Universidade do Minho
Escola de Engenharia

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica

Trabalho realizado sob orientação da
Doutora Paula Alexandra da Silva Jorge
e da
Professora Doutora Maria Olívia Pereira

outubro de 2018

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RESUMO

Péptidos antimicrobianos no combate às principais bactérias patogénicas: novas combinações e estratégias

Tendo em conta o surgimento global de microrganismos resistentes a antimicrobianos, estudos têm-se direcionado para o desenvolvimento de terapias mais eficazes e novos agentes antibacterianos. Agentes alternativos, tais como os péptidos antimicrobianos (AMPs), assim como o uso de combinações de agentes antimicrobianos são opções promissoras e têm sido exploradas para tratar patogénicos dado que podem permitir uma redução no uso de antibióticos em procedimentos médicos e ainda auxiliar na prevenção ou atrasar o aparecimento de bactérias resistentes aos antibióticos. Seguindo estas linhas de estudo, este projeto estuda a atividade antimicrobiana de combinações AMP-antibiótico contra duas principais bactérias: *Pseudomonas aeruginosa* e *Staphylococcus aureus*.

Inicialmente, a suscetibilidade de *P. aeruginosa* e *S. aureus* foi testada em culturas planctónicas contra AMPs incluindo tachiplesina I na forma linear (TP-I-L), magainina II, LL-37 e PALM, e alguns antibióticos, tais como gentamicina, amicacina, levofloxacina, tobramicina e ciprofloxacina. Globalmente, TP-I-L foi o péptido que obteve os melhores resultados apresentando os melhores efeitos bactericida e bacteriostático contra todas as estirpes. Por outro lado, tanto ciprofloxacina como levofloxacina mostraram fortes atividades antimicrobianas contra as estirpes testadas, revelando uma atividade antimicrobiana mais forte contra *S. aureus.* AMPs e antibióticos combinados também foram testados em culturas planctónicas. No geral, os resultados mais positivos obtidos contra a maioria das estirpes foram demonstrados quando se combinou levofloxacina com TP-I-L. A combinação de 8 μg/mL LEV com 8 μg/mL TP-I-L foi a única que revelou tanto atividade bacteriostática como bactericida contra *P. aeruginosa* CI.

Para avaliar a suscetibilidade em biofilme, foi utilizada uma abordagem terapêutica de forma a estudar a capacidade de TP-I-L combinado com levofloxacina para erradicar biofilmes pré-estabelecidos de *P. aeruginosa* e *S. aureus.* Tanto na análise estatística como na análise de significância biológica da ação dos agentes combinados em biofilmes crescidos foram verificados resultados sinérgicos, principalmente em *S. aureus.* Concluindo, a eficácia real das combinações encontra-se mais próxima dos resultados obtidos na análise da significância biológica, embora seja ainda possível que a verdadeira eficácia possa estar entre os resultados obtidos em ambas as metodologias. No geral, as combinações demonstraram ser eficazes contra as duas bactérias, principalmente *S. aureus*.

ABSTRACT

Exploring antimicrobial peptides against major pathogenic bacteria: novel combinations and strategies

Considering the global emergence of antimicrobial-resistant microorganisms, antimicrobial research has been working hard on more effective therapeutics and the development of new antibacterial agents. Alternative agents, such as antimicrobial peptides (AMPs), as well as the use of antimicrobial agents in combination are promising options and have been explored to treat the pathogens as they could allow the reduction of the use of antibiotics in medical procedures and also help in the prevention or slow the emergence of bacteria resistant to antibiotics. Following these lines of study, this work evaluates the antimicrobial activity of AMP-antibiotic combinations against two major bacteria: *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Firstly, the susceptibility of *P. aeruginosa* and *S. aureus* was tested in planktonic cultures against single AMPs, including a linear analogue of tachyplesin I (TP-I-L), magainin II, LL-37 and PALM, and some antibiotics, such as gentamicin, amikacin, levofloxacin, tobramycin and ciprofloxacin. Overall, TP-I-L was the peptide that obtained the best results by showing the best bactericidal and bacteriostatic effects against all the strains. On the other hand, both ciprofloxacin and levofloxacin showed potent antimicrobial activities against the tested strains, revealing a stronger antimicrobial activity against *S. aureus*. Combined AMPs and antibiotics were also tested in planktonic cultures. In general, the most positive outcomes obtained against most of the strains were demonstrated when combining levofloxacin with TP-I-L. The combination of 8 μg/mL LEV with 8 μg/mL TP-I-L was the one revealing both bacteriostatic and bactericidal activity against *P. aeruginosa* CI.

To evaluate biofilm susceptibility, a therapeutic approach was used in order to study the ability of TP-I-L combined with levofloxacin to eradicate pre-established biofilms of *P. aeruginosa* and *S. aureus*. Both in the statistical analysis and the biological significance analysis of the activity of the combined agents on grown biofilms, synergic outcomes were verified, mainly for *S. aureus*. In conclusion, the real effectiveness of the combinations may be closer to the outcomes obtained in the biological significance approach, although it is still likely that the real effectiveness may be between the results obtained in both methodologies. In general, the combinations revealed to be effective against both bacteria, mainly *S. aureus*.

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ABBREVIATIONS AND ACRONYMS

A – Antagonism

Abs – Absorbance

Ad – Additiveness

AK – Amikacin

AMP – Antimicrobial peptide

ANOVA – Analysis of variance

CIP – Ciprofloxacin

CF – Cystic fibrosis

CFU – Colony-forming unit(s)

CI – Clinical isolate(s)

GEN – Gentamicin

DNA – Deoxyribonucleic acid

eDNA – Extracellular DNA

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FBCI – Fractional bactericidal concentration index

FICI – Fractional inhibitory concentration index

h – Hour(s)

I – Indifference

LEV – Levofloxacin

Log – Logarithm

MAGII – Magainin II

MDR – Multi-drug resistant

MHA – Mueller Hinton agar

MHB – Mueller Hinton broth

MIC – Minimum inhibitory concentration

MBC – Minimum bactericidal concentration

ND – Not detected

OD – Optical density

PALM – Lipopeptide palm-KK-NH_2

PS – Polystyrene

R – Resistant

RNA – Ribonucleic acid

rpm – Revolutions per minute

S – Synergy

Sp – Susceptible

TOB – Tobramycin

TP-I – Tachyplesin I

TP-I-L – Linear version of TP-I

TSA – Tryptic soy agar

TSB – Tryptic soy broth

THESIS FRAMEWORK

This section presents a brief contextualization of the main themes explored in this thesis, including the issues related to antibiotic resistance, an historic evolution of antibiotic discovery, the biofilm-related bacteria infections and pathogens associated to the infections, as well as the potential use of antimicrobial peptides to treat these pathogens. In addition, the structure of the thesis is briefly described. The objectives of this thesis are also reported.

Contextualization

Antimicrobial resistance has become a global public health threat as antimicrobial-resistant microorganisms can be found not only in people and animals, but also in food, plants and the environment. Antimicrobial resistance is considered when the drug can no longer inhibit bacterial growth. Since antibiotics were discovered and started being used in therapeutic approaches, it was noticed the development of specific mechanisms of resistance of bacteria (Figure 1) (Levy, 2007).

The exposure to antibacterial agents provides the necessary conditions for the rise and spread of resistant microorganisms (such as bacteria, fungi, viruses and parasites), acquired by the acquisition of mobile genetic elements carrying resistance genes or by mutation, leading to the increasing rates of resistance associated to the use and misuse of those agents (Roca et al., 2015). In order to preserve both human and animal health, and the environment, governments all over the world are paying more attention to this problem as it threatens the effective prevention and treatment of a growing range of infections ("Global Antimicrobial Resistance Surveillance System (GLASS) Report Early implementation," 2016). The current scientific achievements, which involved both the understanding of antimicrobial resistance dynamics and the discovery of new antimicrobials, led to a significant reduction of patients dying from infectious diseases that were fatal before. Despite being essential in medical procedures like major surgeries, organ transplants or cancer chemotherapy, antimicrobial treatments led to the global emergence of antimicrobial resistance and, consequently, to the increment of the cost of healthcare caused by prolonged diseases, incapacities and deaths ("Global Antimicrobial Resistance Surveillance System (GLASS) Report Early implementation," 2016).

Most human bacterial infections involve biofilm-associated microorganisms (Pooi Yin Chung & Khanum, 2017). Biofilms are a common way of bacteria development and growth. Inside the biofilm, there are less external stresses like the action of antimicrobials, making them more resistant to conventional antimicrobial treatments. As they are also resistant to host defense mechanisms, there is no risk of attack by the immune system, what makes these structures problematic when in medical procedures. Thus, these infections are often hard to diagnose and treat, and are associated with the development of infections in indwelling devices, such as intravascular catheters, prosthesis, cardiac devices, and shunts; or linked to tissue-related infections, which include lung infections in patients with cystic fibrosis (CF), chronic otitis media, endocarditis, urinary and biliary tract infections, osteomyelitis, kidney stones, and open wounds (Joo & Otto, 2012; Percival, Suleman, Vuotto, & Donelli, 2015; Reffuveille, de la Fuente-Nunez, Mansour, & Hancock, 2014).

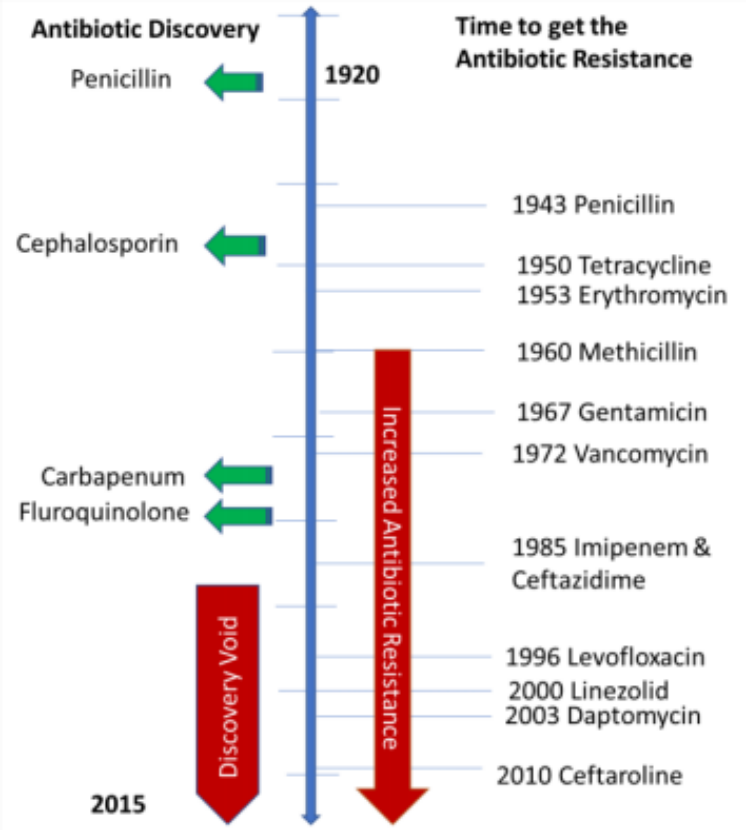

Figure 1 – Historic evolution of antibiotic discovery versus time to get antibiotic resistance (Zaman et al., 2017).

Among the bacteria involved in biofilm-associated infections, there are some that have received most attention. The Gram-negative bacteria *Pseudomonas aeruginosa*, for example, is especially notorious for causing severe chronic infections in cystic fibrosis patients. Likewise, the Gram-positive bacteria *Staphylococcus aureus* is one of the most frequent causes of nosocomial infections related to indwelling medical devices (Joo & Otto, 2012).

Given this scenario and considering the significant growth of antimicrobial resistance, there is an urgent need for the development of new antibacterial agents. Alternative agents, such as antimicrobial peptides (AMPs), are good applicants to treat these pathogens. These peptides are essential molecules of the innate immune system in humans and other organisms that contribute to the first line of defense against infections. They are usually known for their ability to cause cell membrane damage, but can also inhibit cell wall, nucleic acid, and protein biosynthesis (Pooi Yin Chung & Khanum, 2017). Antimicrobial, anti-attachment and antibiofilm properties that have been demonstrated in AMPs, as well as other inherent properties, such as the broad spectrum of activity, are considered to be attractive for the development of a new antibiotic class. AMPs also exhibit low toxicity for eukaryotic cells, due to their specific mode of action, and low incidence of bacterial resistance. Besides, they have shown potent synergistic activity with clinically used antibiotics (Pooi Yin Chung & Khanum, 2017).

Alongside finding new antimicrobials, and in order to guarantee minimal side effects of these agents, fight the emergence of resistance and minimize toxicity, there is an increasing interest about combination studies involving AMPs (Pooi Yin Chung & Khanum, 2017). The use of antimicrobial agents in combination could allow both a reduction in the use of antibiotics in medical procedures and a synergistic effect, which rapidly increases antibiofilm activity and also helps to prevent or slow the emergence of bacteria resistant to antibiotics (Cha, Lee, Choi, Choi, & Park, 2014).

Thesis structure

This document is divided into four main chapters that are divided into different sub-chapters, the bibliography and appendixes.

Chapter 1 introduces the main concepts of the developed work, including the definition, the main characteristics and other related important subjects about biofilms and antimicrobial peptides. Strategies for combating and control bacterial biofilm infections and the main advantages and disadvantages of biofilm development are briefly described. Topics as the use of antimicrobial combinations as a potential solution to slow the emergence of antibiotic resistant bacteria and the rising challenges in the therapeutic use of antimicrobial peptides are also reported.

In Chapter 2, the materials and methods used are explained in detail. There is a brief description of each antimicrobial peptide and antibiotic, as well as some information about the bacteria employed in the laboratory work. Also, all the methods applied in the susceptibility assays, both in planktonic cultures and biofilms, are detailed.

Chapter 3 shows the main results obtained in the different assays performed in this work and their respective discussion. Both the results and the discussion are included in sub-chapters, where each one is related to a different assay.

Finally, Chapter 4 includes the main conclusions of this dissertation, as well as some future prospective paths of work.

Objectives

This work aims to test the anti-biofilm abilities of different agents, including some AMPs like tachyplesin I, magainin II, LL-37 and palm-KK-NH_2, and several antibiotics, such as gentamicin, amikacin, levofloxacin, tobramycin and ciprofloxacin.

The main goal of this project includes the evaluation of the efficiency of novel combinations of AMPs with antibiotics against biofilms of the reference strains *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923, and clinical isolates of *P. aeruginosa* and *S. aureus*.

The susceptibility profiles of *P. aeruginosa* and *S. aureus* to single and combined AMP and antibiotics will be tested in planktonic cultures and in biofilms.

In planktonic cultures, the effect of the combinations will be evaluated through the checkerboard microdilution assay, which will help to choose the best combinations, as well as the concentration of the agents to use in the following tests.

To evaluate biofilm susceptibility, it will be used a therapeutic approach which includes the evalution of the cell viability of 24 h-old biofilms treated with the chosen combinations.

1. INTRODUCTION

In this chapter, the concept of biofilm and its formation are explained. There is also some information about biofilm-related infections and biofilm resistance. A few strategies used to control and fight bacterial biofilm infections are equally described. Some of the advantages and limitations of biofilm development are pointed out. Furthermore, the mechanisms of action of antimicrobial peptides are described, as well as the potential use of antimicrobial combinations as a forthcoming solution to slow the emergence of antibiotic resistance bacteria.

1.1 Biofilms

1.1.1 Definition

Biofilms are structured communities of microorganisms that are connected to a surface and surrounded by the self-produced extracellular matrix, which is formed by polymeric compounds, usually extracellular polysaccharides, proteins and nucleic acids (like DNA) (Rabin et al., 2015; Wu, Moser, Wang, Høiby, & Song, 2015). Biofilms can shelter bacteria from antibiotics and host defenses during infection, playing a significant role in human health, with bacterial infections involving biofilms representing around 65-80 % of all infections, and are involved in multiple difficult to treat and chronic infections (Joo & Otto, 2012; Reffuveille et al., 2014). The formation of these structured complex microbial communities occurs as a response to nutritional cues or starvation, attachment to surfaces or stresses, such as subinhibitory antibiotic concentrations (Reffuveille et al., 2014). When compared with planktonic cells, biofilms represent a physiologically distinct state of bacteria, with hundreds of genes changing expression (Reffuveille et al., 2014).

Biofilm formation is performed in distinct stages (Figure 2) (Berlanga & Guerrero, 2016; Høiby et al., 2011; Joo & Otto, 2012):

(i) bacterial attachment to a surface, which involves planktonic (individual freely moving) bacteria attaching reversibly to the tissue or abiotic surface;

(ii) proliferation and extracellular matrix production, involving different components that may support the adhesion between bacterial cells and also provide protection from antibiotics and host defenses, such as exopolysaccharides, proteins and eDNA;

(iii) biofilm structure maturation, phase where the biofilm becomes thick;

(iv) cell dispersion, where bacterial cells are liberated and then spread to places where new biofilms can be formed.

In order to provide nutrients to cells in deeper biofilm layers, there are water-filled channels in the mature biofilm matrix, which indicate that in addition to the adhesive matrix components that mediate aggregation, biofilm maturation requires cell-cell-disruptive factors (Joo & Otto, 2012).

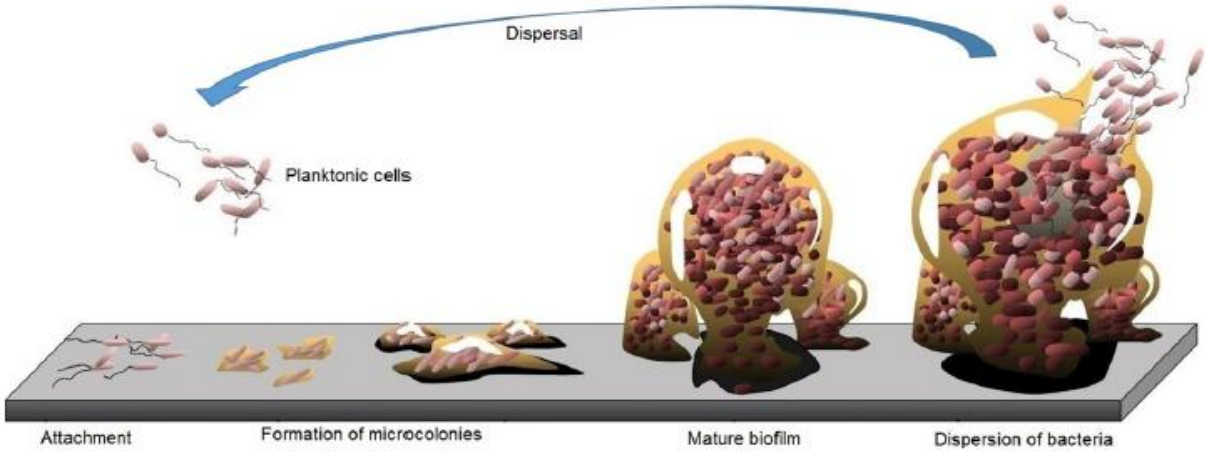

Figure 2 – Bacterial biofilm development stages. In this cycle, bacteria undergo physiological transitions from planktonic cells to sessile cells in building a biofilm, and from sessile cells to dispersed cells in returning to the planktonic state. Adapted from (Pooi Y. Chung & Toh, 2014).

1.1.2 Biofilm-related infections

Bacterial biofilm formation can be found in natural environments with water, in clinical settings, as well as in industrial environments (Yang & Givskov, 2015). Thanks to the progresses verified in medical sciences, there is currently an increment both in the use of antibiotics to treat severe infections as in the number of medical procedures, surgeries and indwelling medical devices. These, together with the development of resistance of many pathogens to antibiotics, induce the emergence of biofilm-related infections (Lebeaux, Ghigo, & Beloin, 2014; Nathan & Cars, 2014). Adding to these issues, there are also some clinical isolates (CI) of opportunistic pathogenic bacterial species, including the Gram-Negative *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* yeasts, particularly *Candida* species, and the Gram-Positive *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Streptococcus* species, which are now resistant to most antibiotics (Table 1) (Nathan & Cars, 2014; Römling & Balsalobre, 2012; Wu et al., 2015).

Biofilm infections are difficult to diagnose and treat, and are associated with the development of medical device-related infections, for example intravascular catheters, prosthetic vascular grafts, cardiac devices, prosthetic joints and shunts (Figure 3). Moreover, they are also linked to tissue-related infections, such as lung infections in patients with cystic fibrosis (CF), chronic otitis media, chronic sinusitis, endocarditis, urinary and biliary tract infections, osteomyelitis, kidney stones, open wounds, and dental plaque, as they are capable of colonizing body surfaces, including the bladder, skin, lungs, and the heart, increasing both patient morbidity and mortality and representing a significant economic concern on healthcare services (Joo & Otto, 2012; Percival et al., 2015; Reffuveille et al., 2014).

Table 1 – Major pathogens associated to biofilm-related infections. Adapted from (Römling & Balsalobre, 2012)

Bacterial species	Biofilm-related infection
Escherichia coli	Acute and recurrent urinary tract infection, catheter-associated urinary tract infection, biliary tract infection
Pseudomonas aeruginosa	Cystic fibrosis lung infection, chronic wound infection, catheter-associated urinary tract infection, chronic rhinosinusitis, chronic otitis media, contact lens-related keratitis
Staphylococcus aureus	Chronic osteomyelitis, chronic rhinosinusitis, endocarditis, chronic otitis media, orthopaedic implants
Staphylococcus epidermidis	Central venous catheter, orthopaedic implants, chronic osteomyelitis
Streptococcus pneumonia	Colonization of nasopharynx, chronic rhinosinositis, chronic otitis media, chronic obstructive pulmonary disease
Streptococcus pyogenes	Colonization of oral cavity and nasopharynx, recurrent tonsillitis

The chronic tissue and device-related infections show high tolerance toward antibiotics, what makes them difficult to treat, exposing the patient to the risk of the infection reappearance (Lebeaux et al., 2014).

Bacterial biofilms are known for their high resistance to many traditional therapies and immune responses, exhibiting superior antibiotic resistance levels when compared with those observed during planktonic growth (Di Luca, Maccari, & Nifosì, 2014). In spite of being considered the most effective measure to control microbial infections, antibiotic treatments are becoming more and more ineffective in biofilm-related infections due to the rise of antibiotic resistance coupled with the biofilm's intrinsic resistance mechanisms. Comparing planktonic bacterial cells and biofilm bacterial cells, studies demonstrated that the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for biofilm bacteria can be approximately 10–1000 times higher (Wu et al., 2015). Bacterial biofilms can show antibiotic resistance through different mechanisms, including: limited antibiotic penetration, as the structure of the matrix of biofilms limits the penetration of antimicrobial agents into the biofilm; horizontal gene transfer between cells and random mutations on genes; limited metabolism and growth rate due to the shortage of oxygen and nutrients in biofilms; persister cells, which have no growth rate or an extremely low growth rate; and efflux pumps, which allow bacterial cells to expel intracellular toxins, including antibiotic drugs (Rabin et al., 2015).

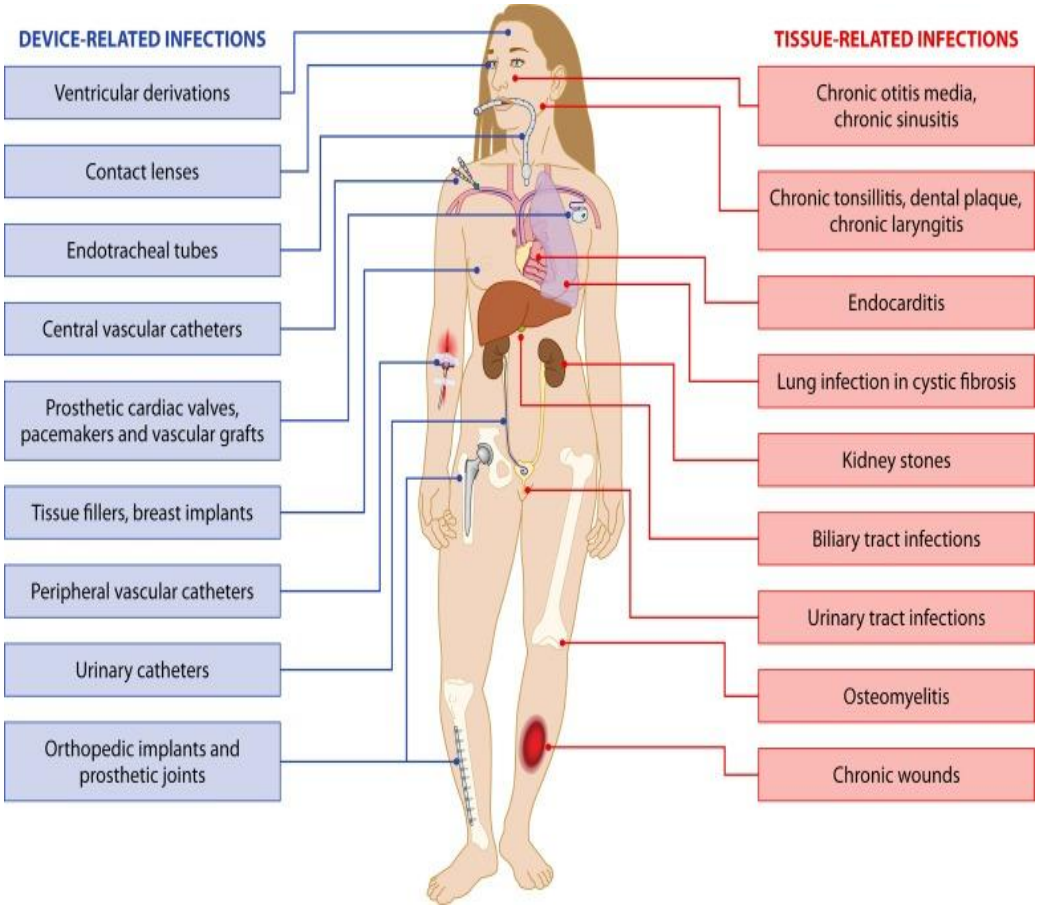

Figure 3 – Examples of biofilm-related infections. Adapted from (Lebeaux et al., 2014).

Biofilm resistance to AMPs changes according to the kind of microorganism resident in the biofilm and seems to be mediated by exopolysaccharides and other extracellular biofilm molecules, which are capable of reducing the activity of these peptides. In order to resist to AMPs and prevent them from reaching their main target, the cytoplasmic membrane, bacteria can adopt distinctive efficient mechanisms, including efflux pumps, secreted proteases or alterations of bacterial surface intended to increase the net positive cell charge to reduce attraction of the typically cationic AMPs, as well as by the inhibition of the AMP-target interaction, and also the modification of the AMP-binding site in target proteins (Pooi Yin Chung & Khanum, 2017; Di Luca et al., 2014; Wright, 2005). Biofilm control can be accomplished through prophylactic and therapeutic strategies. On the one hand, the prophylactic approach can kill planktonic cells in the early biofilm phase, which prevents the development of the biofilm. On the other hand, the therapeutic approach aims to reduce or eradicate mature biofilms (Di Luca et al., 2014).

1.1.3 Strategies for combating biofilm-related infections

As it was overmentioned, microbial biofilm infections are extremely difficult to treat and the conventional antibiotic therapies are not always appropriate. The treatments of the infection can involve the use of a foreign device, which a long term implies its removal for a successful result; or not, when high doses of antibiotic and the combination of antibiotics with distinct killing mechanisms are capable of eradicating the biofilm (Wu et al., 2015). Thus, there are several clinical strategies used to treat the infections, including (Wu et al., 2015):

(i) the removal of foreign bodies and abscess, as the presence of the infected indwelling devices implanted into patients for medical reasons increases significantly the chances of biofilm infection. However, if the infected device cannot be removed or replaced, the biofilm should be treated with antibiotics in order to try to reduce its area, and proceed with the suppressive antibiotic treatment to avoid the biofilm regrowth. Also, the removal of the abscess is crucial as it hinders the penetration of the antibiotic through its wall;

(ii) the change of the infected central venous catheter or dialysis catheter, which is essential in the treatment of catheter biofilm infections and should be followed by a short time therapy involving sensitive antibiotic intravenously so that the bacteria droped in the blood stream can be removed;

(iii) the change of the infected urinary catheter, which should be followed by a sensitive antibiotic treatment in order to reduce the bacteria sheltered in the bladder and in the urinary tract;

(iv) the change of the infected indwelling devices and joint prostheses, that should be combined with a sensitive and aggressive effective antibiotic therapy. In patients with infections placed in biliary stents, endotracheal tubes, dead bones, biliary and urinary stones, the removal of the infected device is also required in order to obtain an effective treatment;

(v) early and aggressive antibiotic treatments against biofilm infections, which is barely performed as biofilm infections are usually difficult to diagnose in the initial stage.

Alongside the overmentioned strategies used to treat and prevent bacterial growth, the current research has also tried different approaches, such as more sophisticated techniques of sterilization and the modification of medical devices, for example the surface modification with hydrogels or antibiotics (minocycline/ rifampicin) (Percival et al., 2015).

1.1.4 Advantages and disadvantages of biofilm development

There are multiple advantages associated to the development of microorganisms in biofilms. In addition to the well known protection against chemicals and antibiotics, biofilms also reveal an increased resistance against UV radiation and dehydration, as well as additional protection from predation and protozoa. These particular features show the evident differences that exist between bacteria grown in biofilm and bacteria grown in planktonic (Xavier, J. B., C. Picioreanu, J. S. Almeida, 2015). These associated microbial communities instigate a wide variety of microbial life and metabolic potential by providing the formation of microniches. Currently, there are applications of biofilms in which they also show their support in the preservation of the health of soil and water, including both the degradation of toxic material in soil and water, and the generation of chemicals and electricity (Lear & Lewis, 2012).
However, biofilm-related infections affect millions of people annually. Limitations related to the matrix and consequently the transport of substances avoids the efficient penetration of the antibiotics in biofilms, reason why it is often associated to antibiotic resistance (Xavier, J. B., C. Picioreanu, J. S. Almeida, 2015). In addition, biofilms have the hability to cause harm to the quality and yield of crops, and also induce biofouling and microbially-induced corrosion (Lear & Lewis, 2012).

1.2 Antimicrobial peptides

1.2.1 What are antimicrobial peptides?

Antimicrobial peptides (AMPs) are powerful antibiotic agents, usually composed by 10-50 amino acids that can be found in all forms of life, but mostly in plants, animals and bacteria (Figure 4), being part of their innate immune system and the first line of host defense. The synthesis of these peptides can be performed through a ribosomal mode (by cleavage of a pro-protein) or a non-ribosomal mode (by multi-modular enzymes) (Patel & Akhtar, 2017). These endogenous peptides are produced by myeloid and epithelial cells in animals (Waters & Smyth, 2015). In bacteria, AMPs are synthesized in ribosomes in order to eliminate antagonists. Regarding the AMPs found in plants, they can be extracted from different parts, including seeds, leaves, flowers, stems and roots. The production of AMPs from bacteria like *Bacillus*, *Paenibacillus* and *Pseudomonas* is already know by their exploration as antibiotics (Patel & Akhtar, 2017).

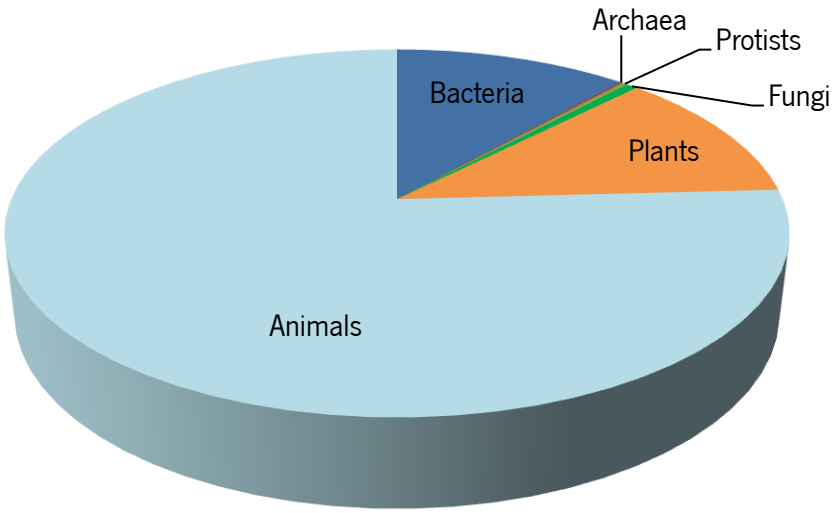

Figure 4 – Sources of AMP (total of 2927 AMPs) from the antimicrobial peptide database. Numbers obtained from http://aps.unmc.edu/AP/, accessed on September 2018.

In the characterization of AMPs, their physiochemical properties (net charge, hydrophobicity, amphipathicity), amino acidic composition, size and conformational structures are taken into account (Pooi Yin Chung & Khanum, 2017; Maria-Neto, de Almeida, Macedo, & Franco, 2015). The major part of natural AMPs own amino acids of L-configuration in their composition and only some have D-amino acids. Considering their structure, AMPs occur in multiple isoforms and can be classified in three groups: β-sheet peptides, α-helical peptides and extended peptides (Figure 5), with β-sheet and α-helical peptides being the most common in nature (Di Luca et al., 2014; Patel & Akhtar, 2017). Table 2 resumes the classification of common AMPs based on their structural features, as well as the main source of extraction of each peptide.

Antimicrobial, anti-attachment and anti-biofilm properties have been demonstrated in AMPs. Furthermore, researchers proved that some AMPs have activity against a wide range of pathogenic microorganisms (Gram-positive and Gram-negative bacteria, and fungi), by interacting with bacterial membranes and creating ion-permeable channels, and consequently, increasing the cytoplasmic membrane permeability and allowing the entrance of AMPs into the cell, which explains how these agents act synergistically with conventional antibiotics against planktonic cells, and by disrupting the bacterial cell membrane, which provides the cell lysis and its death (Mataraci & Dosler, 2012; Waters & Smyth, 2015). AMPs can also inhibit cell wall, nucleic acid, and protein biosynthesis. Other inherent properties, such as a broad spectrum of activity, are considered to be attractive for the development of a new antibiotic class. AMPs also exhibit low toxicity for eukaryotic cells, due to their specific mode of

action. Besides, they have shown potent synergistic activity with clinically used antibiotics (Pooi Yin Chung & Khanum, 2017).

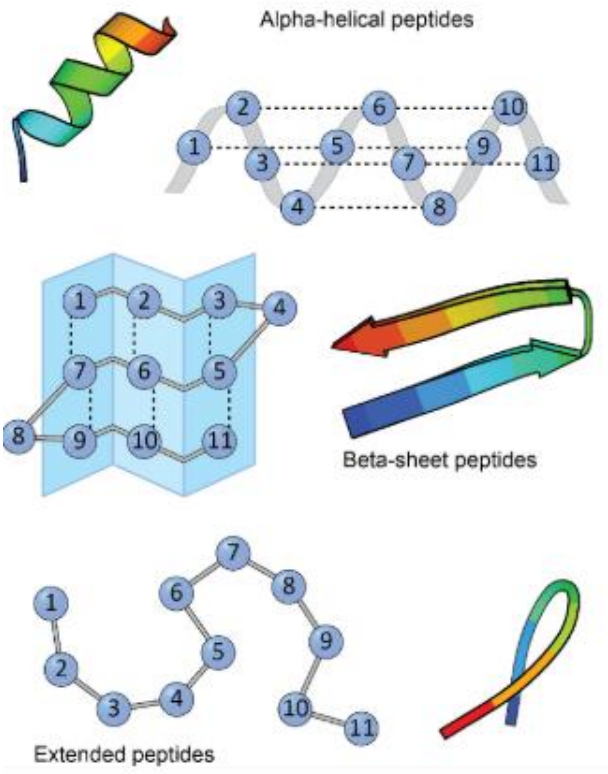

Figure 5 – Classification of the peptides according to the structure. Adapted from (Di Luca et al., 2014).

Microorganisms, including Gram-positive and Gram-negative bacteria, fungi and viruses are affected by the broad-spectrum of action of AMPs. Due to their distinctive mode of action, some AMPs show low tendency of developing resistance and are still effective against multi-drug resistant (MDR) bacteria (Hof, Veerman, Helmerhorst, & Amerongen, 2001; Marr, Gooderham, & Hancock, 2006; Mygind et al., 2005).

Table 2 – Structure and source of common antimicrobial peptides. Adapted from (Kumar et al., 2018)

Category	Peptides	Source
α-helical peptides	Aurein 1–2	Frogs
	Mellitin	Bees
	Brevinin 1	Frogs
	Maculatins	Frogs
	Citropin	Frogs
	Buforin II	Toad
	Cathelicidins	
	• LL-37	Humans
	• BMAP-27,28,34	Bovine
	• Magainins	Frogs
	• Cecropin	Insect
β-sheet peptides	Cathelicidins	
	• Protegrins	Pigs
	• Bactenecin	Bovine
	Defensins	
	• α defensins	Mammals
	• β defensins	Mammals
	• θ defensins	Gorilla
	Tachyplesins and Polyphemusin	Horse Crab
Extended peptides	Cathelicidins	
	• PR-39	Pigs
	• Tritrpticin	Pigs
	• Indolicidin	Bovine
	• Crotalicidin	Snakes

1.2.2 Mechanisms of action of antimicrobial peptides

Most AMPs with intracellular action, including tachyplesin I (TP-I), act on intracellular targets by permeabilizing the bacterial membrane, which affects the transmembrane potential and, consequently, results in cell death (Pooi Yin Chung & Khanum, 2017; Hong et al., 2015). The same does not happen with conventional antibiotics, which mainly act by inhibiting cell wall synthesis or DNA, RNA and protein synthesis. The specific mode of action of AMPs is based on specific attributes like the cationic nature,

size, sequence, hydrophobicity and amphipathicity (Pooi Yin Chung & Khanum, 2017). Their amphipathic properties make them act directly on the membrane of the pathogen; likewise, the accumulation of AMPs on the membrane surface as a result of their selective interaction with the negatively charged surfaces of microbial membranes is related with their cationic nature. Because of these properties, AMPs are generally folded in membrane mimetic environments, where one side of the peptide contains a considerable proportion of hydrophobic residues and the other side is positively charged (Hancock & Lehrer, 1998; Hancock & Sahl, 2006; Zasloff, 2002). Considering the mechanisms of action involved in membrane permeation by AMPs and their molecular targets, AMPs can act by inhibiting the synthesis of macromolecules like DNA, RNA and proteins, binding with DNA/RNA or through the inhibition of the enzymatic activity, and can be classified as: membrane-targeting peptides and intracellular-targeting peptides (Pooi Yin Chung & Khanum, 2017; Di Luca et al., 2014). The mechanism of action of AMPs involves an initial electrostatic interaction which occurs between the outer layer of the bacterial membrane, mainly formed by negatively charged phospholipids (such as phosphatidylglycerol and cardiolipin), and the positively charged AMPs (Kumar et al., 2018). After the initial electrostatic and hydrophobic interactions and reaching the limit concentration, AMPs start to accumulate and settle down at the bacterial membrane surface (Kumar et al., 2018). Some of the mechanisms of action of AMPs encompass the formation of membrane-spanning pores: the 'barrel-stave' and the 'toroidal-pore' models, which depend on the capacity of the peptide to create ordered transmembrane channels; and the carpet model, where the peptides act without the formation of specific pores in the membrane and provide the origin of micelles by breaking the bilayer in a detergent-like manner (Figure 6). Besides membrane permeabilization, AMP can neutralize or disaggregate the lipopolysaccharide, an endotoxin responsible for Gram-negative infections and protect against sepsis (Pooi Yin Chung & Khanum, 2017; Di Luca et al., 2014).

AMPs can be produced by immune cells like neutrophils and macrophages. Adding to the overmentioned direct killing mechanisms of action of AMPs, these peptides can also act by immune modulation, as they are capable of controlling the inflammation and killing bacteria through the activation of immune cells. In order to attract immune cells and control the inflammation it is important the production of an immune response, which can be produced by few AMPs. Thus, these peptides are involved in several events, as they can stimulate the angiogenesis, reduce the expression of proinflammatory chemokines which leads to the reduction of the inflammation, as well as control the expression of reactive oxygen/nitrogen species, and also activate, attract and differentiate white blood

cells. For example, the human AMPs LL-37 and β-defensins are capable of attracting mast cells, leukocytes and dendritic cells (Kumar et al., 2018).

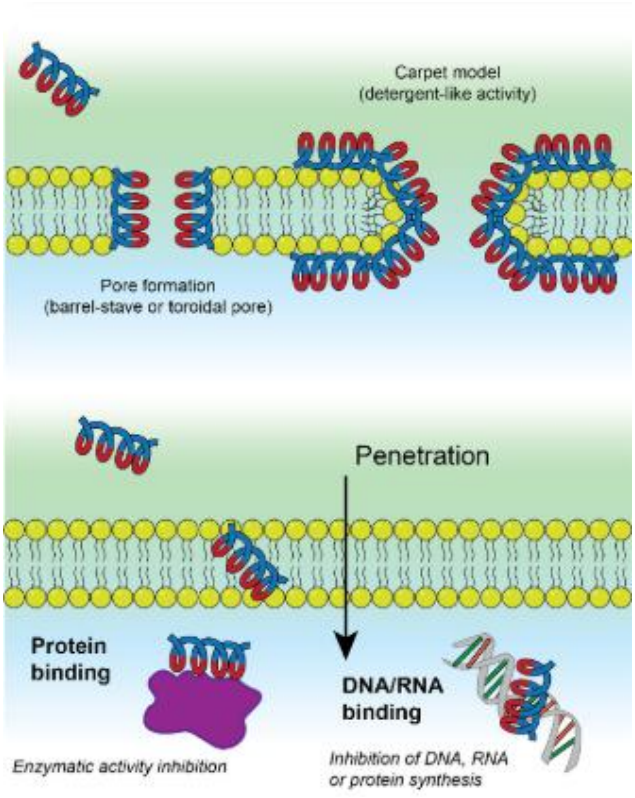

Figure 6 – AMP classification according to the molecular targets. Adapted from (Di Luca et al., 2014).

1.2.3 AMP combinations

Studies have already showed that the combination treatment of antibiotics against biofilm infection is more effective in the treatment of biofilm infections when compared with the conventional antibiotic monotherapy (Wu et al., 2015).

Nevertheless, alongside the recent finding of new antimicrobial agents, the rising growth of the antibiotic resistance demands urgert research in order to find new strategies to fight this public health concern. Researchers are trying to potentializate the action of the antimicrobial agents by searching synergic combinations between the agents. The synergism between the antimicrobial agents could actually retard the emergence of antibiotic-resistant bacteria by forcing bacteria to pass through multiple mutations so that they could become resistant. Therefore, antimicrobial combinations may be a great option as they would allow the increase of the antimicrobial spectrum, slow the emergence of the antimicrobial resistance, potentiate the efficacy of the agents when acting alone and reduce the toxicity (P. Jorge et al., 2016; Naghmouchi, Le Lay, Baah, & Drider, 2012).

Some researches actually showed that the effect of the combination of AMPs with the conventional antibiotics against multidrug-resistant bacteria generally obtained increased effects or synergy outcomes. Thus, these kind of therapies may be an open door in the treatment of multidrug-resistant bacteria infections and also allow the reduction of the dosage and the use of antibiotics in medical procedures, which would immediately decrease the risk of creating other multidrug-resistant bacteria strains (Zhang et al., 2014). Both the discovery of the adequate agents to use in the combinations as the right mechanisms of action to use to treat specific infections are some of the main challenges that remain to these studies (P. Jorge et al., 2016).

1.2.4 Challenges in the therapeutic use of antimicrobial peptides

Although all the potential properties of AMPs and development in the pre-clinical and clinical stages as promising therapeutics, more studies are required in order to identify new natural AMPs, as well as other approaches to improve their activity, as their future use suggests some limitations (Pooi Yin Chung & Khanum, 2017):

(i) in environments with biological fluids like serum and saliva, AMPs exhibit a decrease of their antimicrobial efficacy, which is explained by the high concentrations of salt, anionic proteins and polysaccharides present in biological fluids. The same does not occur in non-physiological conditions, in phosphate buffer for example;

(ii) AMPs demonstrate high levels of viscosity due to the poor physical and chemical features, such as the protein aggregation, formation and reversible self-alocation;

(iii) AMPs reveal susceptibility to proteolytic degradation;

(iv) the high production processes of AMPs are too complex (extraction of the AMP, isolation and purification) and expensive.

2. MATERIALS AND METHODS

This section presents the materials employed in the laboratory work, including the reagents, the equipment, and there is also information about each antimicrobial peptide, antibiotic and bacterial strain. The procedures applied in the susceptibility assays are also explained in detail.

2.1 Antimicrobial peptides

In this work, four different peptides were tested: tachyplesin I linear (TP-I-L) (ProteoGenix, France), magainin II (GenScript, USA), LL-37 (ChinePeptides, China), and palm-KK-NH_2 (Lipopharm.pl, Poland). The solutions were diluted in sterilized water with a concentration of 1 mg/mL and preserved in the freezer (-18 °C).

2.1.1 Tachyplesin I linear

Tachyplesin peptides were initially found in *Tachypleus tridentatus*, in 1988, and are isolated from the hemocytes of horseshoe crabs, where its concentration is really high (approximately 10 mg in the hemolymph of an individual horseshoe crab). This group of peptides, composed of 17 amino acid residues, presents unique primary structures with a solid conformation constrained by two disulfide bridges (Table 3). These molecules adopt an anti-parallel β-sheet connected by a β-turn with an amphipathic structure that might be related with its bactericidal activity. Besides, TP-I presents powerful and broad-spectrum activities against Gram-negative and Gram-positive bacteria and fungi, as it inhibits their growth and also enhances the K+ permeability of bacteria like *Staphylococcus aureus* and *Escherichia coli* (Chen & Chen, 2006; Doherty, Waring, & Hong, 2006; Matsuzaki, 1999). Evidence showed that TP-I can act on intracellular targets and interferes with intracellular functions and normal metabolism by the permeabilization of the bacterial cell membrane without disruption of the membrane, and also binds DNA and RNA, which induces the inhibition of the synthesis of macromolecules, leading to the death of bacteria (Hong et al., 2015).

The version of the peptide used in this study is a linear version of TP-I, as it does not have the two disulphide bridges, what makes its production easier and cheaper, becoming interesting to the development of this kind of peptide antibiotics. In order to ensure that there is no formation of disulphide bonds, the thiol (-SH) groups of the purchased tachyplesin I linear were protected and the cysteine residues were retained. Although this linear tachyplesin I analogue has distinct mechanisms of action, it keeps the membrane disruptive capabilities of the native peptide (P. A. da S. Jorge, 2017).

2.1.2 Magainin II

Magainins were discovered in 1987 and first isolated from the granular gland of the skin of the African clawed frog *Xenopus laevis* (Table 3). In addition to Magainin I and Magainin II, this group of AMPs also

encompasses related peptides such as Peptidyl-glycine-leucine-carboxyamide (PGLa) and the amidated forms of magainins. These molecules act selectively against microorganisms, showing a broad spectrum of antimicrobial activity against bacteria, fungi and protozoa (Matsuzaki, 1999). MAGII possibly targets the regions of the lipid bacterial membrane as it is not required any specific interaction of this peptide with chiral receptors or proteins for its antibacterial activity. This AMP, composed by 23 amino acids, is a positively charged peptide (Table 3). Therefore, by electrostatic attraction, this peptide binds selectively to the negatively charged outer monolayer of the bacterial cytoplasmic membrane, forming a α-helix at the lipid membrane interface, parallel to the membrane surface, inducing pores in the membranes (Karal, Alam, Takahashi, Levadny, & Yamazaki, 2015).

Table 3 – Sequences of amino acids of magainins and tachyplesins. Tachyplesins show unique primary structures with three repeats of a tetrapeptide: hydrophobic amino acid-Cys-aromatic, amino acid-Arg and an amidated C-terminus. Adapted from (Matsuzaki, 1999)

Peptide	Source	Primary structure
α-helical		
Magainin 1	*Xenopul laevis*	H_2N–GIGKFLHSAGKFGKAFVGEIMKS–COOH
Magainin 2		H_2N–GIGKFLHSAKKFGKAFVGEIMNS–COOH
β-sheet		
Tachyplesin I	*Tachypleus tridentatus*	
Tachyplesin II		

2.1.3 Cathelicidin LL-37

The family of cathelicidins peptides owns its name due to the C-terminal region of mature AMPs, integrating a distinctive class of proteins present in the innate immunity of mammals. These precursor molecules are capable of releasing an antimicrobial peptide after proteolytic cleavage (Dürr, Sudheendra, & Ramamoorthy, 2006). LL-37 is the only human cathelicidin-derived AMP, and is released in almost all tissues and organs, including macrophages/monocytes, neutrophils, keratinocytes and various epithelial cells. This peptide is formed by 37 amino acids with the two leading residues being leucines, and it does not have any cysteine residues. LL-37 belongs to the class of α-helical AMPs. This AMP presents a hydrophobic N-terminal domain and a net positive charge of +6 at a physiological pH (Dürr et al., 2006; Kuroda, Okumura, Isogai, & Isogai, 2015).

Studies revealed that LL-37 has an extensive variety of properties, including antimicrobial activity against several microorganisms, activation of cell proliferation, promotion of wound closure and epithelial cells migration (Figure 7). At low concentrations, LL-37 can inhibit the formation of bacterial biofilms, even in the case of microorganisms resistant to conventional antibiotics (Piktel et al., 2016).

Also, researches showed that LL-37 has an important role in complications caused by bacterial infections, particularly protecting against fatal sepsis (endotoxemia). This peptide showed to be active against pathogens like *Escherichia coli*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Patel & Akhtar, 2017).

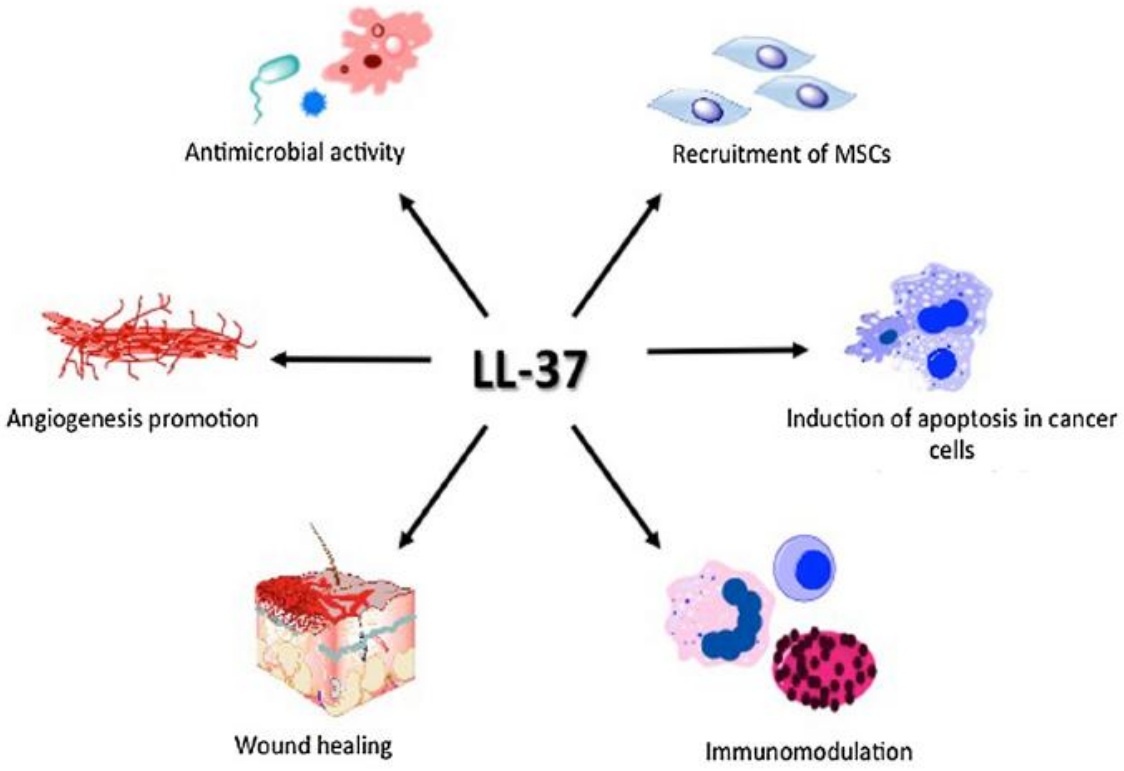

Figure 7 – The multiple properties of LL-37. MSCs: mesenchymal stromal cells. Adapted from (Piktel et al., 2016).

2.1.4 Palm-KK-NH_2

PALM is a synthetic lipopeptide known by its bactericidal and fungicidal activity (Barańska-Rybak et al., 2013). Studies demonstrated that this antimicrobial peptide revealed to be a powerful agent against Gram-positive biofilm showing activity against living forms of Gram-negative strains (Dawgul, Maciejewska, Jaskiewicz, Karafova, & Kamysz, 2014).

2.2 Antibiotics

To evaluate the susceptibility of bacteria, five distinct antibiotic agents were tested by using the disk diffusion susceptibility assay: Gentamicin (GEN), Amikacin (AK), Levofloxacin (LEV), Tobramycin (TOB) and Ciprofloxacin (CIP). These antibiotics, belonging to different families, were already reported in the EUCAST breakpoint table as agents capable of acting against *P. aeruginosa* and *S. aureus* (European Committee on Antimicrobial Susceptibility Testing, 2018). After being tested in the range of concentrations indicated in the EUCAST breakpoint table, *P. aeruginosa* was the only strain that showed resistance against two of them: CIP and LEV. Consequently, these two agents were the chosen ones to proceed the studies and check whether the combinations of both would be able to overcome this resistance or not.

Both antibiotics are Sigma-Aldrich products. Stock solutions of 1 mg/mL were prepared according to the respective documentation, and then preserved at -18 °C. Ciprofloxacin was first dissolved in HCl 0.2 M and then diluted in sterilized water. Levofloxacin was diluted in sterilized water.

2.2.1 Ciprofloxacin

Ciprofloxacin (Figure 8) is the most potent fluoroquinolone, with a broad range of activity against bacteria, where the most susceptible are the aerobic Gram-negative bacilli. This compound shows a powerful antibacterial activity, what makes it a very promising and efficacious drug (Anquetin et al., 2006). Since it was approved, it has been widely studied with more than 250 million patients successfully treated worldwide, and so its safety profile is well documented in many scientific publications. This antibiotic is also interesting as it demonstrates anti-proliferative and apoptotic activities in several cancer cell lines and induces time and dose-dependent growth inhibition and apoptosis of various carcinoma, osteosarcoma and leukemia cell lines (Herold et al., 2002). Various infections, including urinary tract infections, prostatitis, acute and chronic bacterial gonorrhoea, lower respiratory tract infections, acute sinusitis, skin and skin structure infections, bone and joint infections, complicated intra-abdominal infections and blood stream infections, are currently being treated with Ciprofloxacin ("Rationale for the EUCAST clinical breakpoints," 2007).

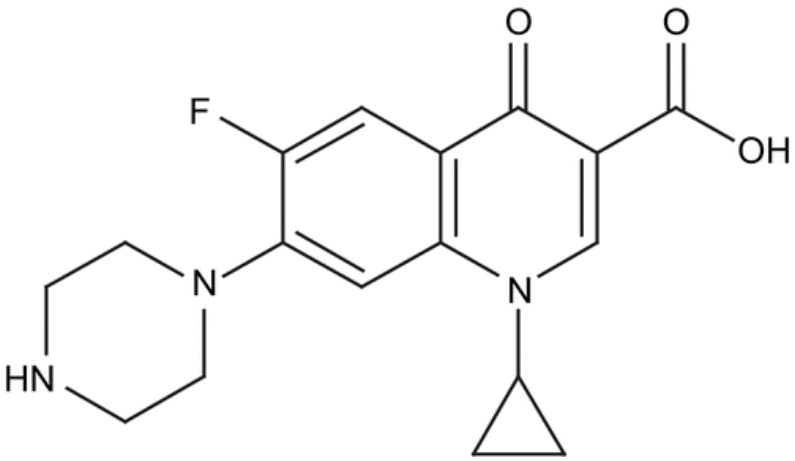

Figure 8 – Chemical structure of ciprofloxacin. Adapted from (Anquetin et al., 2006).

2.2.2 Levofloxacin

Levofloxacin (Figure 9) belongs to a third-generation fluoroquinolone that features broad-spectrum Gram-positive and Gram-negative activity, including bactericidal activity against *P. aeruginosa*. Comparing with ciprofloxacin, a second-generation fluoroquinolone, levofloxacin shows improved activity against Gram-positive organisms. This antibiotic has concentration-dependent bactericidal activity, so that the growth of the rate of bacterial killing increases with drug concentrations (Stockmann, Sherwin, Ampofo, & Spigarelli, 2014).

Because of properties like its broad-spectrum coverage against common bacterial pathogens, the high oral viability, superior tissue penetration and good tolerability, this agent can inhibit an enzyme required in the synthesis of DNA, topoisomerase, which leads to cell death (Waters & Smyth, 2015). Levofloxacin plays a role in the management of community-acquired pneumonia, acute exacerbations of chronic bronchitis, acute sinusitis, as well as bacterial infections in CF lung (Parmar, Meda, & Hennessy, 2007; "Rationale for the EUCAST clinical breakpoints," 2007).

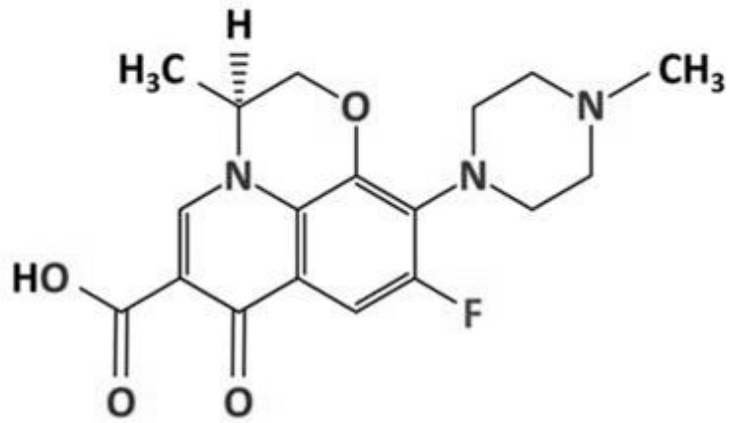

Figure 9 – Chemical structure of levofloxacin. Adapted from (Stockmann et al., 2014).

2.3 Bacteria

2.3.1 *Pseudomonas aeruginosa*

P. aeruginosa is a notorious Gram-negative bacterium that represents the major cause of nosocomial infections, affecting more than 2 million patients every year and is accounted for around 90 000 deaths annually. A significant morbidity and mortality are a consequence of these infections. This opportunistic pathogen is implicated in several biofilm-associated infections related with catheterization and intubation, urinary tract infections, gastrointestinal infections, chronic lung infection of patients with cystic fibrosis, keratitis, otitis media and bacteremia in patients with compromised host defenses. Moreover, both *P. aeruginosa* and *S. aureus* are recognized to be major pathogens associated with ventilator associated pneumonia, a respiratory infection caused by bacteria found on ventilator tubes that likely exposes vulnerable patients to a critical biofilm infection, where the biofilm mode of growth represents the main reason for the antibiotic treatment failure, as it allows the bacteria to survive for decades in the lung (Ciofu, Mandsberg, Wang, & Høiby, 2012; Joo & Otto, 2012; Morita, Tomida, & Kawamura, 2014; Mulcahy, Isabella, & Lewis, 2014).

Nowadays, the presence of *P. aeruginosa* in hospital infections is becoming more and more significant. This metabolic flexible microorganism, pathogenic due to its multiple virulence factors, can grow under both aerobic and anaerobic conditions. Some properties of these bacteria, including the outer-membrane barrier, the presence of multidrug efflux transporters and endogenous antimicrobial inactivation, contribute to their intrinsic resistance to many antimicrobials (Morita et al., 2014). *P. aeruginosa* has multiple mechanisms of response and resistance to antimicrobials, acquiring resistance to individual agents via chromosomal mutations and lateral gene transfer. This organism is intrinsically resistant to β-lactams (penicillins, cephalosporins and carbapenems, except for imipenem) and many other antimicrobial agents, such as fluoroquinolones, aminoglycosides, polymyxins and macrolides. *P. aeruginosa* infections are frequently treated with fluoroquinolones, especially ciprofloxacin (Morita et al., 2014).

2.3.2 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterium that colonizes the nostrils and skin of approximately 30 % of the population. As well as other bacterial pathogens, *S. aureus* can grow as free-floating planktonic cells or as a biofilm, and can be found in 80 % of nosocomial infections, including

acute infections, such as bacteremia and skin abscesses, usually caused by planktonic cells. On the other hand, *S. aureus* biofilms can also cause chronic infections, by attaching and persisting on host tissues (for example, osteomyelitis, pneumonia, bacteremia and endocarditis) (Lister & Horswill, 2014). This opportunistic pathogen is a common human commensal that has become notorious for causing infections by the colonization of indwelling medical devices, including catheters, pacemakers, artificial joints and artificial heart valves, where they become coated with host proteins upon insertion. The matrix-binding proteins on the surface of *S. aureus* favor the attachment to these proteins and the development of a biofilm in these materials. Periodontitis and peri-implantitis, chronic wound infections, chronic rhinosinusitis, and ocular infections are other examples of diseases related with *S. aureus* biofilm formation. Thanks to the range of virulence factors of this pathogen, such as adhesins, toxins and immune evasion proteins, it is capable to develop in multiple places within the body (Crosby, Kwiecinski, & Horswill, 2016; Lister & Horswill, 2014).Therefore, as the infected indwelling devices usually require surgical removal and more time of hospitalization, there has been a raise of the morbidity and mortality that are directly associated to biofilm-related infections and, consequently, more and more expenses related to staphylococcal infections. Thus, in order to reduce the costs associated to *S. aureus* and also to assist the development of new therapeutic strategies, it is urgent a deeper understanding of how this pathogen interacts with the host (Moormeier & Bayles, 2017).

In therapy, β-lactams, fluoroquinolones, aminoglycosides and glycopeptides agents are commonly used. Current therapies are confined to surgical intervention and prolonged antibiotic regiments or addition of antimicrobial compounds to implantable medical devices. Furthermore, studies have been considering the development of an effective vaccine against *S. aureus* biofilm infection by taking into account the selection of the appropriate antigens (Archer et al., 2011).

2.4 Bacterial strains and growth conditions

The reference strains utilized in this work include *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* ATCC 25923. The CI, which are strains isolated from real-life infections, included *P. aeruginosa* U147016-1, which was isolated from an urinary infection at the S. Marcos Hospital in Braga, Portugal, and *S. aureus* GB 2/1, isolated from explanted voice prostheses at the University Medical Center of Groningen, Netherlands.

The media used for bacterial cryopreservation was Tryptic Soy Broth (TSB) (30 g/L) with 20 % glycerol, and all bacteria strains were preserved in storage at -80 °C. Cells were streaked on a TSA plate (30 g/L TSB + 12 g/L Agar) and then grown overnight at 37 °C, when they were necessary.

For planktonic growth, the strains were inoculated in 20 mL of Mueller Hinton Broth (MHB) (21 g/L). Likewise, for biofilm culturing, each strain was inoculated in 20 mL of TSB (30 g/L). After that, the inoculums were incubated overnight (37 °C, 120 rpm) and centrifuged (9000 × g, room temperature, 5 min). The cultures were then re-suspended in MHB or TSB and diluted in order to obtain a concentration of $1x10^6$ CFU/mL or $2x10^6$ CFU/mL, for planktonic and biofilm culturing, respectively. To do this, the optical density (OD) was measured at 620 nm and the cells' concentration was calculated using calibration curves previously established within the group (Table 4).

Table 4 – Calibration curves for the bacterial strains used in this work

Microorganism	Strain	Calibration curve
P. aeruginosa	PAO1	$\mathrm{CFU/mL} = 4 \times 10^9 \times \mathrm{OD}_{620\,\mathrm{nm}} - 1 \times 10^8$
	CI (U147016-1)	$\mathrm{CFU/mL} = 4 \times 10^9 \times \mathrm{OD}_{620\,\mathrm{nm}} - 1 \times 10^8$
S. aureus	ATCC 25923	$\mathrm{CFU/mL} = 9 \times 10^8 \times \mathrm{OD}_{620\,\mathrm{nm}} + 7 \times 10^7$
	CI (GB 2/1)	$\mathrm{CFU/mL} = 4 \times 10^9 \times \mathrm{OD}_{620\,\mathrm{nm}} - 3 \times 10^8$

2.5 Susceptibility assays in planktonic cultures

2.5.1 Disk diffusion susceptibility assay

To determinate the susceptibility profile of bacteria to the chosen antibiotics, antimicrobial susceptibility test discs were used, applying the *in vitro* disk diffusion method. Five different antibiotics common to both bacteria, *P. aeruginosa* and *S. aureus,* were tested: LEV and CIP, belonging to the fluoroquinolones family; and GEN, AK and TOB, which are part of the aminoglycosides class. Initial inoculums with a cell concentration of 1.5 x 10^8 CFU/mL, corresponding to 0.5 McFarland barium sulphate standard, were prepared and spread with a sterile swab as evenly as possible in prepared plates with Mueller-Hinton Agar (MHA) (MHB + Agar 17g/L). With the aid of sterilized tweezers, antimicrobial susceptibility test discs were then placed in the plates with the inoculated medium. After incubation (37 °C, 18h), the diameters of the inhibition zones surrounding the discs were measured in millimeters by visual inspection with the help of a ruler. The obtained values were compared with the

ones set on EUCAST Breakpoint Tables, established for interpretation of MICs and zone diameters to detect whether the bacteria are resistant or susceptible to the antibiotic (Figure 10).

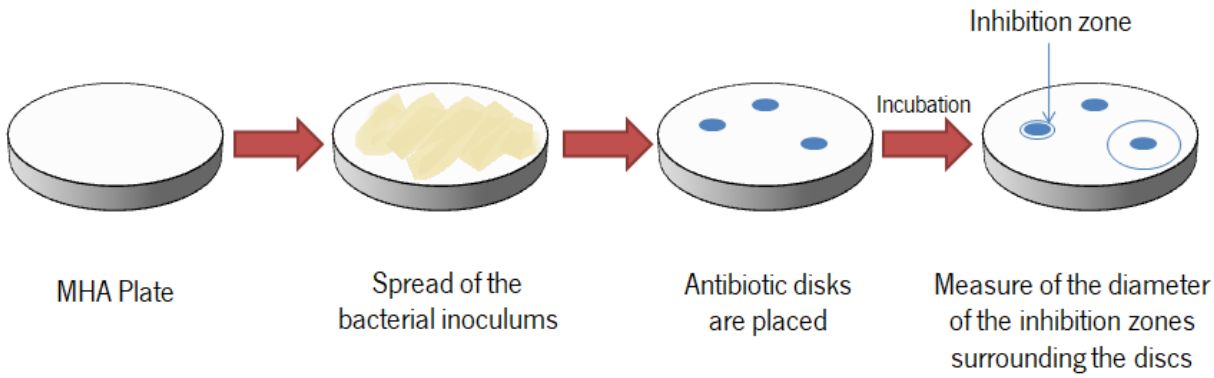

Figure 10 – Antimicrobial drug susceptibility test by disk diffusion method.

2.5.2 Planktonic susceptibility assays of single AMP and antibiotics

The effect of single agents on planktonic cultures was measured by the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). This was determined following the Clinical and Laboratory Standards Institute (CLSI) method by broth microdilution (Figure 11). Basically, 100 µL of sequential dilutions of the single agents in MHB with 100 µL of the prepared bacterial suspensions were put in a round bottom 96-well polystyrene (PS) microtiter plate (Orange Scientific), where both positive (no agent) and negative (no cells and no agent) controls were included. The microtiter plate was then incubated (37 °C, 120 rpm, 24 h) in an orbital shaker (N-Biotek Shaker & Incubator NB-205Q). The growth of the bacterial cells was set by measuring absorbance (Abs) at 620 nm. Thus, the values of the lowest concentrations able to decrease by 99 % the growth of the bacterial cells set the MIC. In the determination of the MBC, 10 µL from each well with no visible growth were added to a TSA plate. After incubation (37 °C, 24 h), the MBC was determined as the values where there was no colony growth, which coincide to the lowest concentrations able to reduce over 99.9 % of the number of viable cells. All the assays were performed at least three times, with two replicates each.

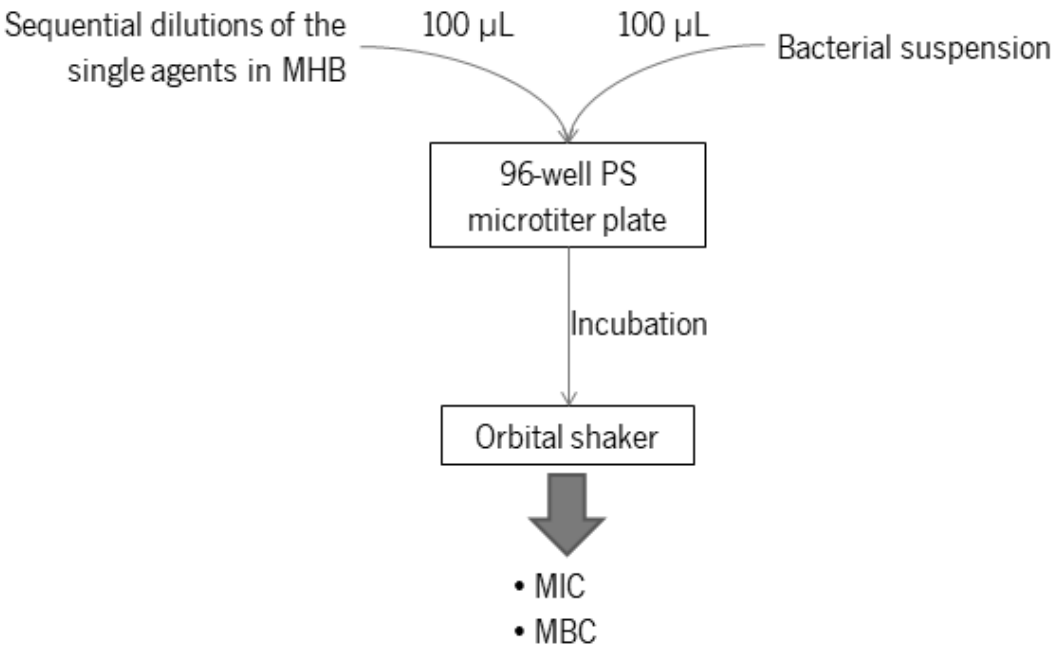

Figure 11 – Diagram representation of broth microdilution method.

2.5.3 Planktonic susceptibility assays of combined AMP and antibiotics

The effect of combined agents on planktonic bacterial cells was measured by the checkerboard microdilution assay (Figure 12). In this test, agents were paired in serial two-fold increasing concentrations, until just below the MIC, following the same conditions previously referred for the MIC and MBC assays. This test measures the *in vitro* interaction of two antimicrobials, identifying whether the antimicrobial combinations can act additively, when the cumulative antimicrobial effect is the total sum of the two antimicrobials acting together, or synergistically, when the effect of the two antimicrobials is greater than the sum of their individual activities. On the other hand, these methods can also identify both antagonistic and indifferent combinations (Doern, 2014).

To evaluate the effectiveness analysis of agent combinations in planktonic bacterial cells, the fractional inhibitory concentration index (FICI) and the fractional bactericidal concentration index (FBCI) were calculated in order to verify whether there was synergy, additiveness, indifference or antagonism between the agents. All the assays were performed with two replicates, at least three times.

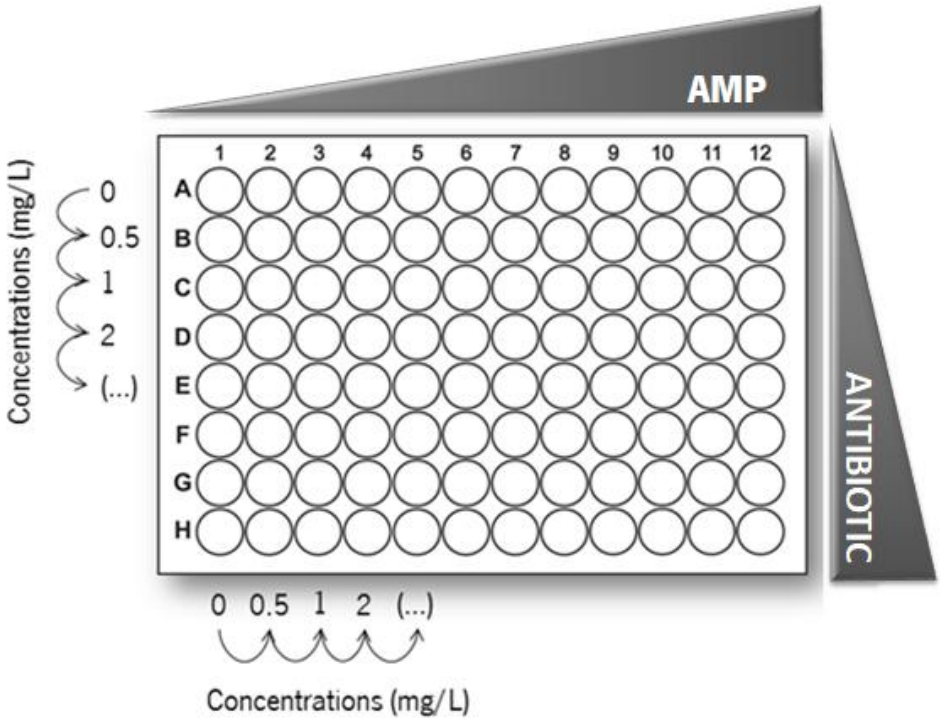

Figure 12 – Illustration of the checkerboard microdilution assay. Adapted from (P. A. da S. Jorge, 2017).

The range of concentrations of the agents used in these assays varied from 0 μg/mL to the respective MIC obtained in the previous assays (planktonic susceptibility assays of single AMPs and antibiotics). Thus, through the equations mentioned below (Equation 1, Equation 2), both MIC and MBC of each individual agent (A_{alone} and B_{alone}), which were determined through the broth microdilution method, were compared with the MIC and MBC obtained for the agent combination ($A_{comb\ A/B}$ and $B_{comb\ A/B}$).

$$FICI = FIC_A + FIC_B = \frac{MIC\ (A_{comb\ A/B})}{MIC\ (A_{alone})} + \frac{MIC\ (B_{comb\ A/B})}{MIC\ (B_{alone})} \qquad \text{(Equation 1)}$$

$$FBCI = FBC_A + FBC_B = \frac{MBC\ (A_{comb\ A/B})}{MBC\ (A_{alone})} + \frac{MBC\ (B_{comb\ A/B})}{MBC\ (B_{alone})} \qquad \text{(Equation 2)}$$

The breakpoint values for FICI and FBCI were read as : 'synergy (S)' (FICI or FBCI ≤ 0.5), 'additiveness (Ad)' (0.5 < FICI or FBCI ≤ 1), 'indifference (I)' (1 < FICI or FBCI ≤ 4) and 'antagonism (A)' (FICI or FBCI > 4.0) (Saiman, 2007).

2.6 Biofilm susceptibility

To evaluate biofilm susceptibility, the study was split into two phases. In a preliminary test, the agents were applied to the different biofilms in order to verify how each agent acts when it is applied alone. The results of this assay helped in the choice of the agent concentrations to be used in the combination tests. In the second phase, the agents were applied in combination on grown biofilms.

2.6.1 Combined agents on grown biofilms

Biofilms were grown following the modified microtiter plate test proposed by Stepanović et al. (Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000) (Figure 13). Basically, 200 µL of each bacterial suspension ($1x10^6$ CFU/mL) were added to a flat-bottom 96-well PS microtiter plate. Then, in order to promote biofilm formation, the plates were incubated (37 °C, 120 rpm, 24 h) in an orbital shaker. Afterwards, the plates were inverted to remove the content of each well. To remove planktonic cells, the wells were washed twice with 200 µL of distilled sterile water.

The agents were tested alone and in combination by adding 200 µL of each solution diluted in TSB to each well. Then, the plates were incubated aerobically (37 °C, 120 rpm) in an orbital shaker.

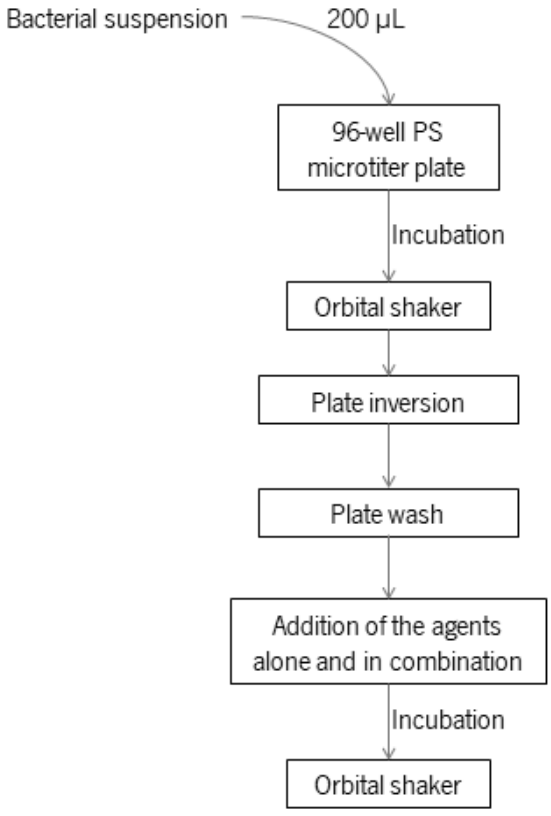

Figure 13 – Diagram representation of the modified microtiter plate test proposed by Stepanović et al..

2.6.2 Cell viability

In order to study the viability of the biofilm cells treated with the combinations, biofilms were detached from the wells by sonication (6 min). The solutions relative to each condition (control, agents, combination of the agents) were vortexed for 30 s and then serially diluted in distilled sterile water. Afterwards, the resulted dilutions were plated on TSA and incubated in an orbital shaker at 37 °C overnight, in order to count *P. aeruginosa* and *S. aureus* CFU (Figure 14).

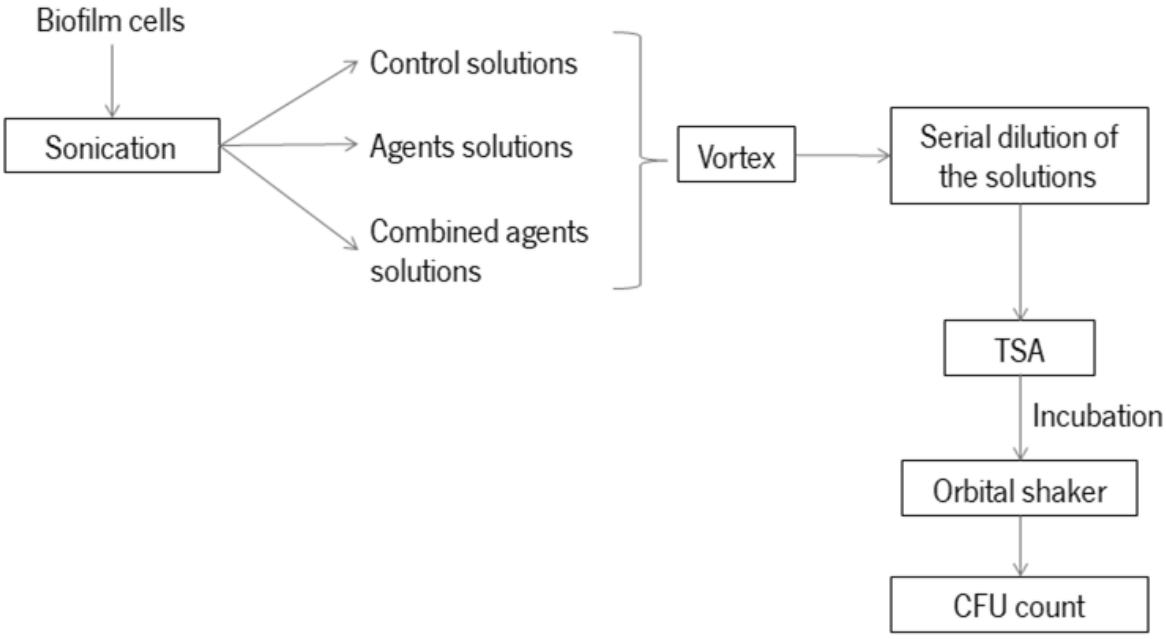

Figure 14 – Diagram representation of procedure adopted to CFU count.

The number of viable biofilm-cells was obtained by the following equation (Equation 3):

$$CFU/cm^2 = \log_{10} \frac{200\mu L \times no.of\ colonies \times dilution\ factor}{10\ \mu L \times 1.53\ cm^2} \qquad \text{(Equation 3)}$$

where 200 µL correspond to the volume of the cells used for biofilm growth, 10 µL to the plated volume of cell solution and 1.53 cm^2 to the surface area of the well occupied by 200 µL and available for biofilm adherence.

2.6.3 Analysis of combined agents on grown biofilms

The effectiveness of combined agents on grown biofilms was measured through the comparison between the action of the most active agent alone and the sum of the individual actions with the action

of the agent combinations. Considering the methodology adopted in a previous study (P. Jorge, Grzywacz, Kamysz, Lourenço, & Pereira, 2017), the analysis of the combined agents was assessed by two different methods (Figure 15).

One of the methodologies is based on the statistical differences verified in cell viability data. In this method, outcomes are concluded as: 'S' – the combined action is superior to the sum of the individual actions; 'Ad' – the combined action is equal to the sum of the individual actions; 'I' – the combined action is equal to the action of the most active agent alone; 'A' – the combined action is inferior to the action of the most active agent alone.

In the other approach, based on the biological significance analysis, there is a comparison based on cell viability between the log reductions of the combinations and that of the most active isolated agent, where the outcomes are defined as: 'S' – ≥ 2 log decrease; 'Ad' – 1 ≤ log < 2 decrease; 'I' – < 1 log decrease; 'A' – ≥ 2 log increase (White, Burgess, Manduru, & Bosso, 1996). All the assays were performed with two replicates, at least three times.

The analysis of the activity of the combined agents in biofilms included the analysis of variance (ANOVA) followed by the Bonferroni's correction method in multiple comparison test for chosen pairs of means. All the assays were realized at least three times. All statistical tests were performed in Graphpad Prism 7.0.

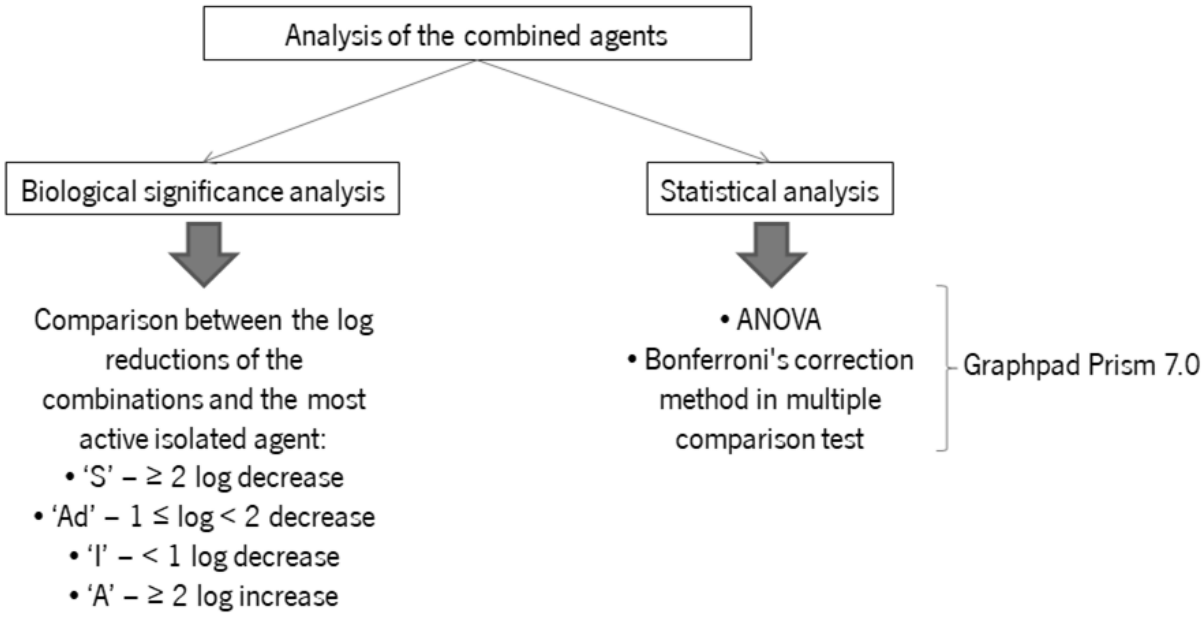

Figure 15 – Representation of the methodology used to evaluate the effectiveness of combined agents on grown biofilms.

3. RESULTS AND DISCUSSION

In this section, the main results obtained in the different assays performed in this work, as well as the discussion related to them are presented.

3.1 Disk diffusion susceptibility assay

The disk diffusion susceptibility assay provided the determination of the susceptibility profile of bacteria to the antibiotics. One preliminary test was done in order to help in the choice of the antibiotics to continue the studies, following the methodology described in sub-chapter 2.5.1.

All the results of the antimicrobial activity obtained in the disk diffusion assay for *P. aeruginosa* PAO1, *P. aeruginosa* CI, *S. aureus* ATCC, and *S. aureus* CI are resumed in Table 5. Through the analysis of this table, it is notable that *P. aeruginosa* CI is the only strain revealing resistance against two of the antibiotics, CIP and LEV, which means that this strain is not inhibited by the antimicrobial concentrations used and there is a great chance of therapeutic failure. The other strains showed to be susceptible against all the antibiotics tested, that is, the infection caused by the isolated bacteria can be adequately treated with a recommended dosage and the antimicrobial can be used for the treatment. Therefore, as all bacteria revealed to be susceptible against GEN, AK and TOB, CIP and LEV were selected to proceed with the experiments. These antibiotics were chosen in order to verify whether their combination with the AMPs would be beneficial when used against the bacteria, by helping to overcome the resistance showed by *P. aeruginosa* CI against these agents. Thus, as *P. aeruginosa* CI is not susceptible to CIP and LEV, the aim is to try to turn this strain susceptible to the combinations of these antibiotics with the AMPs so that the bacterial infections could be properly treated with the right dosage and the combined agents could be used in the treatment.

Table 5 – Outcomes obtained for P. aeruginosa and S. aureus in the disk diffusion assay

Strain	Antibiotic	Zone diameter breakpoint (mm)		Mean diameter (mm)	Outcome
		S ≥	R <		
***P. aeruginosa* PAO1**	GEN	15	15	19.50 ± 0.35	Sp
	AK	18	15	23.50 ± 0.35	Sp
	LEV	22	22	26 ± 0.00	Sp
	TOB	16	16	23.50 ± 0.35	Sp
	CIP	26	26	41.00 ± 0.71	Sp
***P. aeruginosa* CI**	GEN	15	15	18.50 ± 0.35	Sp
	AK	18	15	20.50 ± 1.77	Sp
	LEV	22	22	ND	R
	TOB	26	16	24 ± 0.00	Sp
	CIP	26	26	16.50 ± 0.35	R

Table 6 – Outcomes obtained for P. aeruginosa and S. aureus in the disk diffusion assay (continuation)

***S. aureus* ATCC 25923**	GEN	18	18	23 ± 0.00	Sp
	AK	18	16	24 ± 0.00	Sp
	LEV	22	22	27.50 ± 0.35	Sp
	TOB	18	18	26.00 ± 0.71	Sp
	CIP	21	21	31 ± 0.00	Sp
***S. aureus* CI**	GEN	18	18	28.50 ± 0.35	Sp
	AK	18	16	31 ± 0.00	Sp
	LEV	22	22	35.50 ± 0.35	Sp
	TOB	18	18	34 ± 0.00	Sp
	CIP	21	21	36.50 ± 0.35	Sp

Note: ND – Not Detected; Sp – Susceptible; R – Resistant.

3.2 Planktonic susceptibility assays of single AMP and antibiotics

In this part of the work, the antimicrobial activity of the AMPs and antibiotics was tested on planktonic cultures of *P. aeruginosa* and *S. aureus* through the determination of the MIC, which prevents the visible growth of bacteria, and also the MBC, to verify the lowest concentration of each antibacterial agent required to kill the bacteria. All the assays followed the procedure described in sub-chapter 2.5.2.

The antimicrobial activity of the AMPs was assessed and compared between each other. Table 7 resumes the outcomes obtained for planktonic bacteria with TP-I-L, MAGII, LL-37 and PALM, where the MIC values were generally equal or lower than the MBC values. Only PALM revealed higher values of MIC against the *S. aureus* strains. The agents showed a different range of inhibitory values. TP-I-L was the one that obtained the best results against all the strains, with MICs ranging from 4 μg/mL to 16 μg/mL and MBCs of 32 μg/mL to 128 μg/mL. PALM has also demonstrated some antimicrobial action, with MICs of 64 μg/mL to >128 μg/mL, and MBCs of 128 μg/mL. However, it was less effective against *S. aureus* (MBC and MIC values ≥128 μg/mL). MAGII and LL-37 showed to be inactive against every tested strain (MICs and MBCs >128 μg/mL). Overall, comparing the results obtained for all the strains, the peptides that have exhibited some antimicrobial action (TP-I-L and PALM) showed a stronger antimicrobial activity against *P. aeruginosa* strains.

Considering the susceptibility profiles of the reference strain *P. aeruginosa* PAO1 and the CI, there were noticed very little differences, which were only observed in TP-I-L and PALM. Furthermore, the only difference between the reference strain *S. aureus* ATCC 25923 and the CI was verified in the

antimicrobial activity of TP-I-L. *S. aureus* CI revealed lower values of MIC and MBC for TP-I-L, which means that this peptide has better bactericidal and bacteriostatic effects against the CI strain of *S. aureus* comparing with the reference strain.

Table 7 – Antimicrobial activity of TP-I-L, MAGII, LL-37 and PALM obtained for planktonic P. aeruginosa and S. aureus

	TP-I-L		**MAGII**		**LL-37**		**PALM**	
Strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
***P. aeruginosa* PAO1**	8	32	>128	>128	>128	>128	64	128
***P. aeruginosa* CI**	16	32-128	>128	>128	>128	>128	128	128
***S. aureus* ATCC 25923**	8	64	>128	>128	>128	>128	128	128
***S. aureus* CI**	4	32	>128	>128	>128	>128	128	128

Note: MIC and MBC values are in µg/mL.

Taking into account all data obtained, TP-I-L was the AMP with the best bactericidal and bacteriostatic effects against all the strains. Therefore, as this AMP was the one revealing the most positive results, it was selected to be carried on to the next phases of the study, where it will be tested in combination with the chosen antibiotics against *P. aeruginosa* and *S. aureus* grown biofilms.

Then, the antimicrobial activity of the antibiotics was studied. As shown in Table 8, both CIP and LEV revealed potent antimicrobial activities against most strains, with MICs of 0.125 µg/mL to 0.25 µg/mL. The MBC values for these antibiotics ranged from 0.125 µg/mL to 1 µg/mL. Only *P. aeruginosa* CI demonstrated lower susceptibility by showing a MIC of 4 µg/mL for both antibiotics, MBC of 8 µg/mL for CIP and 32 µg/mL for LEV. Between the two, CIP revealed better bactericidal effect against all the strains, except for *S. aureus* ATCC, where the MBC was the same for both agents. However, the antibiotics showed similar bacteriostatic effects, with the exception of *S. aureus* ATCC, where LEV demonstrated a superior effect.

Table 8 – Antimicrobial activity of CIP and LEV obtained for planktonic P. aeruginosa and S. aureus

	CIP			**LEV**		
Strain	MIC	MIC Interpretation	MBC	MIC	MIC Interpretation	MBC
***P. aeruginosa* PAO1**	0.125	Sp	0.125	0.125	Sp	0.5
***P. aeruginosa* CI**	4	R	8	4	R	32
***S. aureus* ATCC 25923**	0.25	Sp	0.5	0.125	Sp	0.5
***S. aureus* CI**	0.25	Sp	0.25	0.25	Sp	1

Note: MIC and MBC values are in µg/mL. Sp – Susceptible; R – Resistant.

Comparing the susceptibilities profiles of the reference strain *S. aureus* ATCC 25923 and the CI, it was verified little difference between them. However, the same did not succeed with *P. aeruginosa*, where the differences between the reference strain and the CI were notable. Considering the outcomes obtained in the disk diffusion assay, where *P. aeruginosa* CI was the only revealing resistance against both antibiotics while the other strains were susceptible to them, it was expectable that this strain would hold the highest values of MIC and MBC. Thus, both CIP and LEV demonstrated better bactericidal and bacteriostatic effects against the reference strain of *P. aeruginosa*. Consequently, it is expectable that the CI is harder to eradicate than the reference strain and, thus, it is probably necessary to administrate a higher concentration of antibiotic to kill the bacteria.

In general, the antibiotics revealed a stronger antimicrobial activity against *S. aureus* strains. As succeeded with the AMPs, the MIC values were also equal or lower than the MBC values.

In a global analysis of both AMPs and antibiotics effectiveness against planktonic bacteria, there were observed lower values of MIC for conventional antibiotics than those for AMPs, which suggests a stronger antimicrobial activity of the conventional therapy.

3.3 Planktonic susceptibility assays of AMP combined with antibiotics

Combinations of AMP with antibiotics were tested on planktonic cultures of *P. aeruginosa* and *S. aureus* by applying the checkerboard assay. Combination results were estimated through FICI and FBCI determination, which provides information about the relation that exists between the agents, whether there is synergy, additiveness, indifference or antagonism between them. These assays were performed according to the procedure described in sub-chapter 2.5.3.

presents the best outcomes for the agent combinations obtained for both bacteria, for the pair of combinations of the agents TP-I-L with LEV and TP-I-L with CIP (the outcomes obtained for all the combinations tested are illustrated in Appendix I). The FICI and the FBCI values revealed that almost all the strains were not very susceptible to the action of the combinations. The FBCI values seem to indicate more positive outcomes as they showed some additiveness activity against most strains, condition where the cumulative antimicrobial effect corresponds to the sum total of two agents acting together. These conditions, represented by the mentioned combinations, denote that the action of the agents acting together can kill the bacteria (bactericidal effect). The combination of the AMP with LEV demonstrated additive outcomes against all the strains, except for *S. aureus* CI where there were not detected any bactericidal effects. However, the combination of TP-I-L with CIP only showed additive

outcomes when acting against *S. aureus* strains and was not detected any bactericidal activity in the range of the tested concentrations against *P. aeruginosa* strains.

Table 9 – Best outcomes for the combinations of TP-I-L with LEV and CIP against planktonic P. aeruginosa and S. aureus

Strain	TP-I-L + LEV (MIC)	Outcome		TP-I-L + CIP (MIC)	Outcome	
		FICI	FBCI		FICI	FBCI
***P. aeruginosa* PAO1**	16 + 0.25	I	Ad	1 + 0.125	I	ND
***P. aeruginosa* CI**	8 + 8	Ad	Ad	1 + 4	I	ND
***S. aureus* CI**	1 + 1	I	ND	16 + 0.125	I	Ad
***S. aureus* ATCC 25923**	16 + 0.25	I	Ad	16 + 0.25	I	Ad

Note: MIC values are in µg/mL. ND – Not detected in the range of concentrations tested; Ad – Additiveness; I – Indifference.

On the other hand, the FICI values revealed indifference outcomes in both combinations against all the strains, which means that the action of the combinations tested was similar to the action of the most active single agent and there was no bacteriostatic effect, this is the agents are not capable of inhibiting the growth or reproduction of bacteria.

In the range of concentrations tested, the reference strain *S. aureus* ATCC 25923 was the only strain in which both bacteriostatic and bactericidal outcomes for both combinations were detected. When acting against *P. aeruginosa* CI, the combination of TP-I-L with LEV (8 µg/mL TP-I-L + 8 µg/mL LEV) was the one that revealed both bacteriostatic and bactericidal activity. Also, there were no synergistic or antagonistic interactions. Although there are no synergic activities, additive combinations can still be useful. As these combinations enable the use of lower concentrations, they would be helpful in the expansion of the antimicrobial spectrum, as well as in the reduction of the emergence of resistance and toxicity (Saiman, 2007).

Concerning the antibiotics tested in combination with TP-I-L, Table 9 reveals that LEV was the one that obtained the most positive outcomes by showing more activity against the strains. Therefore, both agents TP-I-L and LEV were chosen to be further tested against grown biofilms of the four strains *P. aeruginosa* CI, *P. aeruginosa* PAO1, *S. aureus* CI and *S. aureus* ATCC 25923.

3.4 Biofilm susceptibility

Simulating a therapeutic approach, the combined agents were tested in 24 h-old biofilms and were analyzed according to the cell viability. In this part of the work, biofilm susceptibility was tested by splitting the study into two phases: a preliminary test, where both agents (TP-I-L and LEV) were applied to the different biofilms alone to assist in the choice of the agent concentrations to be used in the combination tests; and a combination test that encompassed a set of assays where the agents were applied in combination on grown biofilms.

3.4.1 Application of TP-I-L and LEV alone on grown biofilms of *P. aeruginosa* and *S. aureus*

Biofilms were evaluated in terms of cell viability following the procedure related in sub-chapter 2.6.2. In this assay, which encompassed the CFU count, the agents TP-I-L and LEV were tested on grown biofilms of *P. aeruginosa* and *S. aureus*.

Figure 16 illustrates the outcomes obtained in the biofilm susceptibility test for TP-I-L and LEV in the range of concentrations of 0–128 μg/mL on grown biofilms of *P. aeruginosa* and *S. aureus*. The illustrated graphics show the variation of the number of viable cells according to the concentration of each agent, for each strain. It is evident that, for both TP-I-L and LEV, there are no typical dose-response curves, and that these vary depending on the agent and the strain. The behavior of the strains was analysed in order to find common concentrations for all the strains where it was evident some decrease on the number of cells. Thus, results show that the number of viable biofilm-cells start to decrease in the range of concentrations of 16–32 μg/mL for LEV against all the strains. For TP-I-L, the number of viable biofilm-cells reduction is only verified in the concentrations of 64–128 μg/mL. Furthermore, in the subsequent combination test – where the combined agents are applied on grown biofilms of *P. aeruginosa* and *S. aureus* – two distinct combinations were chosen: 16 μg/mL LEV with 128 μg/mL TP-I-L and 32 μg/mL LEV with 128 μg/mL TP-I-L.

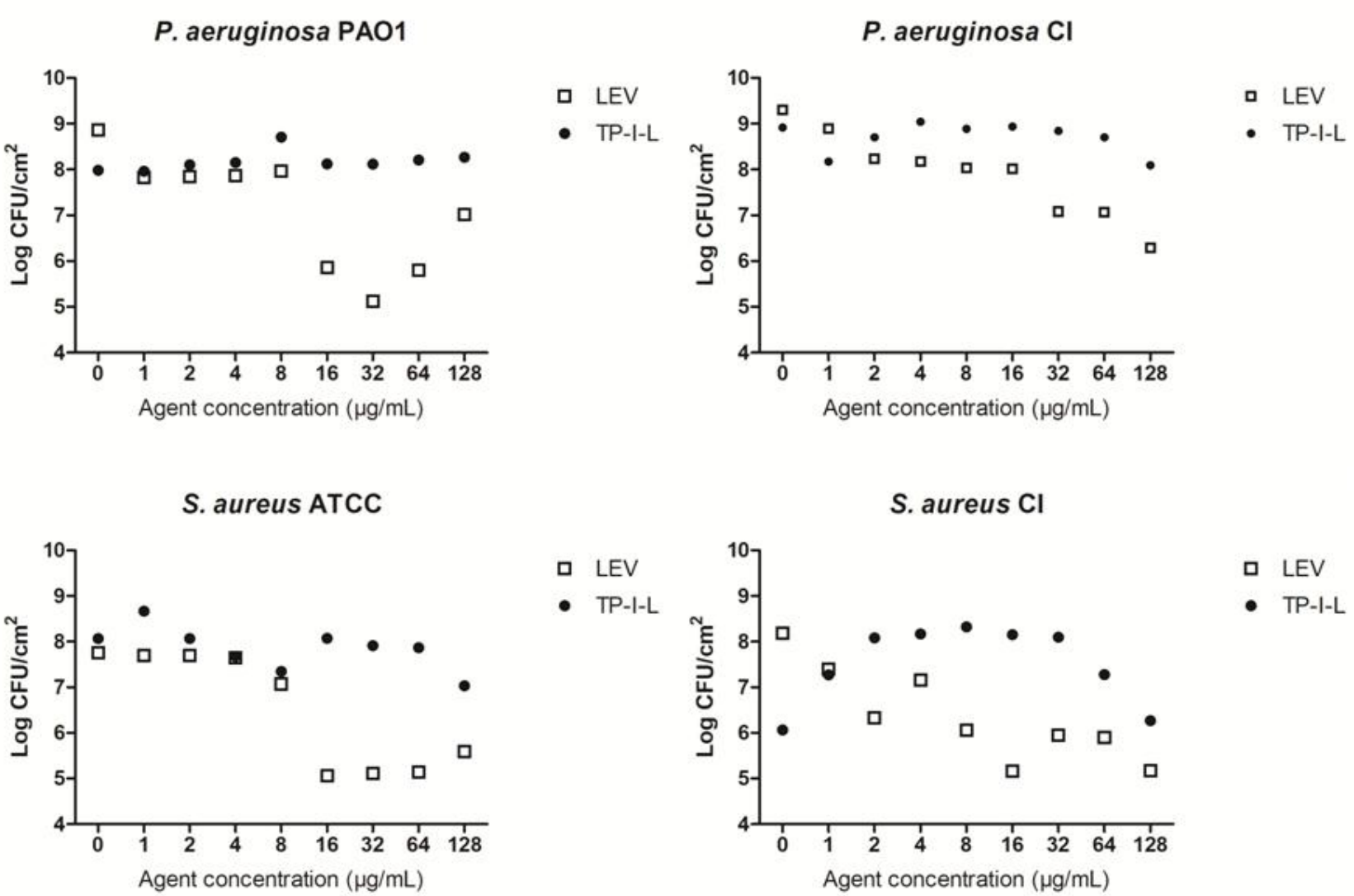

Figure 16 – Results obtained for the agents tested on grown biofilms of P. aeruginosa and S. aureus.

Comparing the results obtained in planktonic and biofilm susceptibility assays, there is an evident increase in the effective concentrations of both agents. TP-I-L varied from a range of concentrations of 4–8 μg/mL in planktonic to 64–128 μg/mL in biofilm. In its turn, LEV demonstrated a variation of 0.125–4 μg/mL in planktonic to 16–32 μg/mL in biofilm. With these evident differences, it is plausible to consider that biofilms are likely more resistant to TP-I-L and LEV when compared with the planktonic mode of growth. In fact, since planktonic bacteria are usually easier to eradicate than bacteria living in biofilm, it is acceptable that the concentrations in biofilm required to its eradication need to be higher than the respective MIC values as planktonic bacteria. Also, after the 24h incubation period, and at the time of treatment application, the number of cells in the biofilms becomes higher than the number of starting cells in planktonic. This can be confirmed in Figure 16, where the number of cells when there is no agent (the concentration of the agent is 0 μg/mL) is higher than 10^6 CFU/mL.

Therefore, this preliminary test was useful to check how TP-I-L and LEV act when applied alone on grown biofilms of *P. aeruginosa* and *S. aureus*, and thus choose the best concentrations of the agents to use in the combination test.

3.4.2 Application of combined agents on grown biofilms of *P. aeruginosa* and *S. aureus*

The outcomes of the combined agents were analyzed as described in sub-chapter 2.6.3. In therapeutic approaches, the effectiveness of agent combinations can be assessed by comparing the action of the individual agents with the action of the combinations. Considering the methodology adopted in a previous study (P. Jorge et al., 2017), the analysis of the combined agents was performed following two distinct approaches: biological significance analysis and statistical analysis. In this test, the agents were applied in combination on grown biofilms of *P. aeruginosa* and *S. aureus*, with a time of treatment of 24h.

Table 10 presents the best outcomes obtained in the analysis of the effectiveness of the agent combinations on grown biofilms of *P. aeruginosa* and *S. aureus* (detailed outcomes are shown in Appendix II). This analysis was performed through the estimation and comparison of the log reduction of each combination and of each isolated agent. Table 9 presents the mean log reductions of all the assays performed, with combination log reductions ranging from 1.17 CFU/cm^2 to 5.30 CFU/cm^2. Despite the high mean standard deviations presented in Table 9, in the analysis of the results, the variability between the different assays was taken into account, since the outcomes were analyzed separately for each test, in order to ensure that related datasets were being compared.

Table 10 – Analysis of the effectiveness of combined agents on grown biofilms

Strain	Combined agents	Log reduction TP-I-L (CFU/cm²)	Log reduction LEV (CFU/cm²)	Log reduction combination (CFU/cm²)	Biological significance analysis	Statistical analysis
***P. aeruginosa* PAO1**	TP-I-L 128 µg/mL + LEV 16 µg/mL	0.41 ± 0.54	1.84 ± 1.25	3.07 ± 1.17	Ad	S
	TP-I-L 128 µg/mL + LEV 32 µg/mL		4.56 ± 0.99	5.11 ± 0.83	I/Ad	I
***P. aeruginosa* CI**	TP-I-L 128 µg/mL + LEV 16 µg/mL	0.45 ± 0.39	0.78 ± 0.94	1.17 ± 1.51	I	I
	TP-I-L 128 µg/mL + LEV 32 µg/mL		1.45 ± 0.4	3.19 ± 0.83	Ad	S
***S. aureus* ATCC 25923**	TP-I-L 128 µg/mL + LEV 16 µg/mL	0.57 ± 0.38	1.60 ± 0.54	3.55 ± 0.69	S	S
	TP-I-L 128 µg/mL + LEV 32 µg/mL		2.55 ± 0.59	4.48 ± 0.78	S	S
***S. aureus* CI**	TP-I-L 128 µg/mL + LEV 16 µg/mL	0.66 ± 0.63	1.81 ± 0.72	3.31 ± 2.32	S	S
	TP-I-L 128 µg/mL + LEV 32 µg/mL		3.78 ± 0.75	5.15 ± 0.18	Ad	S

Note: Ad – Additiveness; I – Indifference; S – Synergy. In the column of the combined agents, the values following the antimicrobial agent are related to the concentration values used in this analysis and are represented in µg/mL.

In general, data obtained varied according to the type of the analysis accomplished: the ones obtained in the statistical analysis were a bit different from those obtained in the biological significance analysis. This can occur because the significant differences detected on a statistical level may not correspond to the same on a biological level. Considering the synergy outcomes obtained in both the biological significance analysis and the statistical analysis, all the tested combinations revealed to be more efficient against *S. aureus* strains. In turn, in the biological significance analysis, it was verified some additiveness in *P. aeruginosa* PAO1 and *P. aeruginosa* CI.

Considering the statistical analysis, the more positive outcomes were obtained with both combinations (TP-I-L 128 µg/mL + LEV 16 µg/mL and TP-I-L 128 µg/mL + LEV 32 µg/mL) mainly for the strains of *S. aureus*, where all the outcomes revealed synergism, condition where the cooperation of the two agents is greater than the sum of their individual activities. For *S. aureus* CI biofilms, the reductions

were between 3.31 CFU/cm^2 and 5.15 CFU/cm^2. Likewise, for *S. aureus* ATCC, the reductions ranged from 3.55 CFU/cm^2 to 4.71 CFU/cm^2. Both *P. aeruginosa* PAO1 and *P. aeruginosa* CI revealed one condition with indifference outcomes, and one with synergic activities. For *P. aeruginosa* PAO1, there was verified a variation in the reductions between 3.07 CFU/cm^2 and 5.30 CFU/cm^2. In its turn, for *P. aeruginosa* CI the reductions ranged from 1.17 CFU/cm^2 to 2.96 CFU/cm^2. Overall, it was verified a higher log reduction when combining TP-I-L with LEV 32 μg/mL than when combining this AMP with LEV 16 μg/mL, which means that the combination of TP-I-L with a higher concentration of LEV is more effective than the combination of TP-I-L with a lower concentration of LEV against all the strains. As it would be expected, higher antibiotic concentrations lead to more favorable outcomes by improving the effectiveness of the combined action of the agents. Thus, the highest log reduction, exceeding a 5 log reduction decrease, corresponds to the most effective combination and was verified when combining TP-I-L 128 μg/mL with LEV 32 μg/mL against *P. aeruginosa* PAO1 biofilms. On the other hand, the least effective combination, corresponding to the lowest log reduction (1.17 CFU/cm^2), was observed against *P. aeruginosa* CI, when combining TP-I-L 128 μg/mL with LEV 16 μg/mL.

Comparing the values obtained for the log reductions of the isolated agents with the ones obtained for the log reductions of the combinations, it is noticeable that the values of the combinations are higher than the ones of the isolated agents. Moreover, with the exception of *P. aeruginosa* CI, all the log reductions were significant (≥ 3 log).

Despite the statistical analysis being a more common approach, the biological significance is a more realistic method and, thus, the one that provides more information whether the combinations are actually efficient or not. Results obtained on statistical approaches are directly dependent to the collected data, which can induce misleading conclusions. Statistical analysis is full with careful study designs and corrections for multiple testing and so is a great help to determine whether the observations are based on a pattern rather than chance, while biological significance determines if the observed difference has any practical outcome in clinical terms. Thus, statistics is only a way of interpreting data of a given experiment.

In summary, on a statistical level, the combinations of TP-I-L with LEV tested in this assay demonstrated synergy effects in the treatment of grown biofilms of both CI and reference strains of *P. aeruginosa* and *S. aureus*. On the one hand, all the combinations revealed synergism in the eradication of *S. aureus* biofilms. However, when acting against the reference strain of *P. aeruginosa*, the lower concentration of LEV (16 μg/mL) showed synergic outcomes, while the highest concentration (32 μg/mL) of this

antibiotic was more effective against the CI of *P. aeruginosa*. The synergism verified between the agents shows the possibility of the expansion of their antimicrobial effect when acting together.

Considering the biological significance approach, the action of the combinations showed to be more efficient against *S. aureus*. *P. aeruginosa* strains also revealed some positive outcomes by showing some addictiveness. There was only one inconclusive outcome verified against *P. aeruginosa* PAO1 biofilms, where the combination of TP-I-L 128 μg/mL with LEV 32 μg/mL revealed indifference outcomes in half of the assays, while the other half revealed additiveness.

As the efficiency of the combinations in the biological significance analysis is more realistic and conservative than the statistical approach, the final results that should be considered remark that the real effectiveness of both combinations when acting against the strains used in this work may be closer to the outcomes obtained in the biological significance approach. Nevertheless, there is still some probability that the real effectiveness of the combinations may be between the results obtained in both methodologies. In general the combinations showed to be efficient, mainly in the treatment of *S. aureus* biofilms.

In spite of not being necessarily complementary features, the two methodologies used in this work should be both performed in order to verify that the conclusions taken by most studies that use the statistical significance approach as a main method may not correspond to the reality, that is, their results may not be so positive as they actually seem.

4. CONCLUSIONS AND FUTURE WORK

This chapter explains the main conclusions obtained from all the assays performed in this experimental work, as well as some future prospective paths of work.

4.1 Conclusions

The emergence of antimicrobial resistance is one of the greatest public health concerns. Because of the interesting properties of AMPs and their potential use as antimicrobial agents, researchers are currently trying to take the most advantage of these agents as a strategy for future treatments and control of infections, alone or in combination with antibiotics. The combination of antimicrobial agents especially the ones that result in synergistic outcomes, may lead to the increment of antibiofilm activities and also assist in the prevention of resistance.

Both the Gram-negative bacteria *P. aeruginosa* and the Gram-positive bacteria *S. aureus* are significant opportunistic pathogens related to multiple nosocomial infections, with an evident concern in the healthcare system. In this work, *P. aeruginosa* and *S. aureus* were selected for the study of the antimicrobial activity of antimicrobial combinations including AMPs and antibiotics, in planktonic and biofilm cells.

When testing the planktonic susceptibility of both reference and CI strains of *P. aeruginosa* and *S. aureus*, TP-I-L was the peptide that obtained the best results by showing the best bactericidal and bacteriostatic effects against all the strains, with MIC and MBC ranging from 4 to 16 μg/mL and 32 to 128 μg/mL, respectively.

Considering the antimicrobial activity of the antibiotics, both CIP and LEV showed potent antimicrobial activities against most strains, except for *P. aeruginosa* CI, for which they demonstrated lower bactericidal and bacteriostatic effects. In general, these antibiotics revealed a stronger antimicrobial activity against *S. aureus* strains.

The combinations of TP-I-L with LEV and CIP were tested on planktonic cultures of *P. aeruginosa* and *S. aureus,* where both FICI and FBCI values revealed that most of the strains were not very susceptible to the action of the combined agents. Basically, FBCI values showed some outcomes of additiveness, while FICI values revealed indifference outcomes in both combinations against all the strains. Also, there were no synergistic or antagonistic interactions. Comparing the antibiotics tested in combination with TP-I-L, LEV revealed the most positive outcomes by showing more activity against the strains, reason why this antibiotic was chosen to be further tested against mature biofilms.

Following a therapeutic approach, the biofilm susceptibility towards the combination of TP-I-L and LEV was evaluated on grown biofilms. Comparing the results obtained in planktonic and biofilm susceptibility assays, it was verified an evident increase in the effective concentrations of both agents in biofilms, which was expectable considering the fact that bacteria living in planktonic are usually easier to eradicate than bacteria living in biofilm, as it was discussed with more detail in the sub-chapter

3.4.1. Both in the statistical analysis and the biological significance analysis of the activity of the combined agents on grown biofilms of *P. aeruginosa* and *S. aureus,* synergic outcomes were verified when combining TP-I-L 128 μg/mL + LEV 16 μg/mL and TP-I-L 128 μg/mL + LEV 32 μg/mL, mainly for *S. aureus*. The combination of TP-I-L with a higher concentration of LEV was more effective than the combination of TP-I-L with a lower concentration of LEV against all the strains. Considering the synergic outcomes obtained, the combinations tested in this work can be capable of improving the effectiveness of the agents.

On the one hand, the biological approach is more conservative than the statistical analysis, which means that the results obtained biologically can indicate the absence of activity when actually there might be some activity. On the other hand, positive results obtained in statistics may not imply significative outcomes on the biological level. Thus, as the biological significance analysis is also more realistic than the statistical approach, the final results that actually should be taking into account denote that the real effectiveness of both combinations when acting against the strains may be closer to the results obtained in the biological significance approach. Still, there is a chance that the real effectiveness of the combinations may be between the results obtained in the different methodologies. In general, there were obtained good results as the combinations demonstrated high log reductions when tested on grown biofilms and the combinations revealed to be effective against both strains, mainly *S. aureus*.

The comparison between both methodologies corroborate with the fact that these are not necessarily complementary properties, although they should be performed to find out that conclusions taken by most studies, the ones that only use the statistical significance analysis, may not be so realistic and positive as they seem.

In this work, it was not verified any outcome of antagonism among the combinations tested.

4.2 Future work

Considering the results acquired in this thesis, more work is still needed in order to draw to a final and solid conclusion. In order to prevent or inhibit biofilm growth, studies including a prophylactic approach should be performed.

Furthermore, it could be interesting to try other times of treatment for biofilms, as well as other concentrations of the agents and other combinations, by testing those agents that also revealed some

antimicrobial activity when tested in planktonic. For example, the combination of TP-I-L with CIP, PALM with CIP or even PALM combined with LEV can eventually generate interesting outcomes.

In a later stage, it would be interesting to realize all the same studies against multi-resistant strains and also test the best combinations in double-species biofilms (*P. aeruginosa* plus *S. aureus*) in order to simulate more realistic infections.

Before therapeutic treatments are implemented, studies including toxicity tests *in vitro* need to be performed in order to evaluate the citotoxicity of the combinations towards mammalian cell lines.

Lastly, biofilms should be studied more deeply by performing a proteomic analysis of both the biofilms treated with the best combinations and the non treated biofilms, in order to try to identify proteins related to virulence and resistance issues. This proteomic analysis could be studied through the combination of a two-dimensional gel electrophoresis (2DE) to analyze and separate the proteins, with a mass spectrometry (MS) to identify them.

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APPENDIX I – COMBINATION OF ANTIBIOTICS WITH AMP IN PLANKTONIC SUSCEPTIBILITY ASSAYS

Table 11 – Best outcomes of the combinations of TP-I with LEV and CIP obtained for P. aeruginosa

Strain	TP-I (MIC)	Antibiotic (MIC)	Outcome	TP-I (MIC)	Antibiotic (MIC)	Outcome
***P. aeruginosa* PAO1**	1	0,125 LEV	-	1	0,125 CIP	I
		0,25 LEV	I,Ad		0,25 CIP	I
		0,5 LEV	I		0,5 CIP	A
		1 LEV	A,I		1 CIP	A
	2	0,125 LEV	-	2	0,125 CIP	I
		0,25 LEV	I		0,25 CIP	I
		0,5 LEV	I		0,5 CIP	A
		1 LEV	A,I		1 CIP	A
	4	0,125 LEV	Ad	4	0,125 CIP	I
		0,25 LEV	I,Ad		0,25 CIP	I
		0,5 LEV	I		0,5 CIP	A
		1 LEV	A,I		1 CIP	A
	8	0,125 LEV	I,S	8	0,125 CIP	I
		0,25 LEV	I,Ad		0,25 CIP	I
		0,5 LEV	I		0,5 CIP	A
		1 LEV	A,I		1 CIP	A
	16	0,125 LEV	I,Ad	16	0,125 CIP	I
		0,25 LEV	I,Ad		0,25 CIP	I
		0,5 LEV	I		0,5 CIP	A
		1 LEV	A,I		1 CIP	A
	1	1 LEV	-	1	1 CIP	-
		2 LEV	-		2 CIP	-
		4 LEV	-		4 CIP	I
		8 LEV	-		8 CIP	I
		16 LEV	I	2	1 CIP	-
		32 LEV	I		2 CIP	-
		1 LEV	-		4 CIP	-

***P. aeruginosa* CI**	2	2 LEV	-	4	8 CIP	I
		4 LEV	-		1 CIP	-
		8 LEV	-		2 CIP	-
		16 LEV	I		4 CIP	I
		32 LEV	I		8 CIP	I
	4	1 LEV	-	8	1 CIP	-
		2 LEV	-		2 CIP	-
		4 LEV	-		4 CIP	I
		8 LEV	Ad		8 CIP	I
		16 LEV	I	16	1 CIP	I
		32 LEV	I		2 CIP	I
	8	1 LEV	-		4 CIP	I,Ad
		2 LEV	-		8 CIP	I
		4 LEV	-			
		8 LEV	Ad			
		16 LEV	I			
		32 LEV	I			
	16	1 LEV	-			
		2 LEV	-			
		4 LEV	-			
		8 LEV	I,Ad			
		16 LEV	I			
		32 LEV	I			

Note: MIC - µg/mL. A – Antagonism; Ad – Additiveness; I – Indifference; S – Synergy.

Table 12 – Best outcomes of the combinations of TP-I with LEV and CIP obtained for S. aureus

Strain	TP-I (MIC)	Antibiotic (MIC)	Outcome	TP-I (MIC)	Antibiotic (MIC)	Outcome
***S. aureus* ATCC 25923**	1	0,125 LEV	-	1	0,125 CIP	-
		0,25 LEV	-		0,25 CIP	-
		0,5 LEV	-		0,5 CIP	I
		1 LEV	A,I		1 CIP	A,I
	2	0,125 LEV	-	2	0,125 CIP	-
		0,25 LEV	-		0,25 CIP	-
		0,5 LEV	-		0,5 CIP	I
		1 LEV	I		1 CIP	A,I
	4	0,125 LEV	-	4	0,125 CIP	-
		0,25 LEV	-		0,25 CIP	-
		0,5 LEV	-		0,5 CIP	I
		1 LEV	I		1 CIP	A,I
	8	0,125 LEV	I	8	0,125 CIP	Ad
		0,25 LEV	I,Ad		0,25 CIP	I,Ad
		0,5 LEV	A,I		0,5 CIP	I
		1 LEV	A,I		1 CIP	A,I
	16	0,125 LEV	I,S	16	0,125 CIP	I,S
		0,25 LEV	I,Ad		0,25 CIP	I,Ad
		0,5 LEV	A,I		0,5 CIP	I
		1 LEV	A,I		1 CIP	A,I
***S. aureus* CI**	1	1 LEV	I	1	0,125 CIP	-
		2 LEV	I		0,25 CIP	I
		4 LEV	A		0,5 CIP	I
		8 LEV	A		1 CIP	A
	2	1 LEV	I	2	0,125 CIP	-
		2 LEV	I		0,25 CIP	-
		4 LEV	A		0,5 CIP	-
		8 LEV	A		1 CIP	-
	4	1 LEV	I	4	0,125 CIP	-
		2 LEV	I		0,25 CIP	I
		4 LEV	A		0,5 CIP	-

		8 LEV	A		1 CIP	-
8		1 LEV	I	8	0,125 CIP	Ad
		2 LEV	I		0,25 CIP	I
		4 LEV	A		0,5 CIP	I
		8 LEV	A		1 CIP	A
16		1 LEV	I	16	0,125 CIP	I,Ad
		2 LEV	I		0,25 CIP	I
		4 LEV	A		0,5 CIP	I
		8 LEV	A		1 CIP	A

Note: MIC - μg/mL. A – Antagonism; Ad – Additiveness; I – Indifference; S – Synergy.

Table 13 – Outcomes of the combinations of PALM with LEV and CIP obtained for P. aeruginosa

Strain	PALM (MIC)	Antibiotic (MIC)	Outcome	Strain	PALM (MIC)	Antibiotic (MIC)	Outcome
***P. aeruginosa* CI**	1	1 LEV	-	***P. aeruginosa* PAO1**	1	0,125 CIP	Ad
		2 LEV	-			0,25 CIP	I
		4 LEV	-			0,5 CIP	A
		8 LEV	Ad			1 CIP	A
		16 LEV	Ad		2	0,125 CIP	I
	2	1 LEV	-			0,25 CIP	I
		2 LEV	-			0,5 CIP	A
		4 LEV	-			1 CIP	A
		8 LEV	-		4	0,125 CIP	I
		16 LEV	I			0,25 CIP	I
	4	1 LEV	-			0,5 CIP	A
		2 LEV	-			1 CIP	A
		4 LEV	-		8	0,125 CIP	I
		8 LEV	-			0,25 CIP	I
		16 LEV	I			0,5 CIP	A
	8	1 LEV	-			1 CIP	A
		2 LEV	-		16	0,125 CIP	I
		4 LEV	-			0,25 CIP	I
		8 LEV	-			0,5 CIP	A
		16 LEV	I			1 CIP	A
	16	1 LEV	-		32	0,125 CIP	I
		2 LEV	-			0,25 CIP	I
		4 LEV	-			0,5 CIP	A
		8 LEV	Ad			1 CIP	A
		16 LEV	I		64	0,125 CIP	I
	32	1 LEV	-			0,25 CIP	I
		2 LEV	-			0,5 CIP	A
		4 LEV	-			1 CIP	A
		8 LEV	-		128	0,125 CIP	I
		16 LEV	I			0,25 CIP	I
		1 LEV	-			0,5 CIP	A

	2 LEV	-	1 CIP	A
64	4 LEV	-		
	8 LEV	-		
	16 LEV	I		
	1 LEV	I		
	2 LEV	I		
128	4 LEV	I		
	8 LEV	I		
	16 LEV	I		

Note: MIC - µg/mL. A – Antagonism; Ad – Additiveness; I – Indifference; S – Synergy

APPENDIX II – BONFERRONI'S CORRECTION METHOD IN MULTIPLE COMPARISON TEST

Table 14 – Results obtained for Bonferroni's correction method in multiple comparison test for selected pairs of means

Strain	Combined agents	Pair of means	Mean Diff.	Summary
***P. aeruginosa* PAO1**	TP1-128+LEV-16	combination action vs sum of the isolated actions	1.2572	***
		combination action vs action of the most active agent alone	1.2325	***
	TP1-128+LEV-32	combination action vs sum of the isolated actions	0.0159	****
		combination action vs action of the most active agent alone	0.7595	**
***P. aeruginosa* CI**	TP1-128+LEV-16	combination action vs sum of the isolated actions	-0.0838	ns
		combination action vs action of the most active agent alone	0.3889	ns
	TP1-128+LEV-32	combination action vs sum of the isolated actions	0.9656	****
		combination action vs action of the most active agent alone	1.3968	****
***S. aureus* CI**	TP1-128+LEV-16	combination action vs sum of the isolated actions	1.2091	****
		combination action vs action of the most active agent alone	1.4989	****
	TP1-128+LEV-32	combination action vs sum of the isolated actions	0.7331	**
		combination action vs action of the most active agent alone	1.6660	****
***S. aureus* ATCC 25923**	TP1-128+LEV-16	combination action vs sum of the isolated actions	1.5127	***
		combination action vs action of the most active agent alone	1.9583	****
	TP1-128+LEV-32	combination action vs sum of the isolated actions	1.3078	***
		combination action vs action of the most active agent alone	1.9775	***

Note: In the column of the combined agents, the values following the antimicrobial agent are related to the concentration values used in this analysis and are represented in µg/mL. ns – not significant. The mean differences are described by the p-value as: $p < 0.0001$ (****); $0.0001 <= p < 0.001$ (***); $0.001 < p < 0.01$ (**); $p > 0.05$ (ns).