a regulatory system where are cooperation between the cells allowing the formation and differentiation of the cells of the biofilm. Thus, evaluating the effect of selected NSAIDs on inhibition of quorum sensing may be an important strategy to prevent and control biofilms [22]. The study of the potential of selected NSAIDs in inhibiting efflux pumps is also an important study, since the inhibition of these pumps increases the availability of the drugs within the cells, simultaneously increasing their effect [124].

Once combination assays are performed, it would be important to evaluate the physical and chemical interaction of NSAIDs with antibiotics by evaluating the stability of the mixture through analytical techniques. In addition, predicting the interaction of these drugs from existing models usually is quick and provides a reasonable degree of classification [106].

For all this, future studies are important to truly understand the potency of these NSAIDs as antimicrobial agents.

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Appendix A

Antibacterial activity and mode of action of selected NSAIDs

A.1 Effect of different doses of the selected NSAIDs on *E. coli* and *S. aureus* planktonic cells growth

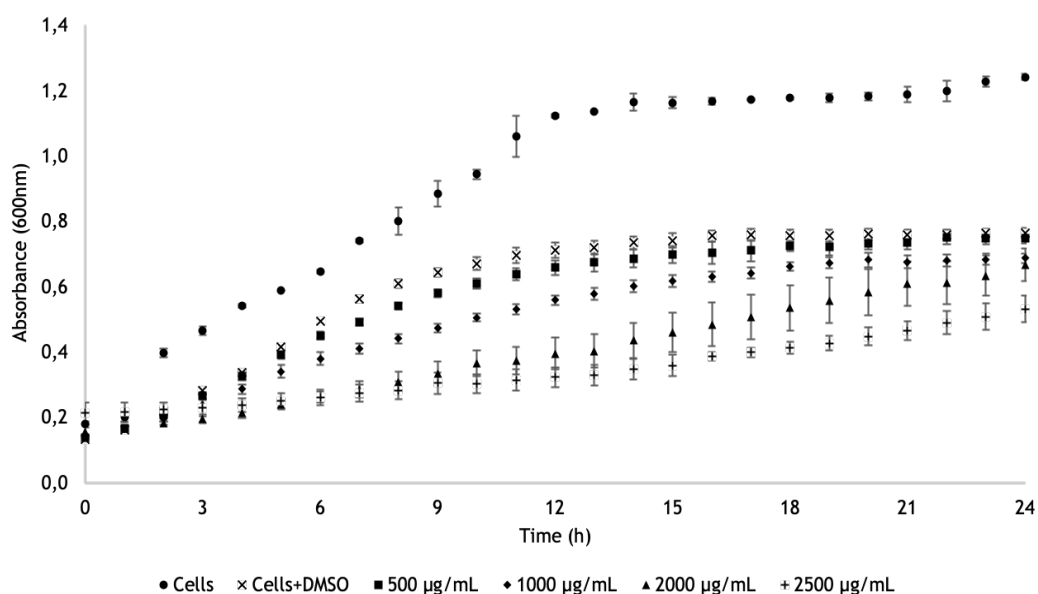


Figure A.1.1: Time kill curves. Absorbance (600 nm) of *E. coli* planktonic cells as a function of PXC concentration without lactose in the medium ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without PXC and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

b

Antibacterial activity and mode of action of selected NSAIDs

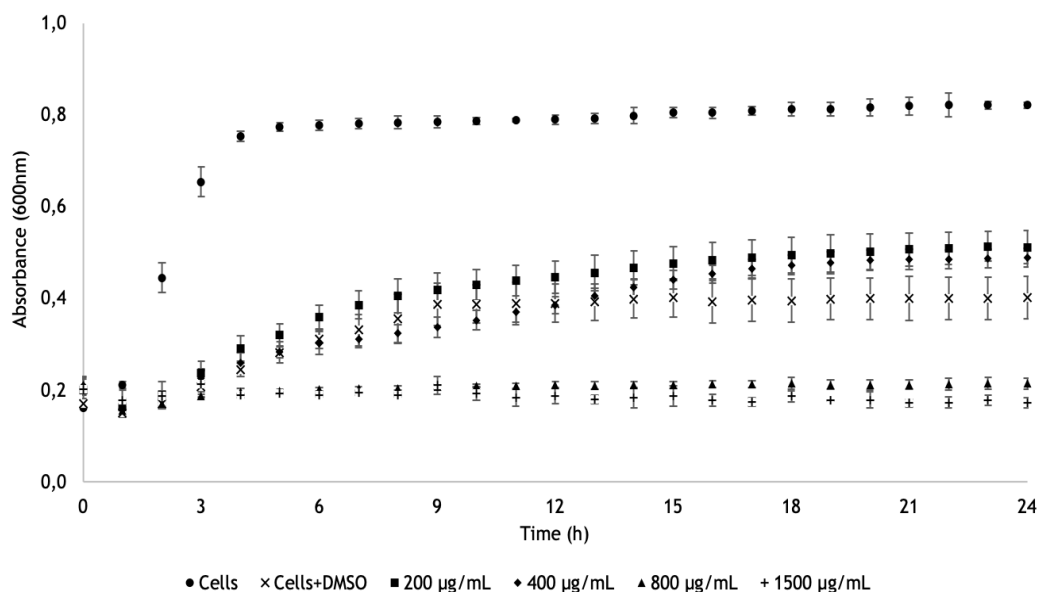


Figure A.1.2: Time kill curves. Absorbance (600 nm) of *E. coli* planktonic cells as a function of PXC concentration with lactose in the medium ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without PXC and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

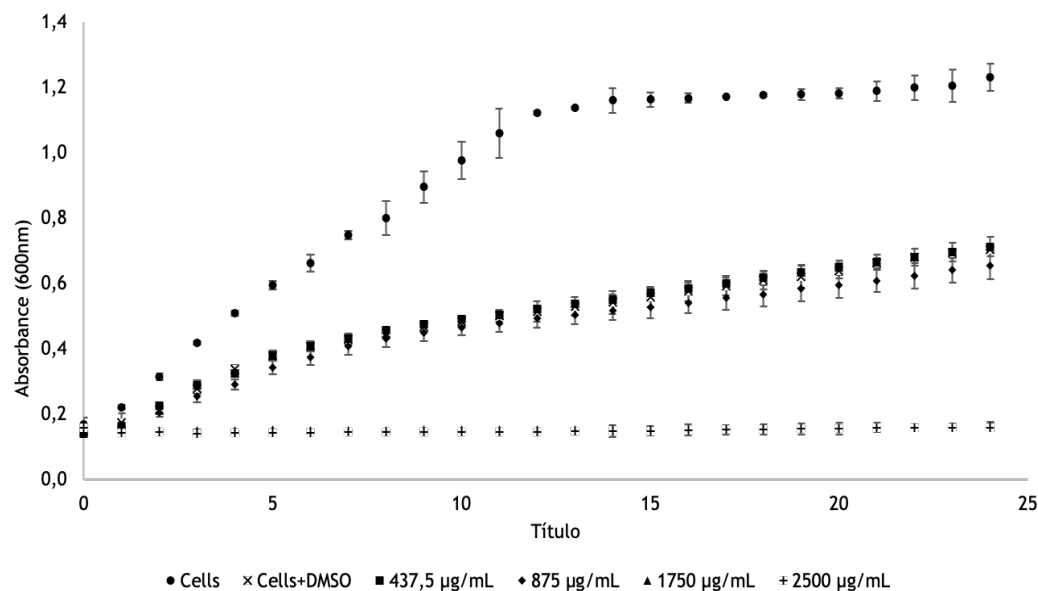


Figure A.1.3: Time kill curves. Absorbance (600 nm) of *E. coli* planktonic cells as a function of ASA concentration ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without ASA and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

A.1 Effect of different doses of the selected NSAIDs on *E. coli* and *S. aureus* planktonic cells growth

c

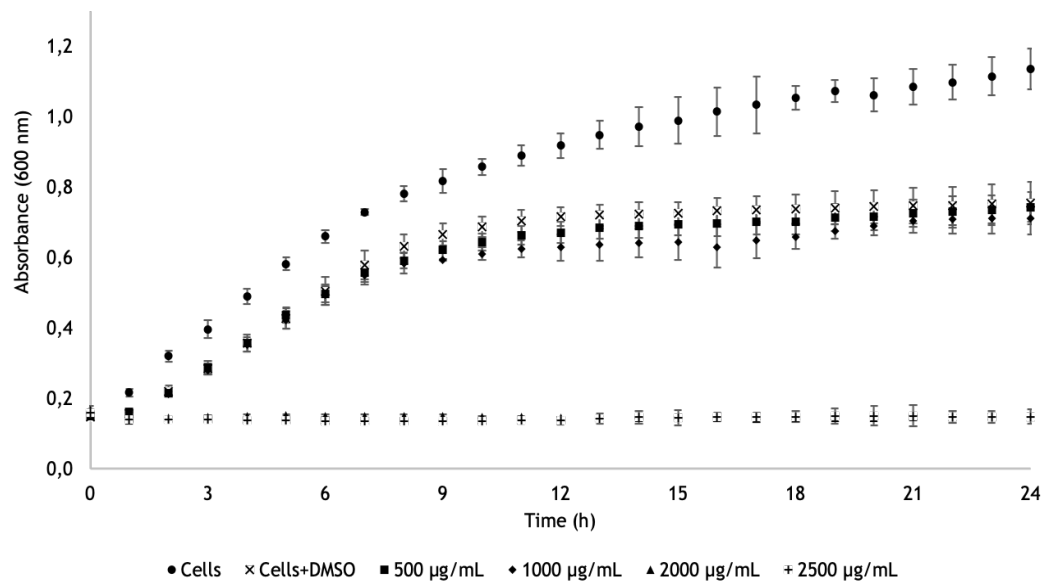


Figure A.1.4: Time kill curves. Absorbance (600 nm) of *S. aureus* planktonic cells as a function of ASA concentration ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without ASA and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

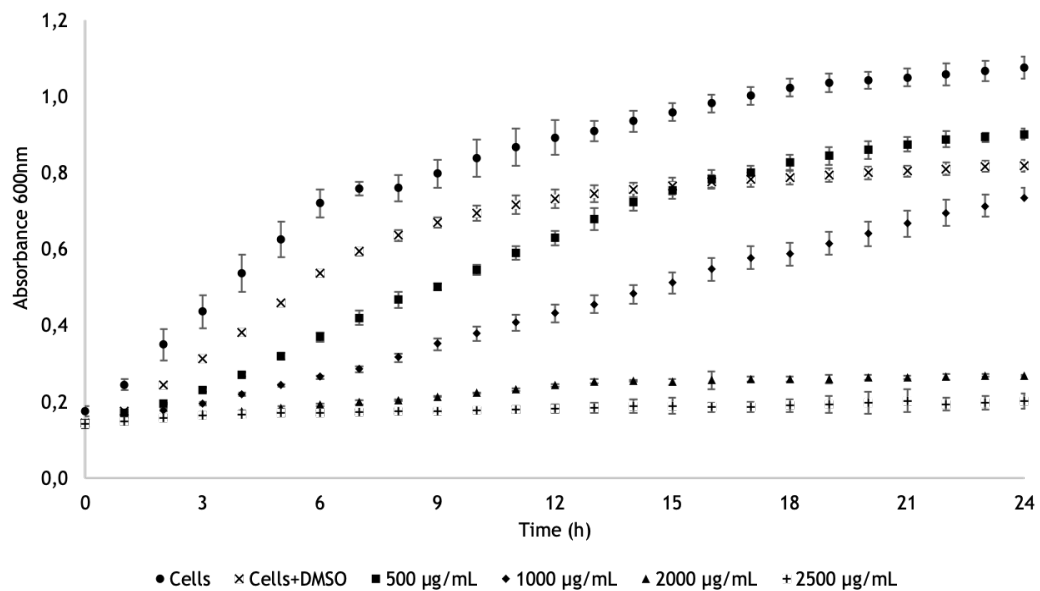


Figure A.1.5: Time kill curves. Absorbance (600 nm) of *S. aureus* planktonic cells as a function of DCF concentration ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without DCF and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

d

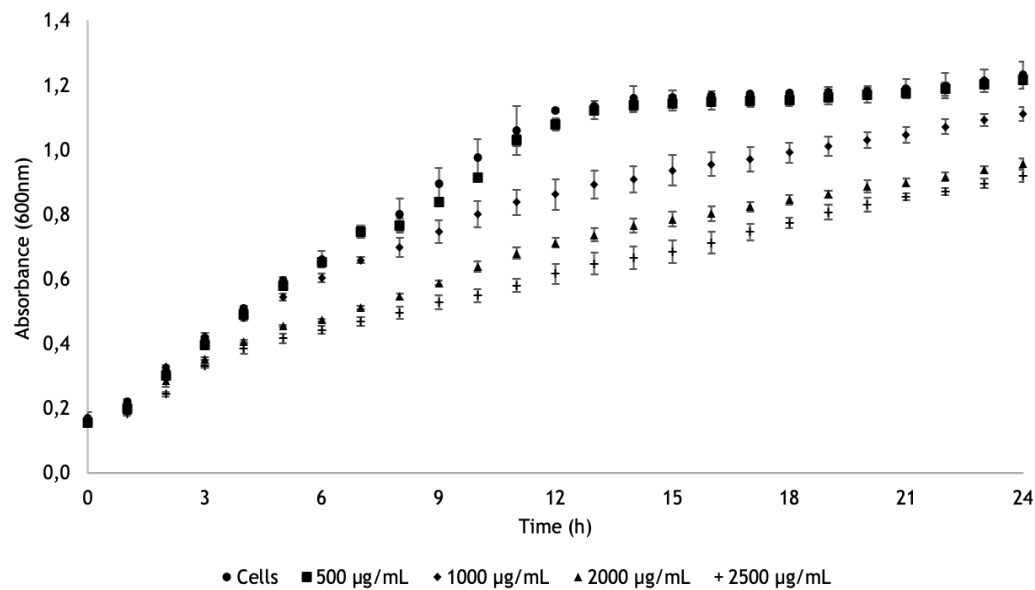


Figure A.1.6: Time kill curves. Absorbance (600 nm) of *E. coli* planktonic cells as a function of NPX concentration ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NPX and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

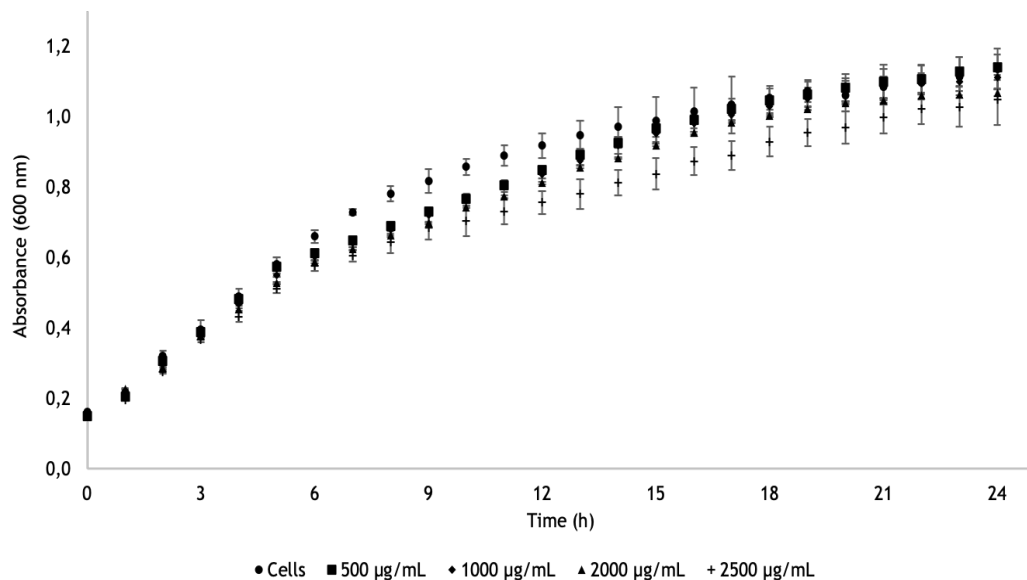


Figure A.1.7: Time kill curves. Absorbance (600 nm) of *S. aureus* planktonic cells as a function of NPX concentration ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and a concentration above the MIC) after 6 h of exposure. Cells without NPX and cells with DMSO were used as negative controls. Data points are from a single experiment with six replicates.

Table A.1.1: Specific growth rate values (h^{-1}) of *E. coli* and *S. aureus* after exposure to PXC, ASA and DCF at four different concentrations (MIC, $5\times$ MIC, $10\times$ MIC and a concentration above MIC), for 24 h. Cells without NSAIDs and cells with DMSO were used as negative controls. '-' means that there was no bacterial growth.

μ (h^{-1})			μ (h^{-1})		
Piroxicam without/ lactose	Cells	0,230	Diclofenac sodium	Cells	0,255
	DMSO	0,212		DMSO	0,225
	500	0,203		500	0,143
	1000	0,163		1000	0,084
	2000	0,086		2000	—
	2500	0,038		2500	—
<i>E. coli</i>	Cells	0,474	Naproxen sodium	Cells	0,233
	DMSO	0,153		500	0,220
	200	0,163		1000	0,211
	400	0,132		2000	0,198
	800	—		2500	0,177
	1500	—		Cells	0,259
Acetylsalicylic acid	Cells	0,233	Naproxen sodium	500	0,245
	DMSO	0,199		1000	0,235
	437,5	0,208		2000	0,221
	875	0,169		2500	0,207
	1750	—			
	2500	—			
<i>E. coli</i>	Cells	0,259	Acetylsalicylic acid	Cells	0,259
	DMSO	0,221		DMSO	0,221
	500	0,218		500	0,218
	1000	0,210		1000	0,210
	2000	—		2000	—
	2500	—		2500	—
<i>S. aureus</i>	Cells	0,259	Acetylsalicylic acid	Cells	0,259
	DMSO	0,221		DMSO	0,221
	500	0,218		500	0,218
	1000	0,210		1000	0,210
	2000	—		2000	—
	2500	—		2500	—

A.2 Effect of different doses of the selected NSAIDs on *E. coli* and *S. aureus* planktonic cells culturability

Table A.2.1: Final log CFU/mL reduction values for NSAIDs at $1/2 \times \text{MIC}$, MIC and a concentration above MIC.

	C (ug/mL)	log reduction
Piroxicam	400	1,20
	800	1,27
	1500	1,34
<i>E. coli</i>	875	0,19
	1750	9,83
	2500	0,90
Acetylsalicylic acid	1000	0,05
	2000	0,92
	2500	1,06
<i>S. aureus</i>	1000	2,50
	2000	3,86
	2500	5,68

A.3 Effect of selected NSAIDs on bacterial cytoplasmic membrane integrity

Table A.3.1: Percentage of PI stained cells for *E. coli* and *S. aureus* after exposure to PXC, ASA and DCF at MIC for 6h. The mean $\pm$ SD for two replicates are illustrated

		% PI stained cells
<i>E. coli</i>	Cells	0,6 $\pm$ 0,6
	DMSO	2,6 $\pm$ 0,8
	ASA	17,2 $\pm$ 2,2
	PXC	24,0 $\pm$ 4,0
<i>S. aureus</i>	Cells	0,2 $\pm$ 0,2
	DMSO	5,0 $\pm$ 0,4
	ASA	21,1 $\pm$ 0,9
	DCF	27,1 $\pm$ 1,3

Table A.3.2: Concentration of K^+ ($\mu\text{g/mL}$) in solutions of *E. coli* and *S. aureus* after 6h of exposure to PXC, ASA and DCF at MIC

	K^+ release ($\mu\text{g/mL}$)			
	<i>E. coli</i>		<i>S. aureus</i>	
	PXC	ASA	DCF	ASA
1/4MIC	$0,18 \pm 0,01$	$0,24 \pm 0,01$	$0,23 \pm 0,01$	$0,17 \pm 0,02$
1/2MIC	$0,32 \pm 0,00$	$0,33 \pm 0,02$	$0,28 \pm 0,02$	$0,18 \pm 0,01$
MIC	$0,36 \pm 0,02$	$0,37 \pm 0,03$	$0,30 \pm 0,03$	$0,22 \pm 0,02$
>MIC	$0,48 \pm 0,01$	$0,43 \pm 0,03$	$0,51 \pm 0,01$	$0,29 \pm 0,02$

Appendix B

Biofilm control activity of NSAIDs

B.1 Effect of dual combination of NSAIDs with selected antibiotics on the control of *E. coli* and *S. aureus* biofilms

Table B.1.1: $\sum C_I$ scoring of the selected NSAIDs (at MIC, 5×MIC and 10×MIC) with TET and KAN (at MIC) for biofilm removal, biofilm inactivation and culturability.

			Biofilm Reduction	Biofilm Inactivation	Culturability
<i>E. coli</i>	PXC+KAN	MIC + MIC	+	+	++
		5MIC + MIC	+	+	++
		10MIC + MIC	++	+	++
	PXC+TET	MIC + MIC	-	++	++
		5MIC + MIC	+	++	++
		10MIC + MIC	++	++	++
	ASA+KAN	MIC + MIC	++	++	+
		5MIC + MIC	++	++	++
		10MIC + MIC	++	++	++
	ASA+TET	MIC + MIC	++	++	++
		5MIC + MIC	++	++	++
		10MIC + MIC	++	++	++
<i>S. aureus</i>	DCF+KAN	MIC + MIC	+	++	+
		5MIC + MIC	++	++	+
		10MIC + MIC	++	++	++
	DCF+TET	MIC + MIC	++	++	++
		5MIC + MIC	++	++	+
		10MIC + MIC	++	++	++
	ASA+KAN	MIC + MIC	++	++	+
		5MIC + MIC	++	++	++
		10MIC + MIC	++	++	++
	ASA+TET	MIC + MIC	++	++	++
		5MIC + MIC	++	++	++
		10MIC + MIC	++	++	++

B.2 Classification of NSAIDs and antibiotics activity on the control of *E. coli* and *S. aureus* biofilms

Table B.2.1: Biofilm control activity classification for NSAIDs (at MIC, 5×MIC and 10×MIC) with TET and KAN (at MIC) for biofilm removal, biofilm inactivation.

		Biofilm Reduction		Biofilm Activity
<i>E. coli</i>	KAN	MIC	Low Efficacy	High Efficacy
	TET	MIC	Low Efficacy	High Efficacy
	PXC	MIC	Moderate Efficacy	High Efficacy
		5MIC	Low Efficacy	High Efficacy
		10MIC	Low Efficacy	High Efficacy
	PXC+KAN	MIC + MIC	Low Efficacy	High Efficacy
		5MIC + MIC	Low Efficacy	High Efficacy
		10MIC + MIC	Moderate Efficacy	High Efficacy
	PXC+TET	MIC + MIC	Low Efficacy	High Efficacy
		5MIC + MIC	Low Efficacy	High Efficacy
		10MIC + MIC	Low Efficacy	High Efficacy
	ASA	MIC	Low Efficacy	High Efficacy
		5MIC	Low Efficacy	High Efficacy
		10MIC	Low Efficacy	High Efficacy
	ASA+KAN	MIC + MIC	Moderate Efficacy	High Efficacy
		5MIC + MIC	Moderate Efficacy	Excellent Efficacy
		10MIC + MIC	Low Efficacy	Excellent Efficacy
	ASA+TET	MIC + MIC	Moderate Efficacy	High Efficacy
		5MIC + MIC	Low Efficacy	Excellent Efficacy
		10MIC + MIC	Low Efficacy	Excellent Efficacy
<i>S. aureus</i>	KAN	MIC	Moderate Efficacy	Moderate Efficacy
	TET	MIC	Moderate Efficacy	Moderate Efficacy
	DCF	MIC	Low Efficacy	High Efficacy
		5MIC	Low Efficacy	High Efficacy
		10MIC	Low Efficacy	High Efficacy
	DCF+KAN	MIC + MIC	Low Efficacy	High Efficacy
		5MIC + MIC	Low Efficacy	High Efficacy
		10MIC + MIC	Low Efficacy	High Efficacy
	DCF+TET	MIC + MIC	Low Efficacy	High Efficacy
		5MIC + MIC	Low Efficacy	High Efficacy
		10MIC + MIC	Moderate Efficacy	High Efficacy
	ASA	MIC	Low Efficacy	High Efficacy
		5MIC	Low Efficacy	High Efficacy
		10MIC	Low Efficacy	High Efficacy
	ASA+KAN	MIC + MIC	Moderate Efficacy	High Efficacy
		5MIC + MIC	Moderate Efficacy	High Efficacy
		10MIC + MIC	Low Efficacy	High Efficacy
	ASA+TET	MIC + MIC	Moderate Efficacy	High Efficacy
		5MIC + MIC	Moderate Efficacy	High Efficacy
		10MIC + MIC	Low Efficacy	High Efficacy