Universidade do Minho
Escola de Engenharia

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Preparation of a Standardized Methodology for the Growth of *Pseudomonas aeruginosa* Biofilms and Evaluation of their Response to Antimicrobial Peptide Strategies

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Abstract

Preparation of a Standardized Methodology for the Growth of Pseudomonas aeruginosa Biofilms and Evaluation of their Response to Antimicrobial Peptide Strategies

Novel antimicrobial products, such as antimicrobial peptides (AMPs), are being investigated to combat clinically relevant biofilms and their growing antimicrobial resistance. These relevant studies, however, lack protocol standardization. Acknowledging the importance of standardized methodologies to ensure the quality of experimental results, this dissertation initiates the establishment of a standardized operational procedure (SOP) for the growth of *P. aeruginosa* biofilms. The proposed SOP was evaluated statistically, firstly in terms of *repeatability* and *ruggedness*. The method showed a *repeatability* greatly influenced by between experimental variance. The variability of the live organism at study or other uncontrollable or yet unknown variable could be the cause. The *ruggedness* of the protocol showed that slight variations to some of the SOP conditions cause differences in the results, meaning that these variables are important in the biofilm growth and must be tightly controlled.

Furthermore, this thesis evaluated the *P. aeruginosa* biofilms' response to prophylactic and therapeutic control approaches using the AMPs colistin, tachyplesin III and lactoferricin B, alone or combined. The analysis conjugates information from biomass, respiratory activity and cell viability measurements. Results show that the prophylactic approach revealed that combinations of colistin and tachyplesin III with lactoferricin B are capable of inhibiting biofilm formation at low concentrations. In the preliminary therapeutic tests, although the results were not as good, the colistin and lactoferricin B combination was able to eradicate most of the biofilm for the strain *P. aeruginosa* ATCC 10145.

Resumo

Preparação de uma Metodologia Padronizada para o Crescimento de Biofilmes de Pseudomonas aeruginosa e Avaliação da sua Resposta a Estratégias de Controlo com Péptidos Antimicrobianos

Novos compostos antimicrobianos, tais como os péptidos antimicrobianos (AMPs), estão a ser investigados para combater biofilmes de relevância clínica e a sua crescente resistência a antimicrobianos tradicionais; no entanto, estes estudos carecem de protocolos padronizados. Reconhecendo a sua importância para garantir a qualidade dos resultados experimentais, esta dissertação inicia o estabelecimento de um procedimento operacional padronizado (SOP) para o crescimento de biofilmes de *P. aeruginosa*. O SOP proposto foi avaliado estatisticamente em termos de *repetibilidade* e *robustez*. O método mostrou uma *repetibilidade* muito influenciada pela variância entre experiências. A variabilidade do organismo vivo estudado ou outra variável incontrolável ou desconhecida pode ser a causa. Por sua vez, a *robustez* do protocolo mostrou que pequenas variações em algumas condições do SOP causam diferentes resultados, o que significa que estas variáveis são importantes no crescimento do biofilme e devem ser bem controladas.

Esta dissertação também avaliou a resposta dos biofilmes de *P. aeruginosa* a abordagens profiláticas e terapêuticas usando os AMPs colistina, taquiplesina III e lactoferricina B, isolada ou combinadamente. A análise conjugou informação da biomassa, da atividade respiratória e da viabilidade celular. Os resultados mostram que na abordagem profilática, as combinações de colistina e taquiplesina III com lactoferricina B são capazes de inibir a formação de biofilme em concentrações baixas. Nos ensaios preliminares terapêuticos, ainda que os resultados não sejam tão bons, a combinação de colistina e lactoferricina B foi capaz de erradicar a maior parte do biofilme para a estirpe *P. aeruginosa* ATCC 10145.

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List of Abbreviations

AMP – antimicrobial peptide

ANOVA – analysis of variance

CBR – CDC biofilm reactor

CDC – centres for disease control and prevention

CF – cystic fibrosis

CFTR – cystic fibrosis transmembrane regulator

CV – crystal violet

CVC – central venous catheter

DMMB – dimethyl methylene blue

EC50 – half maximal effective concentration

e(DNA) – extracellular DNA

EPS – extracellular polymeric substance

ESD – extreme studentized deviate

FDA – fluorescein diacetate

GoF – goodness of fit

GraRS – glycopeptide resistance-associated two-component system

h – hour

IC50 – fifty percent growth inhibitory concentration

K-S – Kolmogorov-Smirnov

LPS – lipopolysaccharide

MBC – minimum bactericidal concentration

MBEC – minimum biofilm eradication concentration

MDR – multidrug-resistant

MIC – minimum inhibition concentration

min - minute

mPE – meta-phenylene ethynylene

MSSA – methicillin-sensitive *Staphylococcus aureus*

MSSE – methicillin-sensitive *Staphylococcus epidermidis*

MRSA – methicillin-resistant *Staphylococcus aureus*

MRSE – methicillin-resistant *Staphylococcus epidermidis*

NNISS – national nosocomial infection surveillance system

OD – optical density

OM – otitis media

PMS – N-methyl dibenzopyrazine methyl sulphate

QS – quorum sensing

QSI – quorum-sensing inhibitor

ROS – reactive oxygen specie

sad – surface attachment defective

SAMP – synthetic antimicrobial peptidomimetic

SD – standard deviation

SOP – standard operating procedure

Sr – *repeatability* standard deviation

STAMP – specifically/selectively targeted antimicrobial peptide

UTI – urinary tract infection

XTT – 3,3′-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3is(4-methoxy-6-nitro) benzene sulphonic acid hydrate

1 Introduction

1.1 Context and Motivation

Biofilm control is a prominent research area as microbial adhesion onto surfaces and the subsequent formation of biofilms are critical concerns for many biomedical applications. It is well known that biofilm-forming bacteria account for about 80% of human bacterial infections [Fey, 2010]. In particular, biofilms cause most of the nosocomial infections and are resistant to traditional treatment with antimicrobials. The resistance towards antimicrobials is, in part, due to the biofilm matrix that acts as a protective shield against external pressures as human defences and antimicrobial agents [Hall-Stoodley *et al.*, 2009]. Particularly, *Pseudomonas aeruginosa* is one of the most common microorganisms found in nosocomial scenarios, being highly related to infections in immune compromised patients due to its biofilm forming capabilities, and for which desirable treatment is still needed [Rodrigues, 2011].

Biofilm infections, along with the development of microbial drug resistance and drug-related toxicity, have encouraged the search of new alternatives to control these healthcare-associated infections. Natural compounds have emerged as an interesting approach to limit the emergence and the spread of resistant microorganisms [Spížek *et al.*, 2010]. The main challenge is to find new natural prophylactic and therapeutic compounds with novel antimicrobial targets and

mechanisms of action. Because of this, antimicrobial peptides (AMPs) have gained widespread interest as good replacements for current antibiotics, particularly considering the fact that these compounds are less likely to induce the development of acquired resistance, due to their mechanism of action with low specificity, and that they have a broad spectrum of activity and a wide availability of sources [Zasloff, 2002; Beckloff *et al.*, 2007; Splith *et al.*, 2011; Wimley *et al.*, 2011]. Recent studies have been reporting the successful control of biofilms based on AMP strategies. These positive outcomes seem to be linked to the AMPs' dual capacity to act both on the cytoplasmic membrane and on intracellular targets, once entered the microbial cell. Moreover, interesting is that some studies have reported AMP activity against biofilms at lower concentrations than those required for planktonic cell killing [Lynch *et al.*, 2008; Jorge *et al.*, 2012]. The combination of innovative and/or conventional compounds may improve the prophylactic and therapeutic efficacy and lower drug dosage, reducing toxic side effects [Ncube *et al.*, 2008; Wei *et al.*, 2011]. Therefore, synergistic studies between AMPs are also a viable route for exploring the anti-biofilm efficacy of these peptides.

Current protocols for biofilm laboratory research lack standardization, and therefore validity, in some ways. The inexistence of standard protocols hampers the quality of the information generated by researchers and makes the reproduction of results in different laboratories difficult. Many times, studies target the same scenario but results are not comparable because they are performed using different methods of analysis, which return diverse biological data and lead to various conclusions [Jackson *et al.*, 2001]. Standard operating procedures (SOPs), attending to *ruggedness*, *repeatability* and *reproducibility*, are fundamental.

1.2 Objectives

This dissertation tests the *repeatability* and *ruggedness* of a method to growth biofilms of *P. aeruginosa* ATCC 10145 in 96 well microtiter plates in order to initiate the establishment of a SOP. Additionally, this thesis evaluates the potential anti-biofilm capabilities of a group of AMPs, namely colistin, lactoferricin B and tachyplesin III, used alone and in combination, in biofilms of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO1 and *P. aeruginosa* CGCT III.

The preliminary developments of a SOP for the growth of biofilms of *P. aeruginosa* ATCC 10145 are presented since the lack of a standard protocol in this area is impairing the comparison and output of results. This evaluation addressed the most commons variables in the laboratory growth of biofilms, such as temperature, speed of agitation, time of growth, cell generation and initial cell concentration, to investigate if slight variations affected the resulted biofilm. This will make possible the establishment of the *ruggedness* of the protocol. All experiments with SOP conditions were repeated at least 17 times with at least 4 replicates, making possible to access the protocol's *repeatability*.

The AMP anti-biofilm evaluation accounts for both prophylactic and therapeutic approaches. First, the evaluation focused on AMP efficacy in inhibiting biofilm growth. Then, the evaluation centred on the capacity of AMPs to eradicate mature biofilms, which are very recalcitrant to treatment, due to the exopolymeric protective matrix, presence of tolerant (persister) cells, among other factors.

1.3 Manuscript Structure

This dissertation is divided into seven main chapters, excluding bibliography and annexes.

Chapter 1 – Introduction

This chapter exposes the context and motivation for the completion of this master's thesis. Also, the objectives of the thesis are detailed and the manuscript's structure is also described.

Chapter 2 – Biofilms

In this chapter, the concept of biofilms is explained by stating its definition and main characteristics. Furthermore, highlight is given to the biofilm role in infections and to its resistance mechanisms to antimicrobial treatment.

Chapter 3 – Antimicrobial Peptides

This chapter reviews the different sources and families of AMPs and their mechanisms of action. A detailed revision of the latest studies of biofilm control with AMPs is given and the biofilm resistance mechanisms towards AMPs are specified.

Chapter 4 – Standard Operating Procedures

The importance of the establishment of standardized protocols is highlighted in this chapter, along with a revision on the existing SOPs for biofilm studies. The main characteristics of a SOP are also detailed.

Chapter 5 – Materials and Methods

In this chapter, the materials to be used are described in detail, focusing on the microorganism to be studied, *P. aeruginosa*, and the AMPs to be used, colistin, tachyplesin III and lactoferricin B. Also, the methods used in the laboratory work for the growth, antimicrobial testing and analysis of the biofilms are explained. The steps for the statistical analysis of the data are detailed along with the tests used for the protocol standardization of the growth of *P. aeruginosa* biofilm.

Chapter 6 – Results and Discussion

This section presents the results derived from the tests for the establishment of the standard protocol for the growth of *P. aeruginosa* biofilm and for the control of these biofilms with the AMPs colistin, tachyplesin III and lactoferricin B, alone and combined.

Chapter 7 – Conclusions and Future Work

Finally, the main conclusions, retrieved from the results and discussion section, are summarized and some follow up work is pointed out.

2 Biofilms

2.1 What are Biofilms?

For a long time, the study of microorganisms was closed off to the study of planktonic, liquid cultures. However, nowadays it is evident that the activity of microorganisms in Nature is mainly associated with surfaces and, thus, research has been shifting its attention to the study of the sessile, adhered mode of life [Rodrigues, 2011]. These organized, structured consortia of microorganisms are known as biofilms, and they are viewed as a form of adaptation that allows bacteria and fungi (one or more species combined) to survive in hostile environments and colonize other areas by cell dispersion [Hall-Stoodley *et al.*, 2009].

Biofilms influence a diverse range of disciplines, ranging from biotechnological and environmental industries to medical applications, and they can have both a positive and a negative effect on modern society. For example, bacterial biofilms can be positively applied to wastewater treatment processes as flocs or granules, yeast aggregates are capable of improving brewing processes, and other biofilms have been applied in bioremediation and fuel technology. However, biofilms also have damaging effects in industry and public health. Bacterial communities are involved in detrimental processes such as biofouling and biocorrosion and biofilm-related infections are a serious concern in modern medicine, representing 65% of the total hospital-acquired infections [Beech *et al.*, 2005; Todar, 2008-2012].

Biofilm formation undergoes essentially four typical stages (Figure 2–1): i) adherence of planktonic cells to the tissue or abiotic surface; ii) accumulation of cells and production of the extracellular polymeric substance (EPS) matrix, which is constituted by proteins, polysaccharides and extracellular DNA (eDNA) resulting from autolysis, and whose presence will ensure structural stability and protection to the biofilm; iii) biofilm maturation with development of towers and water channels and specialized zones; and iv) dispersion of cells and/or parts of the biofilm with subsequent colonization of other locations [Costerton *et al.*, 1999; Donlan *et al.*, 2002; Fey, 2010]. Eventually, bacteria can use type IV pili to move through another bacteria's biofilm and colonize it [Høiby *et al.*, 2010].

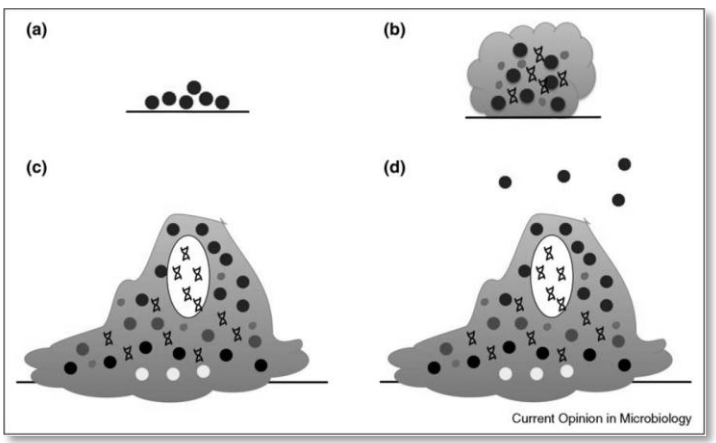


Figure 2-1: Life cycle of a bacterial biofilm. (a) – Adherence; (b) – Accumulation and production of extracellular matrix; (c) – Maturation; (d) – Dispersion [Fey, 2010].

The architecture of a mature biofilm can have many configurations that range from flat homogenous layers of cells to highly organized cell clusters, *e.g.* a mushroom shaped structure containing water-filled channels [Wimpenny *et al.*, 2000]. A mature biofilm results from complex spatial and temporal differentiation of cells in response to environmental signals and cell to cell communications [Bridier *et al.*, 2011]. These dynamic three-dimensional structures maintain a tight

organization through cell signalling. This allows biofilm cells to respond to environmental signals, sense cell density and perform quorum sensing (QS) [Hall-Stoodley *et al.*, 2009]. For example, bacteria in biofilms sense when critical concentration of cells is reached and respond by producing virulence factors, like enzymes or toxins [Høiby *et al.*, 2010].

2.2 Biofilm Related Infections

The aging of worldwide population has increased the number of surgeries, joint replacements and immunosuppressive therapies and, implicitly, the incidence of biofilm-related infections. That is, biofilm infections are more and more related to morbidity and mortality and have become the main cause of emergence and dissemination of antibiotic resistance in the nosocomial scenery [Spížek *et al.*, 2010].

Bacterial biofilms are the cause of among 80% of all bacterial infections, the most common being biomaterial-related infections [Harro *et al.*, 2010]. Biomaterial nosocomial infections are majorly found on central venous and urinary catheters, prosthetic heart valves, orthopaedic devices, cardiac pacemakers, vascular, voice and ocular prostheses, cerebrospinal fluid shunts, contact lenses, among others [Stewart *et al.*, 2001; Fey, 2010; Rodrigues, 2011]. For example, currently about 12% to 25% of mortality in hospitalized patients is due to catheter-related bloodstream infections [Estrela *et al.*, 2010].

In non-surgical devices, such as catheters, the colonization of the surface may be originated by the migration of the microorganisms of the skin in the point of insertion and throughout the catheter. In the case of surgical devices, such as orthopaedic replacements, the

adhesion of the bacteria to the surface will compete with the integration of the material with the surrounding tissue. This last step must be concluded before the 6 h decisive period ends, which is indicated has the time in which the material is most susceptible to colonization [Rodrigues, 2011].

Biofilms are also associated to non biomaterial infections, such as chronic wounds, endocarditis [Stewart *et al.*, 2001; Fey, 2010], periodontitis, chronic urinary tract infections (UTI), recurrent tonsillitis, chronic rhinosinusitis, chronic otitis media (OM) and cystic fibrosis (CF) pneumonia [Hall-Stoodley *et al.*, 2009]. Many of the biofilm producing organisms are opportunistic pathogens, like *P. aeruginosa* or *Staphylococcus epidermidis* [Stewart *et al.*, 2001; Rodrigues, 2011], and the biofilm-associated infections are usually coupled with a chronic condition [Hall-Stoodley *et al.*, 2009]. Microorganisms involved in biofilm human infections are revised by Lynch and Robertson [2008].

2.3 Biofilm Resistance

The administration of antibiotics is the common treatment of infections. However, the widespread, and sometimes unnecessary, use of the antibiotics has led to the selection of multi-drug resistant (MDR) pathogens [Spížek *et al.*, 2010]. Additionally, current antibiotics have been classically developed for treatment of planktonic bacterial populations in acute infection scenarios, being usually ineffective in biofilm (persistent) related infections [Lynch *et al.*, 2010]. Compared to planktonic bacteria, the minimum inhibition concentration (MIC) can be hundreds or thousands of times higher and the resistance to the innate and adaptive immune system is also higher. For example, *Escherichia coli* biofilms require 220 times higher antibiotic concentration to be

eradicated compared with the same strain in the planktonic state [Estrela *et al.*, 2010].

To make matters worse, biofilms' antibiotic resistance does not seem totally linked to the usual resistance mechanisms, and even the most susceptible planktonic bacteria can become a threat when grown in biofilm. The frequency of mutation in biofilms is higher than in planktonic bacteria and there is an increased horizontal gene transmission, which also explains the fast development of the biofilms' antibiotic resistance. However, bacteria detached from biofilms can become rapidly susceptible to antibiotics, suggesting that sometimes no mutations or other genetic modifications are required [Stewart *et al.*, 2001].

Although antibiotic penetration is still feasible, the biofilm matrix can delay it till the expression of resistance-related genes takes place. Also, polymers in the matrix may bind to antibiotics, hindering their action, and antibiotic-degrading enzymes may deactivate them. If the antibiotic is still able to cross the matrix and reach the cells, some biofilms will express efflux pumps in the presence of the antibiotic, preventing its intracellular action [Hall-Stoodley *et al.*, 2009].

Indeed, the three-dimensional structure of a biofilm plays, along with the EPS matrix, a major role in biofilms' resistance to antimicrobials [Bridier *et al.*, 2011]. Researchers have observed that the concentration of oxygen is higher at the surface and lower in the centre of the biofilm, and that the protein synthesis and metabolic activity is higher at the surface and lower or absent in the centre of the biofilm. Due to these oxygen and nutrient gradients throughout the biofilms, nutrient depleted zones can appear and bacteria can enter in a stationary phase-like dormancy and not be affected by antibiotics. Finally, oxidative

stress, caused by an imbalance between the formation of reactive oxygen species (ROS) and the antioxidant system, increases mutability in biofilms and promote antibiotic resistance [Hall-Stoodley *et al.*, 2009; Høiby *et al.*, 2010].

Recent studies have detected that biofilms present resistance to some new alternative treatments as well, as phage resistance and low susceptibility to antibodies [Hall-Stoodley *et al.*, 2009], and resistance to QS inhibitors (QSIs) [Høiby *et al.*, 2010]. Overall, the conclusion is that new compounds with novel mechanisms of action are desired to treat biofilms more effectively, *i.e.* avoiding their natural predisposal to antimicrobial resistance. Most of the antimicrobial products that are being developed are derivatives of already known compounds and target the same resistance mechanisms, so their action can only be somewhat better. Now, the attention is drifting to the deployment of new antimicrobial discovery strategies. For example, the discovery of non-traditional sources of antimicrobials, microbial genome sequencing focused in antibiotic gene expression, metagenomics, and the re-examination of old compounds and investigation of new targets in pathogenic bacteria [Spížek *et al.*, 2010].

Within this scope, natural products stand out because they have a much higher hit rate in high-throughput screens than the combinational libraries of traditional antimicrobials. Moreover, natural products are usually much more complex than synthetic products and present scaffolds with viable and biological validated starting points to design chemical libraries [Spížek *et al.*, 2010]. One group of natural antimicrobial products that have been showing promising results is AMPs, which are discussed in the next section.

3 Antimicrobial Peptides

3.1 Sources of Antimicrobial Peptides

AMPs are short-length peptide antibiotics (between 15 and 30 amino acids), whose majority are cationic, amphipathic, gene-encoded and directed to the cell membrane [Rossi *et al.*, 2008; Melo *et al.*, 2009; Chau, 2010; Splith *et al.*, 2011]. AMPs are usually co-expressed in groups that act together [Lai *et al.*, 2009], but despite their similarities, AMP sequences vary greatly. Usually, AMPs are classified as α helical, β sheeted, extended and looped [Melo *et al.*, 2009] or ribosomally and non-ribosomally synthesized [Rossi *et al.*, 2008].

AMPs come from a variety of sources and in many forms. Their widespread distribution throughout the animal and plant kingdoms suggests that AMPs play a fundamental role in the evolution of complex multicellular organisms. Despite their ancient lineage, AMPs have remained effective defensive weapons [Zasloff, 2002]. Indeed, it has been proposed that AMPs and AMP-directed resistance mechanisms have co-evolved, leading to a host-pathogen balance that has shaped the existing AMP portfolio [Peschel *et al.*, 2006].

AMPs are part of the innate immune system of animals and plants, but can also be found in microbes, like bacteria and fungi [Rossi *et al.*, 2008; Melo *et al.*, 2009; Chau, 2010; Splith *et al.*, 2011]. Mammalian AMPs are generally expressed and easily induced in epithelial surfaces to repel assault from bacteria, viruses, fungi and

parasites [Lai *et al.*, 2009]. AMPs have also been found in glandular cells of amphibian skin, fishes and most classes of invertebrates. In plants, AMPs help in the adaptation to stressful environmental conditions: plant cells act as recognition sites against pathogen-derived metabolites (elicitors), leading to the accumulation of AMPs in the affected plant tissue [Kido *et al.*, 2010]. Additionally, microbes, such as bacteria and fungi, produce AMPs as a defence mechanism and as a competitive advantage against other microorganisms, sometimes of the same species [Sang *et al.*, 2008].

Synthetic AMPs, produced by de novo synthesis or by modification of existing AMPs, emerged as an alternative to reduce production costs [Wimley *et al.*, 2011]. Recently, some reviews [Vooturi *et al.*, 2010; Giuliani *et al.*, 2011] have reported the engineering of AMP mimetics or peptidomimetics, non-peptide molecules which aim to retain and improve the basic features of AMPs [Giuliani *et al.*, 2011].

A resume of the sources and classifications of the various AMPs is available in Table A of Annex I.

3.2 Action Mechanisms of Antimicrobial Peptides

AMPs have been recognized as promising candidates for replacing classical antibiotics due to their multiple mechanisms of action and low specificity in terms of molecular target, which reduces the chance of acquired resistance [Zasloff, 2002; Beckloff *et al.*, 2007]. Moreover, compared with conventional antimicrobials, which are generally active only against bacteria or fungi, AMPs exert activity against a broad spectrum of microorganisms, such as both Gram-negative and Gram-positive bacteria, including drug-resistant strains,

parasites, enveloped viruses and even some cancer cells [Sang *et al.*, 2008; Chau, 2010; Splith *et al.*, 2011; Wimley *et al.*, 2011]. AMPs are also cell specific and are able to distinguish host from non-host cells based on their charge [Beckloff *et al.*, 2007]. Besides their antimicrobial action, AMPs can also influence processes in support of antimicrobial properties, like cytokine release, chemotaxis, antigen presentation, angiogenesis and wound healing[Lai *et al.*, 2009].

Indeed, the number of published papers considering AMPs has risen considerably in the last years, including the studies of AMPs applied to biofilms (Figure 3–1).

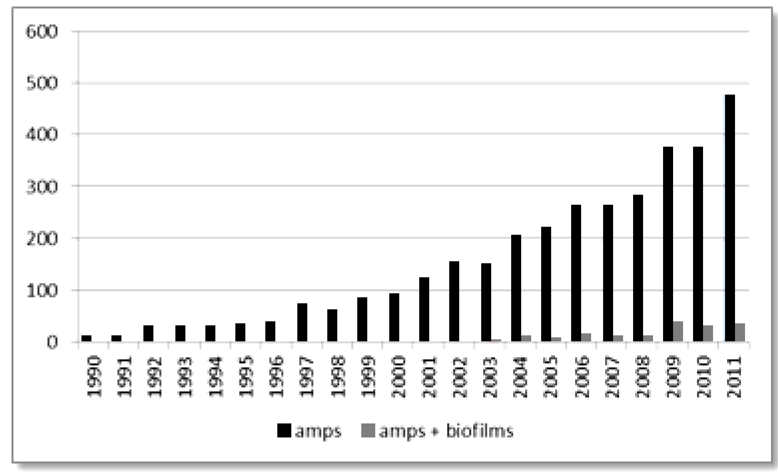


Figure 3-1: General statistics on the number of publications on AMPs (black bars) and AMP applications to biofilms (gray bars) in PubMed [Jorge *et al.*, 2012].

Conventional antibiotics usually act by inhibition of cell wall, DNA, RNA and protein synthesis [Sang *et al.*, 2008; Chau, 2010]. On the other hand, most AMPs permeabilize microbial membranes, inducing either a large-scale failure or small defects that dissipate the transmembrane potential, which results in cell death [Sang *et al.*, 2008; Wimley *et al.*, 2011]. This mechanism of action does not depend on the recognition of chiral targets and, therefore, all D-enantiomers are equally active, giving AMPs broad action spectrum [Podda *et al.*, 2006].

AMP mechanisms of action are divided into pore and non-pore models [Wimley *et al.*, 2011]. Pore models account for the formation of membrane-spanning pores, namely the *barrel stave pore model* [Rapaport *et al.*, 1991] - in which AMPs interact to form a hydrophilic channel - and the *toroidal pore model* [Ludtke *et al.*, 1996] - in which AMPs affect the curvature of the membrane. In turn, non-pore models comprise: the *carpet model* [Gazit *et al.*, 1996], which is the most cited model and accounts for the parallel deposition of AMPs on the membrane, causing global bilayer destabilization due to a detergent-like effect; the *detergent model* [Ostolaza *et al.*, 1993] that explains catastrophic collapse of the membrane using high concentrations of AMPs; the *molecular shape models* [Bechinger *et al.*, 2006], in which AMP-lipid interactions can be portrayed with phase diagrams; *the lipid clustering model* [Epand *et al.*, 2009], in which AMPs induce lipid phase separation; the *sinking raft model* [Pokorny *et al.*, 2002], in which AMP activity is described in terms of binding, insertion and perturbation; and, the *interfacial activity model* [Rathinakumar *et al.*, 2008], which is used to explain, predict and engineer the activity of AMPs. All aforementioned models imply the need to reach a certain threshold concentration of AMPs in the membrane prior to disruption [Melo *et al.*, 2009].

Some AMPs act by alternative means, like binding to DNA, inhibiting cell wall, DNA, RNA and protein synthesis, autolysin and inhibiting enzyme activity [Sang *et al.*, 2008]. The type of mechanism of AMPs can dictate their application fields. For example, it has been noticed that AMPs targeted to the membrane are better suited to be used in surface coating instead of AMPs that act at an intracellular level [Bagheri *et al.*, 2012].More details about the mechanisms of action of AMPs are available in the reviews of Melo *et al.* [2009], Splith *et al.* [2011], Wimley *et al.* [2011], Park *et al.* [2011] and Nguyen *et al.* [2011].

3.3 Anti-Biofilm Antimicrobial Peptide Strategies

Biofilm control can be achieved in three ways: i) reduction of the planktonic population; ii) prevention of the initial adhesion of cells to the surface; and iii) removal of the established biofilm. Studies on biofilm-forming bacteria or yeasts in the planktonic state may open routes to the first strategy. However, in vivo application of the first strategy is quite complicated, given that the planktonic population in the body is widespread and identification of the presence of the planktonic biofilm-forming bacteria is difficult; so, microbial adhesion must be prevented as the next step.

Conceptually, the easiest method for preventing microbial attachment is by pre-treating the surfaces. This can be achieved by impregnating the surface with an antimicrobial agent or using functionalized coatings that allow a localized antimicrobial delivery [Zilberman *et al.*, 2008; Shukla *et al.*, 2010; Yala *et al.*, 2011]. As reviewed by Glinel *et al.* [2012], several AMP-based coatings have been tested successfully.

When surface pre-treatments are not effective, biofilms may form. Then, strategies based on the administration of antimicrobials, to the infected live tissue (antibiotic treatments) or the non-living surface (disinfectant treatments), must be applied to kill the biofilm-growing microorganisms. A summary of the latest biofilm control studies using AMPs is depicted in Table B of Annex I.

The analysis of Table B showed that the AMPs tested on biofilms come from various natural sources, such as humans (AMP-IBP5; HBD3; LL-37; α-MSH), mammals (BMAP-28; cathelicidin WAM1), amphibians (aurein 2.5; magainin I; phylloseptin-1), fishes (chrysophsin-1;

pleurocidin), arthropods (tachyplesin III), bacteria (gramicidin A; lacticin 3147; nisin) and plants (Tn-AFP1). Non-natural AMPs are classified into mimetics (peptoid 1; peptoid 1-C134mer; (RW)4D) and synthetic (F2,5,12W; KSL; PTP-7; Tet213; SAMPs Ltx5, Ltx9 and Ltx10; omiganan pentahydrochloride; STAMPs C16G2, M8G2, C16-33, M8-33 and G10KHc). A substantial part of the tested AMPs is synthetic, which means that improving AMP optimal performance is nowadays becoming an important issue. Most of the microorganisms tested are bacteria probably due to their ubiquity in Nature and their frequent association with infectious diseases and biofilms.

It is also noteworthy that most biofilm-related studies, as seen in Table B, cover mainly biofilm growth in the presence of AMPs, *i.e.* prophylactic strategies meant to prevent biofilm formation, rather than testing AMPs against pre-established biofilms, *i.e.* therapeutic strategies meant to treat existing biofilms. This suggests that prevention of biofilm formation is possibly the current favourite research strategy in the combat of nosocomial infections. However, more work must be done in order to evaluate anti-biofilm efficacy of AMPs on mature biofilms.

One of the characteristics that seems to be linked to the anti-biofilm efficacy of some AMPs is their dual capacity to act both on the cytoplasmic membrane and on intracellular targets, once entered the cell. For example, it is thought that the synthetic AMP meta-phenylene ethynylene (mPE), designed based on magainin and active at nanomolar concentrations against *Streptococcus mutans* biofilms, acts both as an membrane-active molecule, inhibiting lipopolysaccharides (LPSs), similarly to magainin, and as an intracellular antibiotic by binding to DNA at equimolar ratios [Beckloff *et al.*, 2007]. Another example is pleurocidin, which is thought to inhibit nucleic acid and protein synthesis without damaging *E. coli* cytoplasmic membrane at low

concentrations [Patrzykat *et al.*, 2002], but it is able to cause membrane leakage and pore-like channels at higher concentrations [Mason *et al.*, 2006].

Also, interesting is that some studies have reported AMP activity against biofilms at lower concentrations than those required for planktonic cell killing. This is the case of the synthetic AMP NA-CATH:ATRA1-ATRA1 and the natural AMP LL-37, both from the cathelicidin family, that are effective against *Staphylococcus aureus* and *P. aeruginosa* biofilms, respectively. These AMPs are thought to act internally on the bacteria, affecting gene expression essential for the development of biofilms [Overhage *et al.*, 2008; Dean *et al.*, 2011]. Actually, in *P. aeruginosa*, the AMP LL-37 alters the expression of biofilm related genes, such as type IV pili, rhamnolipid and Las QS systems, at sub-antimicrobial levels, and genes, associated with the assembly of flagella, involved in initial adherence during biofilm formation, were found to be down regulated [Overhage *et al.*, 2008]. LL-37 is also capable of inhibiting initial biofilm attachment (58 - 62%), suggesting that peptides of this kind may be interacting with bacterial adhesins as part of their anti-biofilm mechanism [Dean *et al.*, 2011]. Another study also showed that the AMP 1037 directly inhibits biofilms by reducing swimming and swarming motilities, stimulating twitching motility, and suppressing the expression of a variety of genes involved in biofilm formation in *P. aeruginosa* (*e.g.* PA2204) [de la Fuente-Núñez *et al.*, 2012].

In fact, anti-adhesion may be one of the great properties of anti-biofilm AMP abilities, which allows them to be used as an effective pre-treatment strategy. The AMP nisin, which is known to interfere with cell wall synthesis and form membrane pores [Peschel *et al.*, 2006], retards biofilm formation without inhibiting *S. aureus* growth when immobilized

in multi-walled carbon nanotubes [Qi *et al.*, 2011]. Another example is the cathelicidin-2 derived peptide, F2,5,12W, which suppresses *S. epidermidis* biofilm formation at a concentration four times below the MIC, which reflects decreased initial adhesion of the bacteria [Molhoek *et al.*, 2011].

Anti-attachment capabilities may be related to binding of DNA as well. DNA binding may facilitate the detachment or disruption of biofilm structures, since it has been reported that eDNA is involved in cell-cell attachment [Allesen-Holm *et al.*, 2006]. This is the case for cationic AMPs and peptoids [Lobo *et al.*, 2003; Otvos, 2005; Hale *et al.*, 2007]. For example, the development of *P. aeruginosa* biofilms is disrupted by the enzyme DNase I [Whitchurch *et al.*, 2002].

Matrix disruption in biofilms may also be a target for AMPs. It is thought that the peptoid 1-C134mer, which as a hydrophobic tail and is active against *P. aeruginosa* biofilms, interacts strongly with and disrupt the hydrophobic matrix due to its surfactant like nature, facilitating deeper penetration [Kapoor *et al.*, 2011].

Additionally, AMPs have some organism-specific features. For example, lactoferrin inhibits biofilm formation in *P. aeruginosa* due to its iron-chelating properties, increasing surface motility and causing the bacteria to wander around the surface, forming thin and flat biofilms [Singh *et al.*, 2002]. In turn, the inhibition of *Porphyromonas gingivalis* and *Prevotella intermedia* biofilms by lactoferrin is independent of the iron status of the protein. Lactoferrin may interact with the cell surface of these bacteria and interfere with their adherence [Wakabayashi *et al.*, 2009], since this peptide was already reported to interfere with the binding of *P. intermedia* to subepithelial matrix proteins, as well as fibroblasts and epithelial cells [Alugupalli *et al.*, 1994; 1995].

3.4 Biofilm Resistance to Antimicrobial Peptides

Although the development of resistance to AMPs is rare, some studies have reported this phenomenon. General mechanisms for microbial resistance to AMPs, which are valid both for planktonic and sessile states, include mutations that affect the structure and charge distribution of the cytoplasmatic membrane, modifications in the lipopolysaccharide structure of Gram-negative bacteria, and active pumping of the AMPs out of the cell [Altman *et al.*, 2006]. Specifically, it has been reported that Gram-negative bacteria have evolved mechanisms to remodel the composition of the outer membrane through modification of the LPS molecules [Miller *et al.*, 2005], which impairs LPS-binding AMPs.

Biofilm structure is another factor correlated with biofilm resistance to AMPs. For instance, the increased survival of *E. coli* biofilms when treated with colistin is not related directly with biofilm forming ability, but rather to the organization of the biofilm. Also, there is some evidence that biofilm formation in *E. coli* induces tolerance to AMPs due to changes in intra-biofilm physiochemical gradients [Folkesson *et al.*, 2008].

AMP activity over intracellular targets is countered by genetic mutations. Interestingly, in *S. aureus*, the glycopeptide resistance-associated two-component system (GraRS), which is involved in up-regulation of biofilm production, was reported to mediate the resistance of the planktonic cells to AMPs [Herbert *et al.*, 2007]. In CF, where *P. aeruginosa* biofilms cause pneumonia, results show that colistin kills the stalk subpopulation (deeper layer with low metabolic activity) preferentially, whereas the metabolically active cap-forming subpopulation in the upper layer becomes colistin resistant due to the

up-regulation of the *pmr* and *mexAB-oprM* genes [Haagensen *et al.*, 2007; Pamp *et al.*, 2008].

4 Standard Operating Procedures

Biofilms research has grown greatly in recent years, with an ever growing number of publications, but results and conclusions are sometimes contradicting. Results comparison is pivotal to validate individual experiments as well as consolidate research across laboratories. However, most of the current protocols for laboratory research lack standardization, and therefore validity, at some extent. Specifically, the inexistence of standard protocols hampers the quality of the information generated by researchers and makes the comparison of results produced in different laboratories difficult. Many times, studies focusing the same scenario are not comparable because they are performed using different methods of biofilm growth or analysis, which output very different biological data and may lead to various conclusions [Jackson *et al.*, 2001]. In the case of biofilm-related studies, *e.g.* methodologies for cell growth, antimicrobial susceptibility and final biomass/cell activity vary from paper to paper, making it impossible to compare results conveniently.

To enable inter-laboratory evaluations and ensure results' transparency, some validated protocols, the so-called SOPs, have been presented for biofilm growth, biofilm detection and quantification and for antimicrobial testing on biofilms

The SOPs proposed for laboratory biofilm growth are specific for: growth of mix biofilms of *P. aeruginosa* ATCC 700829, *Pseudomonas fluorescens* ATCC 700830 and *Klebsiella pneumoniae* ATCC 700831, which can be analyzed in terms of structure and viable cell counts using

a flat-plate, open channel reactor [Jackson *et al.*, 2001]; growth of *S. epidermidis* ATCC 35984 biofilms on polycarbonate coupons in the CDC biofilm reactor (CBR) [McLeod *et al.*, 2010]; and the growth of *P. aeruginosa* biofilms using the CBR [Goeres *et al.*, 2005; EPA/OPP, 2011].

Biofilm detection and quantification SOPs are available for: quantification of *P. aeruginosa* biofilm grown with high shear and continuous flow using CBR [ASTM, 2007b]; quantification of a *P. aeruginosa* biofilm grown using a drip flow biofilm reactor with low shear and continuous flow [ASTM, 2008]; colorimetric microtiter model for the detection of *S. aureus* biofilms [Toté *et al.*, 2008]; quantification of microbial biofilms grown in microtiter plates (CV, Syto9, fluorescein diacetate (FDA), resazurin, XTT and dimethyl methylene blue (DMMB) assays) [Peeters *et al.*, 2008]; harvesting and disaggregating steps [Hamilton *et al.*, 2009]; optimized quantification of enterococci biofilms using microtiter-plates [Extremina *et al.*, 2011]; and quantification of *P. aeruginosa* biofilm grown with medium shear and continuous flow using rotating disk reactor [ASTM, 2012].

Currently, no SOP is available for anti-biofilm AMP-based strategies. However, there are some works on general antimicrobial testing on biofilms: evaluating: resistance of *S. aureus* biofilms cells to disinfectants [Luppens *et al.*, 2002]; biofilms susceptibility using a microplate alamar blue assay for *S. epidermidis* [Pettit *et al.*, 2005]; and disinfectant efficacy on a laboratory hot tub model on planktonic bacteria and biofilms [Goeres *et al.*, 2007].

4.1 Desired Characteristics for a SOP

A well-established protocol, like a SOP, has to respect several conditions that attest its validity, namely: *reasonableness*, *relevancy*, *validity*, *ruggedness*, *repeatability* and *reproducibility* [Hamilton, 2010]:

i) A SOP is *reasonable* if it can be conducted within practical limitations, such as time, labour and material and if it is easily understandable and requires conventional and inexpensive laboratory material.

ii) In biological test methods, *relevancy* correlates with the capacity to emulate the real-world environment where the biological phenomenon in study occurs. Specifically, in Microbiology, test *relevancy* has motivated a shift in testing, from planktonic microbes to biofilms.

iii) A SOP is *valid* if the data results are unbiased, which means that the observed values equals (or approximates greatly) the true values. In Microbiology, however, the true values are usually unknown (*e.g.* log reductions, MICs, ODs, *etc.*).

iv) A SOP is *repeatable* if the results within the same experiment and between experiments have low variance.

v) *Ruggedness* of a SOP is encountered when results are not affected by small deviations from the SOP conditions.

vi) Finally, a SOP is *reproducible* if similar results are obtained by different operators and in different locations/laboratories [Hamilton, 2010].

Next, a detailed explanation of *repeatability*, *ruggedness* and *reproducibility* is given because they will be assessed in this dissertation and in future work.

4.2 *Repeatability*

The term *repeatability* accounts for random errors of the measurements and includes the contributions from any part of the procedure that varies within a run, *e.g.* gravimetric and volumetric errors, heterogeneity of the test material and variation in the chemical treatment stages of the analysis [Thompson *et al.*, 2002]. For the establishment of *repeatability*, the following conditions need to be satisfied: same measurement procedure; same observer; same measuring instrument, used under the same conditions; same laboratory; and procedure repetition over a short period of time [Taylor *et al.*, 1994].

Typically, errors in *repeatability* can be detected by inspecting replicate dispersions, but formal methods should be employed to assert these errors. Dispersion analysis can be done recurring to box plots. These plots show minimum to maximum whiskers, the bottom and top of the box are always the 25th and 75th percentile (lower and upper quartiles, respectively), and the band near the middle of the box is always the 50th percentile (median). In the case of formal methods, generally, the analysis of variance (ANOVA) is the selected method for quantifying *repeatability* [Engineered Software, 1999]. The ANOVA assesses the variance (σ^2) within experiments and between experiments. The two variances given in the output table (designated mean square (*MS*)) are: between experiment/group variance (σ^2_{btw}), which shows the differences between group means; and within group variance (σ^2_e), which shows the differences among data within the same group. The first variance, σ^2_{btw}, can be explained by a systematic variation due to treatment or by chance due to non-systematic individual differences in data or experimental error. The second variance, σ^2_e, can only be explained by the later explanation. The

repeatability standard deviation (*Sr*) was calculated as follows: $Sr = \sqrt{\sigma^2{}_{\mathrm{e}} + \sigma^2{}_{\mathrm{btw}}}$. *Sr* is interpreted as the difference between a single experiment and the mean across many independent, identical experiments. Small *Sr* values indicate good *repeatability*. *Sr* can further be analysed in terms of the percentage of influence that each variance ($\sigma^2{}_{btw}$ and $\sigma^2{}_{e}$) had. It is known that the between group variance is estimated by $\sigma^2{}_{\mathrm{btw}} = \sigma^2{}_{\mathrm{e}} + 3\sigma^2{}_{\mathrm{t}}$, in which $\sigma^2{}_{\mathrm{t}}$ is the variance due to treatment effect and whose value we can determine. Total variance ($\sigma^2{}_{\mathrm{total}}$) is the sum of $\sigma^2{}_{e}$ and $\sigma^2{}_{t}$. The percentage of the error variance and treatment variance relatively to the total variance can be now easily calculated [NIST, 2012b].

4.3 *Ruggedness*

Ruggedness is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure [Thompson *et al.*, 2002]. The aim is to identify the factors that strongly influence a method's measurements and estimate how tightly those factors need to be controlled, *i.e.* to determine the degree of control required for the experimental procedures [ASTM, 2007a]. *Ruggedness* testing is usually done within a single laboratory and on uniform material, so that the effects of changing only the factors are measured. Deliberately, small changes are introduced into the procedure and the effect on the results is examined. The factors to be tested can be quantitative (continuous, like pH or temperature), qualitative (discrete, like the manufacturer or batch of a reagent) or "mixture" factors (*e.g.* the fraction of solvents in a mixture) [Heyden *et al.*, 2001]. Examples of the factors that a *ruggedness* test for biofilm experiments should address are: the

instrument(s)'s calibration, the operator(s); the brand of reagent(s) or the concentration of reagents; the pH of solutions; the temperature of a reaction; and, the time allowed for completion of a process [Thompson *et al.*, 2002].

The factors are examined in an experimental design, which is selected as a function of the number of factors to investigate. The most common designs applied are the fractional factorial [Voelkel, 2004] and the Plackett–Burman [Plackett *et al.*, 1946] designs. *Ruggedness* can be quantified by ANOVA [Goeres *et al.*, 2005] and multiple comparison testing to assess if the variations in the factors cause statistical significant differences in the results. The results can be easily analyzed through box ploting. The significance from the post-hoc multiple comparison can be given in the plots to help graphic analysis.

4.4 *Reproducibility*

Reproducibility relates to the ability of the procedure to be reproduced by others, namely in different laboratories. In *reproducibility* testing, the results of a collaborative study between laboratories are usually summarized by a *reproducibility* standard deviation (S_R), which can be no smaller, and is usually significantly larger, than *Sr* [Goeres *et al.*, 2005]. S_R includes between-laboratory and within-laboratory variations.

Between-laboratory variation arises from factors such as variation in calibration standards, differences between local interpretations of the protocol, changes in equipment or reagent source, or environmental factors, such as differences in average climatic conditions. Collaborative trials directly estimate the variance of

between-laboratory biases [Thompson *et al.*, 2002; Annis *et al.*, 2005; Wallmann *et al.*, 2006; Huys *et al.*, 2010]. This is important to identify the causes of the differences among laboratories so that they may be controlled. Otherwise they will be summed into S_R [AOAC, 2002].

5 Materials and Methods

5.1 Microorganisms

5.1.1 P. aeruginosa Relevance in Biofilm Infections

P. aeruginosa is a member of the Gamma Proteobacteria class of Bacteria. It is a Gram-negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. The genus *Pseudomonas* is cleaved into eight groups and *P. aeruginosa* is the type species of its group, which contains 12 other members. *P. aeruginosa* is a free-living bacterium, commonly found in soil and water. However, it occurs regularly on the surfaces of plants and occasionally on the surfaces of animals [Todar, 2008-2012].

This bacterium is one of the favourite model organisms to study biofilm formation. Several reasons justify the preference for *P. aeruginosa*. First, there are many molecular tools available for this organism, including DNA arrays for genome studies. Second, the genus *Pseudomonas* has simple requirements for growth, tolerating a wide range of temperatures (4 - 42°C), facilitating laboratory experiments. Third, it is considered a representative organism for a diverse and important group of bacteria [Kjelleberg *et al.*, 2007]. Fourth, it is highly related to nosocomial infections. Actually, according to data from the US Centres for Disease control and Prevention (CDC) and the National Nosocomial Infection Surveillance System (NNISS), *P. aeruginosa* is the

second most common cause of nosocomial pneumonia, third most common cause of urinary tract infections and seventh most common cause of nosocomial bacteraemia [Aksoy *et al.*, 2008].

Specifically, biofilms of this bacteria are commonly related to cystic fibrosis pneumonia, an infection that afflicts patients with the recessive genetic disease CF [Costerton *et al.*, 1999]. The genetic defect in CF leads to the loss of the CF transmembrane regulator (CFTR) chloride channel in the apical membranes of epithelial cells [Cutting *et al.*, 2005]. This defect leads to persistent bacterial infections of the lungs. Most CF patients are colonized with *P. aeruginosa*, and eventually they succumb to the lung damage inflicted by the persistent bacterial infection, with a median life expectancy of about 30 years [Costerton *et al.*, 1999]. *P. aeruginosa* biofilms also cause dermatitis, soft tissue infections, bone and joint infections, contact lenses infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immune suppressed [Todar, 2008-2012].

P. aeruginosa is intrinsically resistant to many antimicrobial agents, including most β-lactams, the older quinolones, chloramphenicol, tetracycline, macrolides, co-trimoxazole and rifampin. The most important anti-pseudomonal agents include some β-lactams (ticarcillin, ureidopenicillins, piperacillin, cefoperazone, ceftazidime, cefepime, aztreonam, imipenem, and meropenem), aminoglycosides (gentamicin, tobramycin, netilmicin, and amikacin), and fluoroquinolones (of which ciprofloxacin remains the most active compound). Polymyxins, such as the AMP colistin, are also active, but they are usually considered only for MDR strains due to their higher toxicity [Aksoy *et al.*, 2008].

Also, pandrug resistant *P. aeruginosa* isolates, which are defined as resistant to carbapenems or to all antibiotics available for clinical use, are being reported with growing frequency. Most cases are seen in patients who were previously infected with *P. aeruginosa* and had been treated with long courses of multiple anti-pseudomonal antibiotics [Aksoy *et al.*, 2008].

Several traits of *P. aeruginosa* have been related to its capability of biofilm formation. The study of two *P. aeruginosa* mutants defective in the initial steps of biofilm formation, called "sad" (surface attachment defective) mutants, revealed that these bacteria need flagella, type IV pili and, therefore, motility to adhere well to the plastic surface used at the time [O'Toole *et al.*, 1998; Costerton *et al.*, 1999]. Indeed, it seems that the attachment itself can initiate the synthesis of the extracellular matrix. Studies with *P. aeruginosa algC*, *algD*, and *algU::lacZ* reporter constructs showed that the transcription of these genes, required for synthesis of the extracellular polysaccharide alginate, is activated after attachment to a solid surface [Davies *et al.*, 1995; Costerton *et al.*, 1999].

5.1.2 Strains, Preservation and Culture

In this work, there were used three strains of *P. aeruginosa*, namely: *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO1 and *P. aeruginosa* CGCT III. The use of three different strains enhances the study, comparing the response of each strain to antimicrobial treatment. All strains are conserved in a cryogenic stock at -80°C in Tryptic Soy Broth (TSB) media (30 g L^{-1} - Merck) with 20% glycerol. The strains were activated by streaking the cells in a Petri dish, or plate, containing TSA (TSB with Agar 12g L^{-1} - Merck). The plates were then incubated overnight at 37°C and then stored at 4°C. Each plate was used

for a week to pick colonies for the biofilm experiments. The colonies in TSA plates were not used as a stock and a fresh plate was grown each week from the cryogenic stock.

Cells were grown in TSB, whose composition comprehends: casein peptone (pancreatic), 17 g L^{-1}; dipotassium hydrogen phosphate, 2.5 g L^{-1}; glucose, 2.5 g L^{-1}; sodium chloride, 5 g L^{-1}; soya peptone (papain digest.), 3 g L^{-1}. TSB is considered a nonselective rich media and is commonly used to grow bacterial biofilms. The media was prepared with distilled water and autoclaved at 121°C for 15 min. prior to use.

5.2 Antimicrobial Peptides

5.2.1 Tachyplesin III

Tachyplesins are a group of AMPs isolated from horseshoe crabs' hemocytes. Tachyplesin III was first isolated from Southeast Asian horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda* [Muta *et al.*, 1990]. This peptide consists of 17 amino acids (KWCFRVCYRGICYRKCR-NH2) along with two disulfide bridges, six cationic residues and it has a unique arginine α-amide at the COOH terminal end. Tachyplesin III is considered a representative AMP with cyclic β-sheet and it exhibits broad-spectrum activity against both Gram-negative and Gram-positive bacteria, fungi and enveloped viruses, including extracellular HIV-1, at low concentrations. Because it is a potent and relatively small peptide, tachyplesin III is considered in structure/activity studies addressing novel infection therapeutics [Nakamura *et al.*, 1988; Hirakura *et al.*, 2002; Cirioni *et al.*, 2007; Minardi *et al.*, 2007]. Furthermore, tachyplesin III also plays a role in

pro-inflammatory response due to the formation of complexes with bacterial LPS [Nakamura *et al.*, 1988]. Recently, Hong *et al* revealed that tachyplesin I acts on the membrane by binding to the interface of anionic lipid membranes and undergoes fast uniaxial diffusion to cause membrane defects [Hong *et al.*, 2011].

5.2.2 Colistin

Polymyxins are non-ribosomally synthesized by the Gram-positive bacterium *Bacillus polymyxa*. They present themselves as pentabasic decapeptides and contain a cycloheptapeptide ring with a C9 or C10 hydrophobic fatty acid chain through a α-amide linkage [Mogi *et al.*, 2009]. Colistin (polymyxin E) was discovered in the late 1940s but soon its popularity diminished due to its nephrotoxicity and neurotoxicity. Recently, colistin's toxicity was dismissed and it has been proposed for the treatment of severe infections due to MDR Gram-negative bacteria [Li *et al.*, 2006; Lim *et al.*, 2010].

Commercial preparations of colistin consist of a mixture of colistins A and B, which differ from one another by their fatty acid residues [Landman *et al.*, 2008]. Colistin is bactericidal and its mechanism of action consists in binding to the LPS in the outer membrane of Gram-negative bacteria, disrupting it [Martti, 2010]. Today, it is primarily used in the last resort treatment of infections caused by *P. aeruginosa*, *Acinetobacter baumannii* and *K. pneumoniae* [Lim *et al.*, 2010]. Sadly, resistance to colistin has already been reported among all three of the mentioned organisms. Although the exact mechanism that causes colistin resistance is not clear, it is hypothesized that the *PmrA-PmrB* and *PhoP-PhoQ* genetic regulatory systems may play a role [Lim *et al.*, 2010].

5.2.3 *Lactoferricin B*

Lactoferricin B is an AMP with 25 amino acids released by pepsin cleavage of bovine lactoferrin, an 80 kDa iron-binding glycoprotein that is part of the immune system. Similar to other AMPs, lactoferricin B exerts its antimicrobial activity via a pore-forming mechanism in the microbial membrane [Hwang *et al.*, 1998]. It also inhibits bacterial growth by impairing intracellular activities without destroying membrane integrity or penetrating the cytoplasmic membrane [Haukland *et al.*, 2001]. It has been observed that lactoferricin B inhibits DNA, RNA and protein synthesis of *E. coli* at a sub-lethal concentration and induces filamentation during an SOS-response in bacteria [Ulvatne *et al.*, 2004]. These results suggest that lactoferricin B has multiple intracellular targets in bacteria. However, the exact intracellular targets of this AMP are still unknown [Tu *et al.*, 2011].

5.2.4 *AMP Storage and Solutions*

Colistin (Sigma), tachyplesin III and lactoferricin B (ChinaPeptides Co. Ltd) were purchased in a white powder formula. The three peptides were solubilised in phosphate buffered saline (PBS) with a pH of 7.2 and stored at -20°C as stock solutions of 1mg mL^{-1}. PBS is composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. The pH was adjusted to 7.2 by addition of HCl.

5.3 Phenotypic Analysis of Biofilms

5.3.1 Total Biomass Evaluation by Cristal Violet Staining

Biomass of the grown biofilms was quantified by the crystal violet (CV) staining method adapted from Stepanović *et al.* [2000]. CV is a basic dye that binds negatively charged surface molecules and polysaccharides in the EPS matrix [Peeters *et al.*, 2008]. The biofilms were fixed with 200 µL of pure methanol (Vaz Pereira, Portugal) per well for 15 min. Next, the plates were emptied, air dried and the fixed bacteria were then stained for 5 min with 200 µL of CV (Merck, Portugal) per well. Excess stain was rinsed off under running tap water. After the plates were air dried, the dye bound to the adherent cells was re-suspended with 200 µL of 33% (V/V) glacial acetic acid (Merck, Portugal) per well. The obtained solution was measured for its OD at 570 nm using a spectrophotometer with microtiter plate reader (Tecan, Model Sunrise-basic Tecan, Austria).

5.3.2 Respiratory Activity Evaluation by XTT Method

The respiratory activity of the biofilms was measured through the 3,3′-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3is(4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) colorimetric assay, modified from Stevens and Olsen [1993]. XTT is a colourless or slightly yellow compound that becomes brightly orange when reduced. This reduction is carried out by a mix of cellular effectors. The sensitivity of the XTT assay is greatly improved by the usage of an intermediate electron carrier, N-methyl dibenzopyrazine methyl sulphate (PMS). PMS helps drive XTT reduction and the formation of its formazan derivative, which is soluble and can be used in real-time assays [ATCC, 2011]. In bacteria,

XTT is reduced by enzymes of the respiratory chain localized in the cytoplasmic membrane [Peeters *et al.*, 2008].

Biofilms were washed as described above and 200 µL of a combined solution of 150 µg mL^{-1} XTT (Sigma-Aldrich) and 10 µg mL^{-1} PMS (Sigma) was added to each well. Afterwards, the plates were left to incubate for 3 h, at 37°C and 120 rpm, in the dark. The biofilm activity was determined through measurement of the OD at 490 nm.

5.3.3 Cell Viability Determination by CFU Counting

Colony-forming unit (CFU) is an estimate of viable bacterial numbers [Bitton, 2011]. In theory, one viable cell, defined as able to multiply via binary fission, can give rise to a colony through multiplication. However, having solitary cells can be tricky, and most likely the progenitor of the colony was a mass of cells deposited together. Also, many bacteria grow in chains (*e.g. Streptococcus*) or clumps (*e.g. Staphylococcus*). Fortunately, this is not the case with *Pseudomonas.* So, generally, the estimation of microbial numbers by CFU will undercount the number of living cells for these reasons.

In order to determine the number of CFUs, the grown biofilms were detached by sonication (220 V, 50/60 Hz, 6 min) and were later serially diluted. After plating the serial dilution on TSA, the plates were incubated at 37°C overnight prior to enumeration. The number of viable bacterial cells was expressed as log (CFU cm^{-2}). To calculate this value, the formula $CFU\ cm^{-2} = \frac{200\ \mu\text{L} \times \text{no.of colonies} \times \text{dilution factor}}{10\ \mu\text{L} \times 1.53\ \text{cm}^2}$, where 200 µL correspond to the volume of a well, 10 µL to the volume of cell solution used to plate the cells in TSA and 1.53 cm^2 to the wells' area.

5.4 General Steps of the Statistical Analysis

The statistical analysis of the data retrieved from the work done for this dissertation followed five consecutive steps: i) identification and removal of outliers; ii) check if the data follows a normal distribution; iii) check if the data has homogeneity of variance; iv) analysis of variance, to assess if there are statistical differences in the experiments; v) multiple comparisons tests to assess which experiments are different. The statistical analysis was performed in Microsoft Excel© and GraphPad© software.

5.4.1 Outlier Identification

There are several approaches to outlier detection. Grubbs' test [Grubbs, 1969; Stefansky, 1972], also called the extreme studentized deviate (ESD) method, is probably the most popular method to identify an outlier. Although it is designed to detect only one outlier at a time, Grubbs' method can be extended to multiple detection quite easily: if an outlier is found, it is removed and the remaining values are tested again.

Grubbs' test is based on the assumption that the data, except the potential outlier(s), are sampled from a Gaussian distribution. The test calculates the ratio Z (difference between the outlier and the mean) divided by the *SD*. If Z is large, the value is far from the others. Critical values of Z for a data set of N samples are tabled; if the calculated Z is greater than the critical value, then the p value is less than 0.05 and the sample is considered an outlier [GraphPad-Software, 2012a].

However, Grubbs' test has a drawback which is caused by the presence of pairs of outliers, *e.g.* if one outliers is present, it is detected; however, the presence of a second outlier prevents the outlier test from

finding the first one [GraphPad-Software, 2012c]. This is called masking. For this reason, some outliers may have to be manually identified.

5.4.2 *Normality*

The ANOVA, the *t* test, and many other statistical tests assume that sampled data come from a population following the Normal distribution. Biological data never follow a Gaussian distribution precisely, because a Gaussian distribution extends infinitely in both directions, and so it includes both infinitely low negative numbers and infinitely high positive numbers. However, many kinds of biological data follow a bell-shaped distribution that is approximately Gaussian [Utts *et al.*, 2007; GraphPad-Software, 2012b].

Formal procedures that assess the underlying distributions of a data set are called Goodness of Fit (GoF) tests. The Kolmogorov-Smirnov (K-S) test [Chakravart *et al.*, 1967] is an example of these tests. K-S GoF test uses the cumulative distribution function (CDF) approach, therefore belonging to the class of "distance tests". K-S is among the best distance tests adequate for small samples and is widely computerized in statistical packages. The test starts by sorting the data and estimating the parameters for the normal distribution (mean and standard deviation (SD)). Next, the test calculates the theoretical function and the empirical function at each data point. If the difference between the assumed or theoretical distribution and the empirical distribution is small, then the assumed distribution is likely correct [RAC, 2003].

5.4.3 Homogeneity of Variance

Analysis of variance, like ANOVA, and *t* tests assume that the data to be analyzed come from populations that have equal variances. In this case, the sample is called homoscedastic. This assumption is not very important when all the groups have the same (or almost the same) number of subjects, but is very important when sample sizes differ.

Levene's test [Levene, 1960] is used to test if samples have equal variances. One advantage of Levene's test is that it does not require normality of the underlying data. However, Levene´s test is not included in the common software like GraphPad, Excel or Minitab. However, it is possible to perform Levene's test in Excel following a few steps. A new table was created where each value was defined as the absolute value of the difference between the actual value and median of its group. Then, a one-way ANOVA was run on this new table. By subtracting each value from its group median, the difference between group averages is eliminated. If the ANOVA comes up with a small *p* value, equal variance is not guaranteed [GraphPad-Software, 2012d].

5.4.4 Analysis of Variance

ANOVA [Snedecor *et al.*, 1980] provides statistical testing that tells us whether or not the means of several data samples are all equal, and therefore it is a generalized *t* test to more than two groups. This test assumes that the data is normally distributed and so it is considered a parametric test. There are several types of ANOVA: the one-way ANOVA is used to test for differences among two or more independent groups (means); the factorial ANOVA, *e.g.* two-way ANOVA, is used to measure the effect of more than one independent variable (in the case of two-way ANOVA, two variables) on the response variable; the

repeated measures ANOVA is used when the same subjects are used for each treatment; and the multivariate analysis of variance (MANOVA) is used when there is more than one response variable. The most common test used is the one-way ANOVA, which compares all means simultaneously and informs if means differ. The one-way ANOVA is further divided into fixed-effect ANOVA and random-effect ANOVA: fixed-effect ANOVA is used to test for differences among the means of the particular groups from which data was collected; random-effect ANOVA assumes that there was a random selection of groups from an infinite (or at least large) number of possible groups, and that the aim is to reach conclusions about differences among all the groups, even the ones not included in the experiment. Graphpad and Excel used only fixed-effect ANOVA, since it is more common with biological data analysis. If the ANOVA leads to significant results (low *p*-value) it means that at least one of the means is different from the others. To assess which group(s) of data is causing the difference, there must be a follow up with multiple comparison procedures.

However, data is not always normally distributed and other tests of variance have to be applied. Nonparametric tests are used when assumptions about the underlying dataset fail or are questionable. In the case of variance analysis, the nonparametric equivalent of the ANOVA test is the Kruskal-Wallis test [Kruskal *et al.*, 1952]. As in ANOVA, when the Kruskal-Wallis test leads to significant results (low *p*-value) it means that at least one of the samples is different from the others. The test does not identify where the differences occur or how many they are.

5.4.5 Multiple Comparisons

The analysis of variance, either parametric or nonparametric, tests the null hypothesis "population means of all of the groups/treatments are equal" to the alternate hypothesis that is "at least two population means are not equal". However, most of the times, researchers want to know which groups differ from the others and how much is that difference. The tests used after analysis of variance to assess these differences are called post-hoc tests. Tukey's test [NIST, 2012a] is a multiple comparison procedure generally used in conjunction with ANOVA (post-hoc) to find which means are significantly different from one another, by comparing all possible pairs of means. In GraphPad, the multiple comparison test available after performing the non-parametric Kruskal-Wallis is the Dunn's test [Dunn, 1964] and not Tukey's. So, for non-normal data, the multiple comparison test used is Dunn's instead of Tukey's.

5.5 Establishment of a Standardized Operating Procedure

5.5.1 Preliminary Studies

The design of a SOP for anti-biofilm AMP studies is based on previous work of our research group [Lopes, 2010; Lopes *et al.*, 2011; Machado *et al.*, 2012]. Specifically, the protocol followed for biofilm growth was a modified microtiter plate test proposed by Stepanovic´ *et al.* [2000]. However, some modifications were made. As in many protocols, time of growth appeared as "overnight" growth, which is not appropriated in a SOP since it is open to interpretation what the actual

number of hours is. So, a previous study was made in which the time of overnight growth of the inoculums was tested (see section 5.5.4) to choose a time interval, which came to be 14 h. Also, the first generation of the cells was also chosen in this section, instead of the usual stock of cells on TSA plate for a month with weakly re-growth, hence using up to the fourth generation. The results of these two tests and the discussion of the choice can be found in the subsection 6.2.

5.5.2 Biofilm Formation of P. aeruginosa ATCC 10145

The initial protocol for the formation of the biofilms of *P. aeruginosa* ATCC 10145 was chosen based on previous works carried out with these conditions. The conditions were later modified to attest for the *ruggedness* of the protocol. Overnight cultures (14 h) of *P. aeruginosa* ATCC 10145 in TSB (37°C, 120 rpm) were centrifuged (9,000 g; room temperature; 5 min) and re-suspended in TSB, until reaching 2x10^{6} CFU mL^{-1} (by optical density (OD) 640 nm measurement). 100 µL of the bacterial suspensions prepared previously were transferred to a 96 well microtiter plate at which 100 µL of TSB was added, making a total of 200 µL per well and diluting the initial cell concentration to half (1x10^{6} CFU mL^{-1}). The plates were then incubated aerobically on a horizontal shaker at 120 rpm, at 37°C, for 24 h to promote biofilm formation. Afterwards, the content of each well was removed by plate inversion and the wells were washed twice with 200 µL of sterile water to remove any planktonic/non-attached cells. The plates were air dried and the remaining attached bacteria were analyzed in terms of adhered biomass, metabolic activity and number of viable cells.

5.5.3 Repeatability Evaluation Study

A series of experiments were conducted to estimate the *repeatability* of the procedure to be proposed. A total of 19 experiments were carried out for biomass and respiratory activity determination, with 8 replicates, and 17 experiments for viable cell determination, with 4 replicates. To assess *repeatability*, the one-way-ANOVA was performed and *Sr* calculated.

5.5.4 Ruggedness Evaluation Study

For the *ruggedness* tests, the settings of eight operational factors were purposely altered – temperature of growth (inoculum and biofilm), agitation speed (inoculum and biofilm), time of growth (inoculum and biofilm), initial cell concentration and cell generation. Three settings were selected for each factor (Table 5-1).

Table 5-1: The three settings for each of the six operating conditions studied in the *ruggedness* test

Operating Conditions	Settings		
	Low	Medium	High
Cell generation	**1st**	2nd	3rd
Time of growth (inoculums) (h)	**14**	18	22
Time of growth (biofilm) (h)	22	**24**	26
Initial cell concentration (biofilm) (CFU mL^{-1})	$4x10^5$	**$1x10^6$**	$2.5x10^6$
Temperature (inoculums and biofilms) (^oC)	35	**37**	39
Agitation speed (inoculums and biofilms) (rpm)	100	**120**	140

Note: SOP values are shown in bold.

A complete factorial experimental design for testing all combinations of the settings would entail 3^8=6561 experiments plus some replicates for purposes of calculating *Sr*. Instead of running a

complete factorial design, due to limited time for experimentation, each factor was altered alone.

The goal in these experiments is:

i) Assess if the non-use of fresh cells from cryogenic stock changes biofilm phenotype and the changes that occur in growing generations. Here, the 1st generation is considered the one from cryogenic stock, the 2nd generation is the one that is re-streaked a week later and the 3rd one is re-streaked from the 2nd a week after.

ii) Assess if slight variations to the time of inoculum growth and of biofilm growth affects biofilm phenotypes and in which extent.

iii) Evaluate if slight variations to the initial cell concentration to form the biofilms causes statistical differences in the final biofilm phenotype.

iv) Assess if cells activated at a different temperature (temperature of inoculum growth) from the one used to grow the biofilm would produce different biofilm phenotypes. In the case of biofilm's temperature of growth, variations were done to assess if this would implicate different biofilms after 24h of growth.

v) Test if variations in the agitation speeds in the growth of the inoculum and of the biofilm caused the final biofilm to present different phenotypes.

5.6 Control of Biofilms with AMP Combinations

5.6.1 Prophylactic Approach

As stated previously, prophylactic strategies are meant to prevent biofilm formation and experimentation covers biofilm growth in the presence of AMPs. This method is used as a first advance in the study of biofilm control to assess the capability of the antimicrobials, in this case AMPs, to prevent the attachment of the cells to the surface of adhesion or to prevent biofilm growth and maturation itself. AMPs that show good prophylactic capabilities can be used in surface coatings and also later tested to assess their therapeutic value.

In this work, prophylaxis of the AMPs colistin, lactoferricin B and tachyplesin III was assessed by altering the biofilm growth protocol described previously. Besides *P. aeruginosa* ATCC 10145, these tests were also performed in *P. aeruginosa* PAO1 and *P. aeruginosa* CGCT III. The conditions of the proposed SOP were met, aside from the time of overnight growth of the inoculums, which was ≈ 17 h instead of 14 h. Also, when the 100 µL of the bacterial suspensions were transferred to the 96 well microtiter plates, 100 µL of the AMP solution in the double concentration of choice was added, instead of TSB. Therefore, the AMPs could act on the cells before biofilm is formed. Afterwards, biofilms were analysed by the three methods already described. All biomass and respiratory activity experiments were conducted at least 3 times with at least 6 replicates; cell viability was repeated at least 1 time with 4 replicates, due to lack of time.

5.6.2 *Therapeutic Approach*

Therapeutic strategies meant to treat existing biofilms and experiments are conducted by putting in contact a mature biofilm and the antimicrobial in question. As referred, mature biofilms are rather tricky to overcome mainly due to the presence of the EPS matrix, among other factors already explained. Therapeutic studies are in demand since prevention of biofilm formation not always works and patients are colonized by these structures, being afterwards hard to eradicate.

In this experiment, the three AMPs colistin, lactoferricin B and tachyplesin III were again tested against mature biofilms of the three strains *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO1 and *P. aeruginosa* CGCT III. The protocol for biofilm growth was preformed as described; however, alterations were performed previously to phenotypic analysis. After the content of each well was removed by plate inversion and the wells were washed twice with 200 µL of sterile water to remove any planktonic/non-attached cells, 200 µL of AMP solution in the concentration of choice was added. Time of contact varied between 30 min and 2 h. Afterwards, the content of each well was again removed by plate inversion, the wells washed once with 200 µL of sterile water and phenotypic analysis was conducted.

6 Results and Discussion

6.1 *Repeatability* of the SOP

The *repeatability* of the proposed SOP required statistical evaluation of results concerning biomass, respiratory activity and cell viability methods of biofilm analysis (see section 5.5.3 for details). Table 6-1 resumes the outcomes of the statistical tests performed on the data from the *repeatability* experiments.

Outliers were found for the three methods used to analyse biofilms. Some of the outliers eliminated in the biomass method were not identified by the Grubb's test due to the masking effect (more details in section 5.4.1). For this reason, some of the outliers in the data set from the biomass method were manually discarded.

Normality was calculated for each experiment at a time, so the p value stated for normality in Table 6–1 was the lowest encountered in all experiments; most experiments had a $p > 0.15$.

In the case of homoscedasticity, in the cell viability dataset, homogeneity of variance was not encountered in the unit log(CFU cm^{-2}), so the log was retrieved and homogeneity of variance was found with the data in CFU cm^{-2}. Literature states that when Levene's test fails, often the best approach is to transform the data to logarithms or reciprocals, restoring equal variance [GraphPad-Software, 2012d].

Table 6-1: Statistical evaluation of the *repeatability* of the proposed SOP

		Method		
		Biomass	**Respiratory Activity**	**Cell Viability**
Number of	**Experiments**	18	18	17
	Replicates	8	8	4
	Data Points	144	144	68
	Outliers Removed	11*	3	2
Normality		Yes ($p \geq 0.03$)	Yes ($p \geq 0.05$)	Yes ($p \geq 0.02$)
Homogeneity of Variance		Yes ($p > 0.05$)	Yes ($p > 0.05$)	Yes ($p > 0.001$)**
Mean		1.16	0.172	4.46×10^{6}
SD		0.370	0.057	1.64×10^{6}
ANOVA	***p* value**	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	σ^2_e	0.030	0.002	1.02×10^{12}
	σ^2_{btw}	0.911	0.016	7.06×10^{12}
	σ^2_t	0.294	0.005	2.02×10^{12}
	σ^2_{total}	0.324	0.006	3.03×10^{12}
	Sr	0.970	0.131	2.84×10^{6}

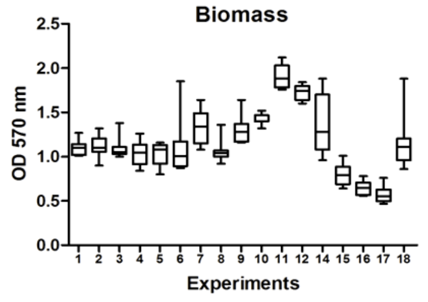
Note: Mean and SD values are in OD570nm and OD490nm units for CV and XTT methods, respectively, and in CFU cm^{-2} for CFU method. All values are presented with at least 3 significant digits * 9 out of 11 outliers were manually identified. ** Homogeneity of variance was found with the data in CFU cm^{-2}.

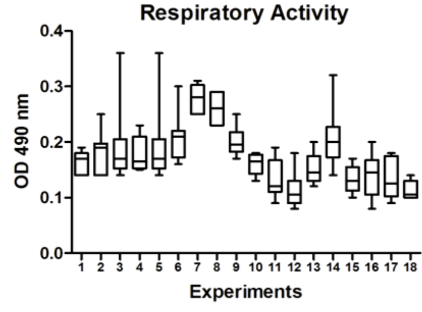
The ANOVA test revealed that it is not possible to guarantee data *repeatability* in any of the three methods of biofilm analysis, *i.e.* biomass, respiratory activity and cell viability methods. The *p*-value encountered for the three methods ($p < 0.0001$) was statistically very significant, which means that the null hypothesis (that is, the equality of means for the different experiments) is rejected with a 99.99% confidence level. This means that at least one experiment was different from the others, regardless the method of biofilm analysis. Analyzing the *Sr* values, it is visible that these are higher than desirable, since they are not far apart from the mean values. For biomass analysis, the *Sr* is

0.97, indicating that 90.7% of variability is attributed to differences between experiments and 9.32% to error variability within experiments. For respiratory activity, *Sr* is 0.131, indicating that 75.7% of variability is due to differences between experiments and 24.3% due to errors within experiments. Finally, *Sr* is 2.84×10^6 for cell viability, with 66.5% of variability due to differences between experiments and 33.5% to errors within experiments. It is evident that the differences between the experiments played the major part in the variability of the three methods. That is, even though in the same experiment the data was not very variable, when repeated the data was not similar, suggesting that the operating procedure is still affected by uncontrolled, unaccounted, or uncontrollable variability. However, before hypothesizing causes or countermeasures, it is necessary to assess if these differences are present in only one experiment or more.

To know how many experiments present significant differences, a Tukey's multiple comparison test was performed on the three datasets and all pairs of means were compared (Table C, Table D and Table E in Annex II). As observable in box plot representation (Figure 6–1), several experiments are quite different from the rest. The median values for some experiments are very different, as seen by the line in the box plots, along with the general data dispersion given by the boxes.

Several reasons can be pointed out to justify this *repeatability* problem. Although the conditions of the proposed SOP were controlled, since experiments are dealing with living organisms, variability is indulged at some extent, *i.e.* it is likely that even in the most controlled environment results show discrepancies, which are inherent to the evolving and behaviour of the microorganism community rather than the operating procedures. In fact, the first 5 to 6 experiments had low variability, which leads to believe that the error factor appeared later.





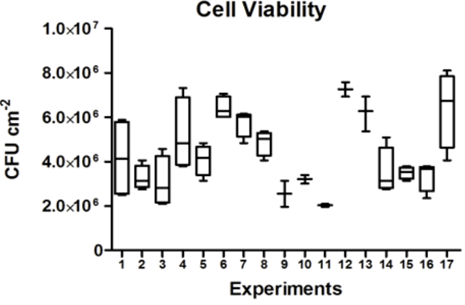


Figure 6-1: Data dispersion of the datasets for *repeatability* for the three methods of analysis. Each plot represents experimental data as box-plots.

Also, the phenotypic test methods here described represent themselves a source of variability since no SOP is yet proposed for them. Random and uncontrollable variables, such as ambient temperature and humidity, age of the reagents used and differences between batches of reagents can influence the results. Actually, one of the conditions of the *repeatability* testing was not met because experiments were not completed in a short period of time due to material and time limitations. Since the same operator was involved in every testing,

following all steps equally every time, it is unlikely that the error has a human source.

6.2 *Ruggedness* of the SOP

6.2.1 *Cell Generation*

Generally, the viable storage period of bacteria is increased as the storage temperature decreases; however, the time that a culture remains viable in a given storage condition is also dependent upon the bacterial strain. It is inevitable that cells die during storage but this should be minimized as much as possible. Bacterial cultures that are used regularly (daily or weekly) can be stored on agar plates at 4°C from 4 to 6 weeks [Thermo-Scientific, 2012]. It is common to researchers to re-use these plates with colonies for about a month and to recur to cryogenic stocks only monthly. However, at 4°C, cells are still growing and their metabolism is not suspended, as it happens at lower temperatures. So, colonies still grow without nutrient replacement for a week, and may start excreting toxic products or even suffer some adaptation to the conditions.

In the Kruskal-Wallis and Dunn's test results (Figure 6–2), there are some differences in the phenotype of the biofilms originated from the three generations, mainly in terms of biomass ($p < 0.05$ for 2nd and 3rd generations) and respiratory activity ($p < 0.01$ for 3rd generation). In both cases, the biomass and respiratory activity tend to increase from generation to generation. It seems that the older the cells are, the more active they become. Since the initial cell concentration used was the same, the number of viable cells in the end was not very different.

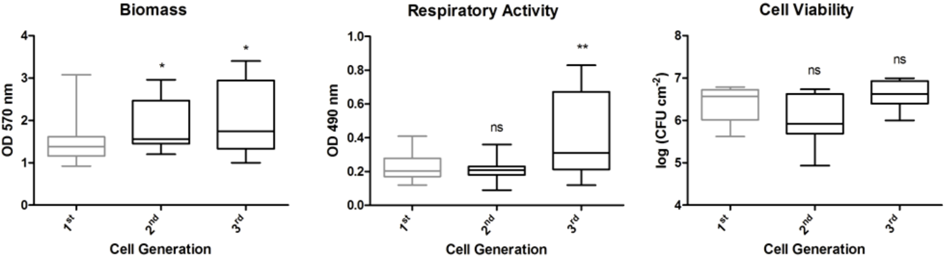


Figure 6-2: Influence of the cell generation on the biofilm phenotype. Each plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

The experiment showed that in order to obtain similar results, the biofilm growth protocol must assume the use of only one cell generation, logically the first one. Re-streaking of the cells is not a step to be considered in the growth of *P. aeruginosa* ATCC 10145 biofilms in microtriter plates.

6.2.2 Time of Inoculum Growth

The time of growth of the inoculums is not always specified and is often referred as "overnight" growth, and researchers have to give this their personal interpretation and choose the number of hours. Sometimes, the number of hours is not followed in different experiments because researchers do not give much importance to this early step of the progress.

The Kruskal-Wallis and Dunn's test results for biomass, respiratory activity and cell viability show that different times of inoculum growth do not influence greatly the results (Figure 6–3).

Statistically differences were only found in biomass for the 22h time of growth ($p < 0.05$). Inoculums have the purpose of activating the cells that were preserved and dormant and to allow the cells to adapt to

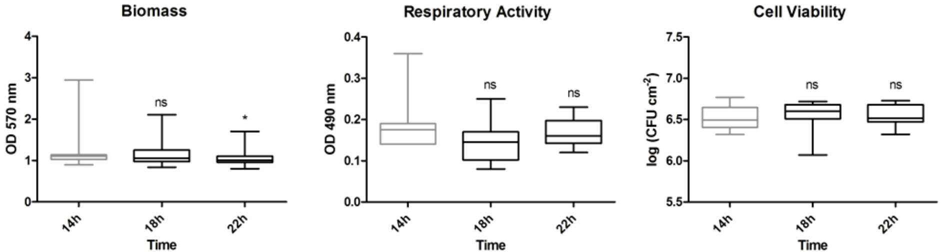


Figure 6-3: Influence of the time of inoculum growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

the media. The growth of the inoculums is usually stopped just before reaching the stationary phase of bacterial growth. This way, cells are still in the log phase of growth, where they are most actively proliferating. *P. aeruginosa* has its log or exponential phase between 8h to 15h of growth time [Guadarrama *et al.*, 2005; Schleheck *et al.*, 2009]. This means that 22h of inoculum growth may cause cells to enter stationary and even to the death phase. This may explain why the biomass is lower at 22h.

6.2.3 Time of Biofilm Growth

The time of growth of the biofilm is probably one of the major variables when trying to obtained similar phenotypes. In terms of biomass, the expected was to obtain growing values of OD throughout time, since the cells would have more time to divide and produce extracellular matrix. However, the Kruskal-Wallis and Dunn's test results obtained (Figure 6–4) show a tendency to augment from 22h to 26h and from 24h to 26h but from 22h to 24h, the values drop. This, however, is not considered statistically significant, as seen in the plot by the Dunn's test results. In the case of respiratory activity and cell viability, the values, although not significantly different, tend to increase. The viable

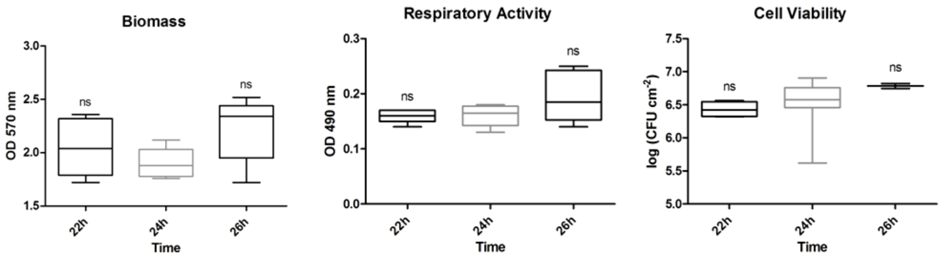


Figure 6-4: Influence of the time of biofilm growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

cells had more time to divide, hence the growing values. In the case of respiratory activity, is could be a representation of the growing stress in the cells due to lack of nutrients or due to the oxidative stress suffered by mature biofilms, or simply because the cell number is higher.

6.2.4 Initial Cell Concentration

Measuring cell concentration is not an exact method and calibration curves vary between laboratories and between spectrometers and different wavelengths can be used. The use of different, yet close, initial cell concentrations showed no statistical differences in the Kruskal-Wallis and Dunn's test for any of the three methods of analysis (Figure 6–5). This means that small differences in cell number and matrix production are not sufficient to delay biofilm formation and reduce or enhance significantly the final cell number, respiratory activity or biomass.

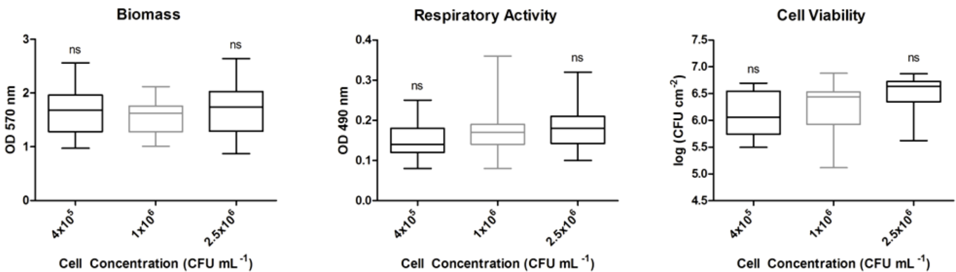


Figure 6-5: Influence of the initial cell concentration on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

6.2.5 Temperature of Inoculum Growth

Temperature of inoculums growth can greatly influence the metabolic state at which cells are introduced in the new media to grow and form biofilms. Differences from the temperature of inoculums growth to the temperature of biofilm growth caused statistically significant differences to be encountered in the three methods of analysis after Kruskal-Wallis and Dunn's test (Figure 6–6).

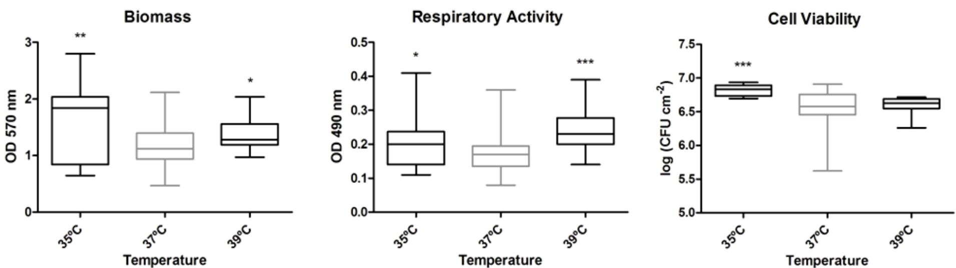


Figure 6-6: Influence of the temperature of inoculum growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

Biomass and respiratory activity seem to be lower at 37°C of inoculation. One explanation is related to the fact that this test was not conducted in the same day. Since the protocol seems to lack

repeatability, mainly between experiments, the differences found may be, at some extent, explained by day variations.

This happened due to equipment deficiency since the analysis of two different temperatures in the same day would require two identical incubators. Since the *repeatability* was assessed after these tests, there was no way to know that these differences would occur. Also, these tests were scattered in time due to, once again, equipment usage limitations, and conditions were not allowed to be changed often.

6.2.6 Temperature of Biofilm Growth

The temperature of biofilm formation can affect the cell's metabolic state and hence affect the rate of biofilm formation, among other factors. Statistical differences were found in the three methods described after Kruskal-Wallis and Dunn's test analysis (Figure 6–7).

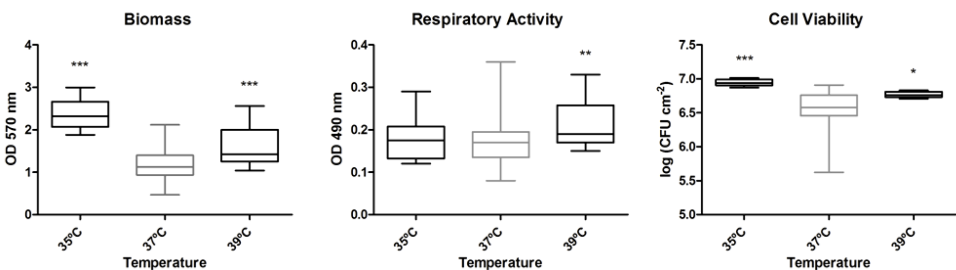


Figure 6-7: Influence of the temperature of biofilm growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

The major differences are in biomass ($p < 0.001$, first plot in Figure 6–7) and for cell viability at 35°C ($p < 0.001$, third plot in Figure 6–7). Once again, the differences may be due to *repeatability* problems and experiment execution in different days may cause comparison to be faulty.

6.2.7 Agitation Speed of Inoculum

The agitation speed of a cell culture can influence its metabolic state, since it is related to the degree of oxygen solubilisation into the media. Also, too much agitation can make the sheer forces to damage the cells [Najafpour, 2007]. Small variations of the agitation speed on the inoculums growth caused only statistical differences in the biomass of the biofilm at 140 rpm, after Kruskal-Wallis and Dunn's test analysis (Figure 6–8).

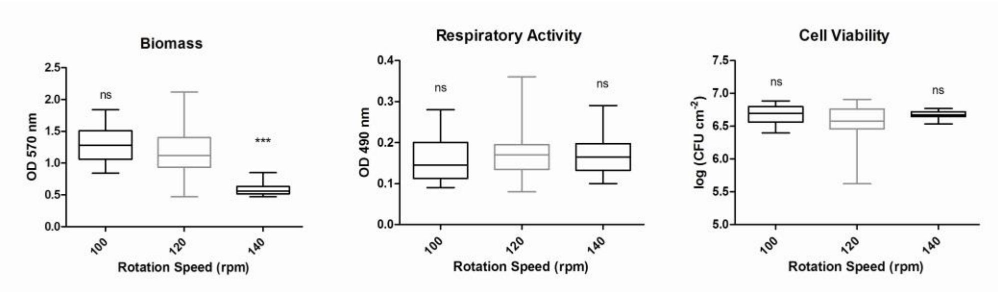


Figure 6-8: Influence of the rotation speed in the inoculum growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

Biomass was lower ($p < 0.001$) when the inoculum was grown at 140 rpm. We can see that this variable does not have great influence on the resulting biofilm in the other cases. However, as in the case of the analysis of temperature influence, these experiments were not conducted in the same day, and this difference could be explained by the data day variations.

6.2.8 Agitation Speed of Biofilm

Possibly, the agitation speed during biofilm growth can influence its formation due to oxygen solubilisation differences and the sheer forces. Indeed, statistical differences were found, after Kruskal-Wallis

and Dunn's test analysis, for biomass, respiratory activity and cell viability only for 140 rpm (Figure 6–9).

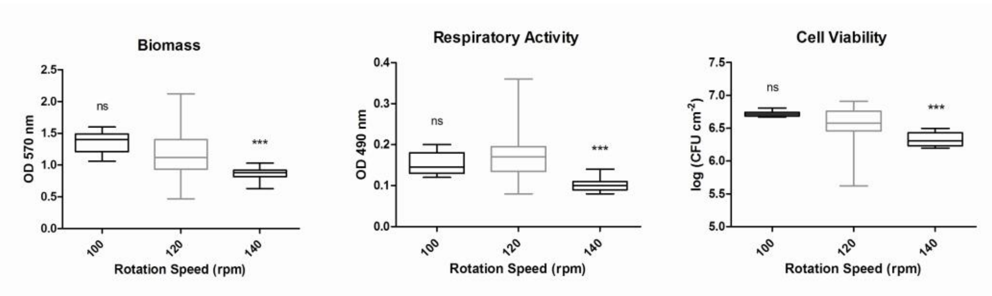


Figure 6-9: Influence of the rotation speed in the biofilm growth on the biofilm phenotype. The plot shows the data as box-plots. The control (SOP condition) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

The plot shows that biomass decreases with speed. This may be explained by the damage of the cells by the sheer forces and due to the impairing of the attachment of the cells to the surface if the agitation is too high. Even after cell adhesion, biofilm formation could be delayed because the sheer force would remove the cells from the upper layers. Respiratory activity seems reduced. As the agitation speed rises, the solubilised oxygen increases, which makes this a not expected result. However, maybe the agitation sheer forces cause some stress in the cells or maybe just because there was less biofilm. The number of viable cells also decreased with agitation, probably due to the difficulties in attachment, as referred.

However, instead of recommending the lowest possible agitation for the growth of biofilms, the agitation is needed in order to imitate the real life environment where biofilms are grown.

6.3 Control of Biofilms with AMP Combinations

6.3.1 Prophylactic Approach with Colistin and Lactoferricin B

The growth of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilms in the presence of the AMPs colistin and lactoferricin B, alone or combined, is characterized in terms of biomass formation, respiratory activity and cell viability (Figure 6–10).

In the case of the individual action of the AMPs, 1 µg mL^{-1} of colistin produced better results than 8 µg mL^{-1} of lactoferricin B, *i.e.* it was more effective, requiring lower concentration to inhibit biofilm formation:

i) In terms of biomass, colistin caused a reduction of 51% in *P. aeruginosa* ATCC 10145 and 35% in *P. aeruginosa* CGCT III. In *P. aeruginosa* PAO 1, the results were weaker, since there was an increase of 15% in biomass.

ii) For respiratory activity, the reductions were of 35% in *P. aeruginosa* ATCC 10145, 7% in *P. aeruginosa* PAO1 and 73% in *P. aeruginosa* CGCT III.

iii) Viable cells were reduced by 1.8 log in *P. aeruginosa* ATCC 10145, 0.23 log in *P. aeruginosa* PAO1 and 2.4 log in *P. aeruginosa* CGCT III.

These outcomes are in accordance to the MIC and MBC values [Lopes, 2010]: lactoferricin B has a MIC of 2 µg mL^{-1} and a MBC of 8 µg mL^{-1}; and colistin as a MIC of 2 µg mL^{-1} for *P. aeruginosa* 10145 and of 1 µg mL^{-1} for the other two strains, and a MBC of 4 µg mL^{-1}. So, in planktonic state, colistin is effective in half the concentration of lactoferricin B.

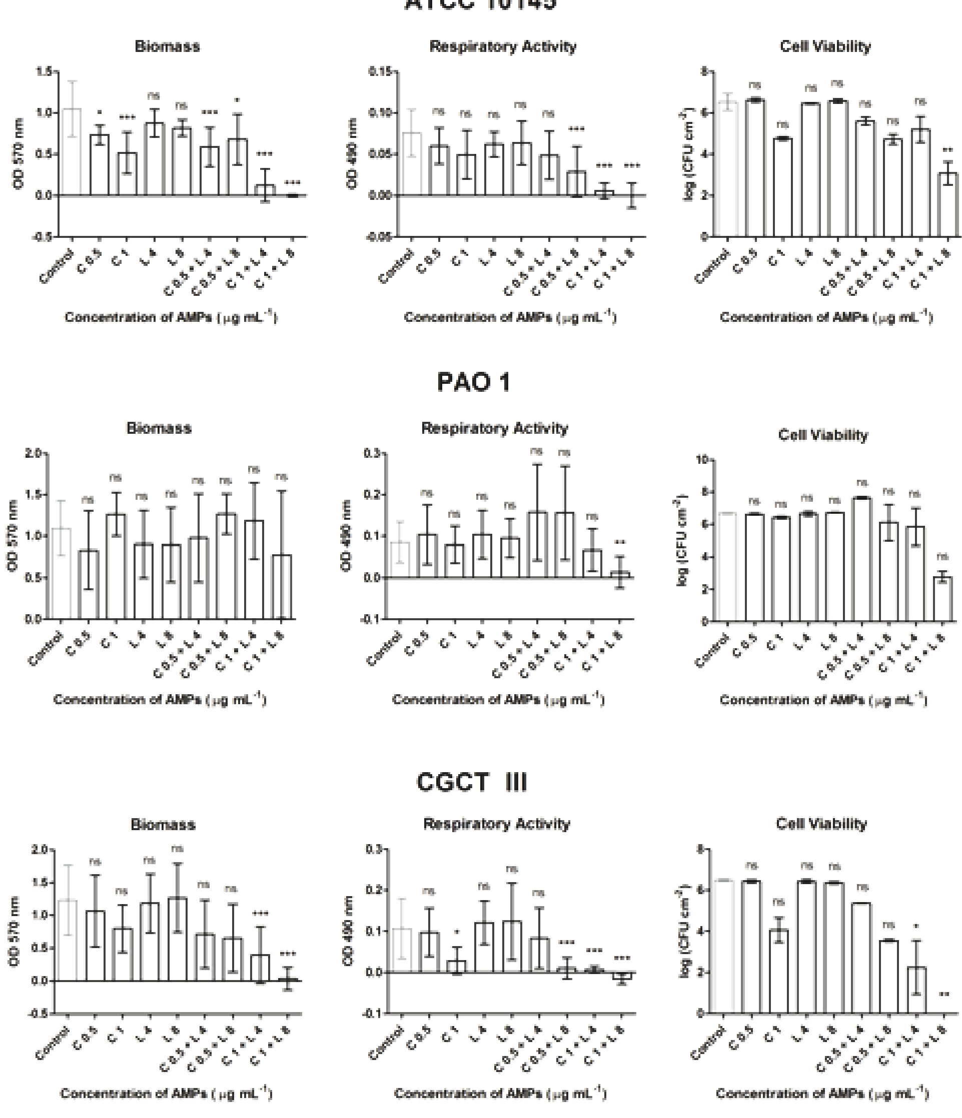


Figure 6-10: Inhibition of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilm formation with the AMPs colistin and lactoferricin B, alone and combined. Bars show the mean value and vertical lines show the SD value. The control experiment (no treatment) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns). "C" stands for colistin and "L" for lactoferricin B.

The combination of colistin (1 µg mL^{-1}) with lactoferricin B (8 µg mL^{-1}) showed the best results for all AMP solutions used, namely:

i) A total inhibition of biofilm formation, in terms of biomass and respiratory activity for *P. aeruginosa* ATCC 10145 and *P. aeruginosa* CGCT III, and a 29% biomass inhibition and 84%

respiratory inhibition for the less susceptible *P. aeruginosa* PAO 1.

ii) The number of viable attached cells suffered also a noticeable decrease with a 3.5 log reduction in *P. aeruginosa* ATCC 10145, a 3.9 log reduction in *P. aeruginosa* PAO 1 and total (6.5) log reduction in *P. aeruginosa* CGCT III.

Overall, the *P. aeruginosa* CGCT III strain was the most susceptible to treatment combining colistin and lactoferricin B. This biofilm susceptibility assay showed that *P. aeruginosa* biofilms are not able to grow in the presence of the combination of 1 μg mL^{-1} of colistin and 8 μg mL^{-1} of lactoferricin B, with the exception of the *P. aeruginosa* PAO 1 strain. However, even with no visible biomass through the CV method, there were still some viable cells that were capable of attachment to the surface of the microtiter plate. This might indicate that these AMP combinations act mainly on the bacterial cell and not on the adhesion mechanisms/proteins. The adhered cells did not produce the exopolymeric matrix or the matrix was too thin and was washed out during the removal of the planktonic cells from the microtiter wells. Furthermore, the absence of respiratory activity for all the strains means that even though some cells were still present, there they were not metabolically active.

Both AMPs are known to act through the destabilization of the bacterial membrane [Hwang *et al.*, 1998; Martti, 2010], and lactoferricin B is also known to act on intracellular targets [Haukland *et al.*, 2001; Ulvatne *et al.*, 2004]. This may explain why the synergetic outcome was advantageous, since colistin may be helping lactoferricin B to enter more rapidly inside the cell.

6.3.2 Prophylactic Approach with Tachyplesin III and Lactoferricin B

The growth of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilms in the presence of the AMPs tachyplesin III and lactoferricin B, alone or combined, is characterized in terms of biomass formation, respiratory activity and cell viability in Figure 6–11.

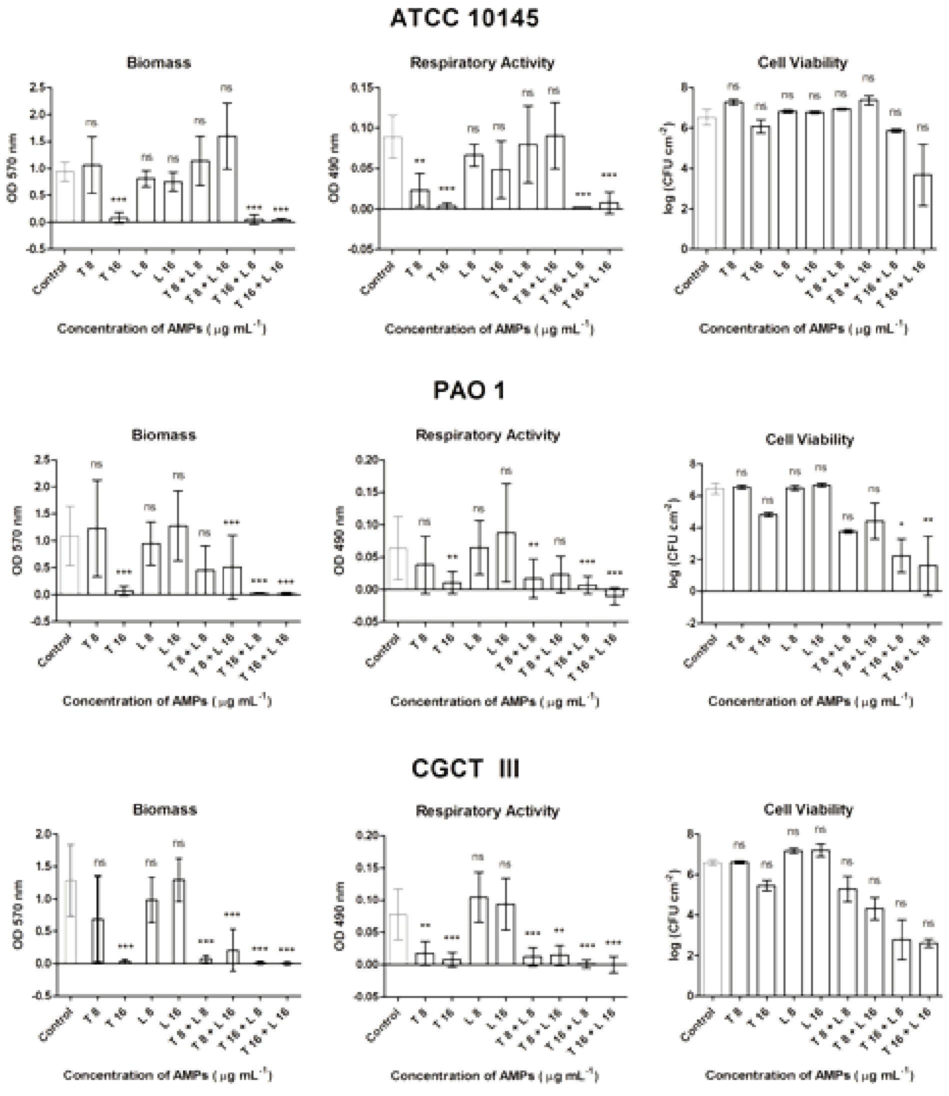


Figure 6-11: Inhibition of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilm formation with the AMPs tachyplesin III and lactoferricin B, alone and combined. Bars show the mean value and vertical lines show the SD value. The control experiment (no treatment) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns). "T" stands for tachyplesin III and "L" for lactoferricin B.

Tachyplesin III alone had better results than lactoferricin B, *i.e.* it was more effective in preventing biofilm growth in the same concentrations. The MIC values for tachyplesin III and lactoferricin B are the same, 2 µg mL^{-1}. However, the MBC concentration is 4 µg mL^{-1} for tachyplesin III and 8 µg mL^{-1} for lactoferricin B. In principle, tachyplesin III seems more effective on biofilms than lactoferricin B. In fact, 16 µg mL^{-1} of tachyplesin III showed the following results:

i) Biomass was reduced by 91% in *P. aeruginosa* ATCC 10145, 94% in *P. aeruginosa* PAO 1 and 98% in *P. aeruginosa* CGCT III.

ii) Respiratory activity suffered a reduction of 96% in *P. aeruginosa* ATCC 10145, 83% in *P. aeruginosa* PAO 1 and 90% in *P. aeruginosa* CGCT III.

iii) Cell viability was reduced only by 0.5 log in *P. aeruginosa* PAO 1, 1.6 log in *P. aeruginosa* PAO 1 and 1.1 log in *P. aeruginosa* CGCT III.

Interestingly, cell viability was not significantly reduced, as we would expect given the low biomass. This might indicate that tachyplesin III acts on the cell and not on the adhesion mechanisms/proteins. The adhered cells did not produce the exopolymeric matrix or the matrix was too thin and was washed out during the removal of the planktonic cells from the microtiter wells; and the cells although viable, had their metabolic activity strongly reduced.

The addition of lactoferricin B in the same concentration was able to improve the later results:

i) Biomass was reduced by 96% in *P. aeruginosa* ATCC 10145, 99% in *P. aeruginosa* PAO 1 and 100% in *P. aeruginosa* CGCT III.

ii) The respiratory activity was totally inhibited for the three strains.

iii) The viable cells were reduced by 4.2 log in *P. aeruginosa* ATCC 10145, 4.9 log in *P. aeruginosa* PAO 1 and 4 log in *P. aeruginosa* CGCT III.

These results suggest synergy between the two AMPs since lactoferricin B alone had very poor results, with some values being higher in its presence than in the control for the three methods. As referred, lactoferricin B acts both on the membrane [Hwang *et al.*, 1998] and on intracellular targets [Haukland *et al.*, 2001; Ulvatne *et al.*, 2004], and tachyplesin III is known to act on the membrane [Hong *et al.*, 2011]. The action of tachyplesin III may be helping lactoferricin B exert its action inside the cell.

6.3.3 Therapeutic Approach with Colistin and Lactoferricin B

This section presents some preliminary studies in *P. aeruginosa* biofilm treatment with the AMPs colistin and lactoferricin B. Since good outcomes were observed for the inhibition of biofilm growth in the previous section, the concentrations used here were the same and 2-fold higher, in order to assess if higher concentrations had better therapeutic outcomes.

The first approach tested two different treatment durations: 2h and 30 min (Figure 6–12). Note that the concentrations used on CGCT III were not the same, but as stated, these are preliminary results that will have to be further investigated. It is interesting to notice that in the three strains, a longer time of treatment (2h) caused the values of biomass to be higher than the ones for a short treatment (30 min). This could be explained by an adaptation of the cells to the AMP treatment occurring after 30 min. Also, the effect of the AMPs might be slow and

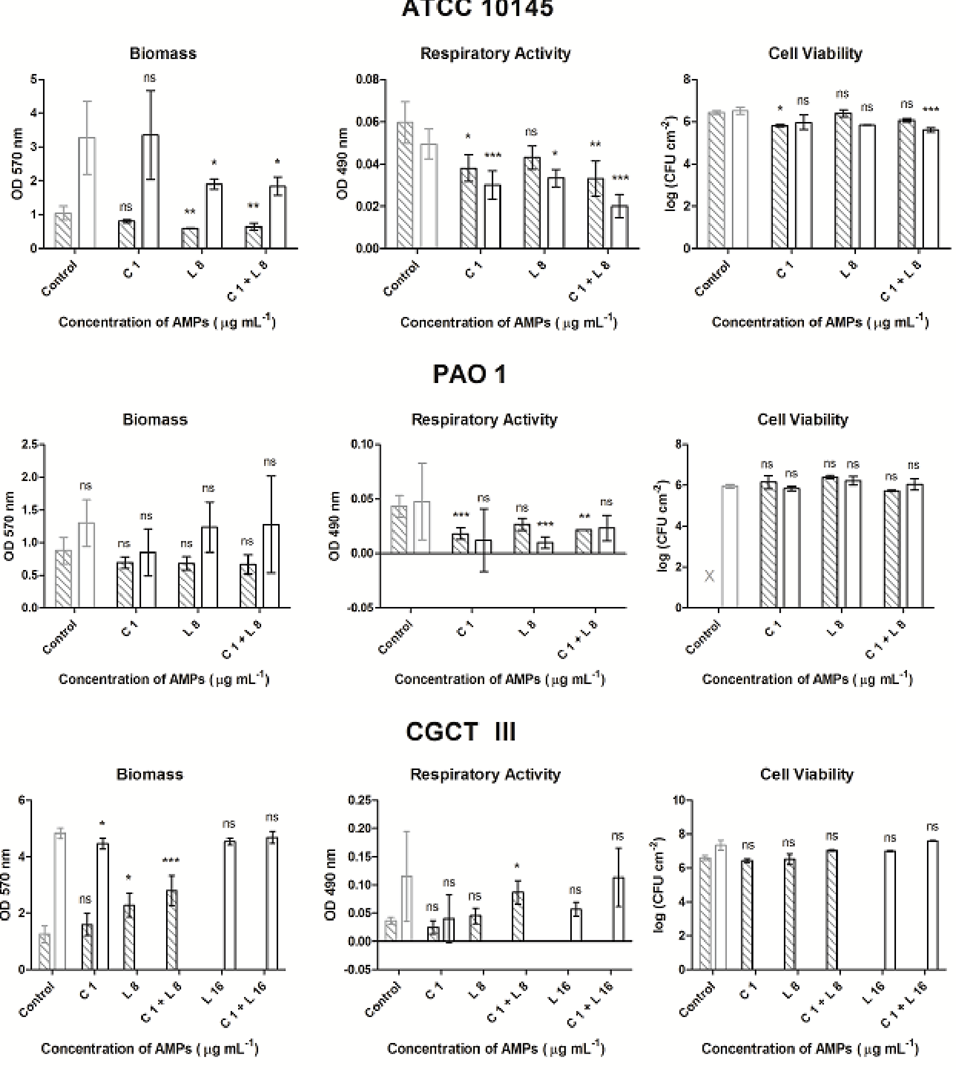


Figure 6-12: Treatment of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilms with the AMPs colistin and lactoferricin B, alone and combined, for 30 min or 2h. Bars show the mean value and vertical lines show the SD value. The control experiment (no treatment) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns). "C" stands for colistin and "L" for lactoferricin B. The control value for cell viability in *P. aeruginosa* PAO1 is missing (X).

not effective in the times given; so, the biofilm can be further growing during the 30min and 2h, hence the higher results in the second case.

In fact, in the case of *P. aeruginosa* CGCT III the values of biomass, respiratory activity and cell viability increase in the presence of the AMPs, comparing with the control. It is possible that the synergism

observed in the prophylaxis method could become an antagonism in the action of these two peptides over mature biofilms.

The treatment appears to result in good statistical differences in *P. aeruginosa* ATCC 10145 with 1 μg mL^{-1} of colistin and 8 μg mL^{-1} of lactoferricin B:

i) Diminution of biomass by 38% after 30 min and 43% after 2h.
ii) Reduction of respiratory activity by 45% after 30 min and of 60% after 2h.
iii) Low reduction of cell viability only by 0.37 log after 30 min and 0.9 log after 2h.

Given these results, the next step chosen was to use the longer time of treatment (2h) but increase the AMPs' concentration to see if there was an improvement in biofilm eradication (Figure 6–13). The best results were found for *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO 1 with the combination of 2 μg mL^{-1} of colistin and 16 μg mL^{-1} of lactoferricin B:

i) Eradication of biomass by 65% in *P. aeruginosa* ATCC 10145 and 52% in *P. aeruginosa* PAO 1.
ii) Eradication of respiratory activity by 83% in *P. aeruginosa* ATCC 10145 and 54% in *P. aeruginosa* PAO 1.
iii) Eradication of viable cells by a 1.6 log in *P. aeruginosa* ATCC 10145 and 0.88 log in *P. aeruginosa* PAO 1.

P. aeruginosa CGCT III did not suffer very significant reduction in any concentration; however, the best one was with 1 μg mL^{-1} of colistin, which caused 8% eradication of biomass and 65% reduction of respiratory activity. The value for cell viability is still to be assessed.

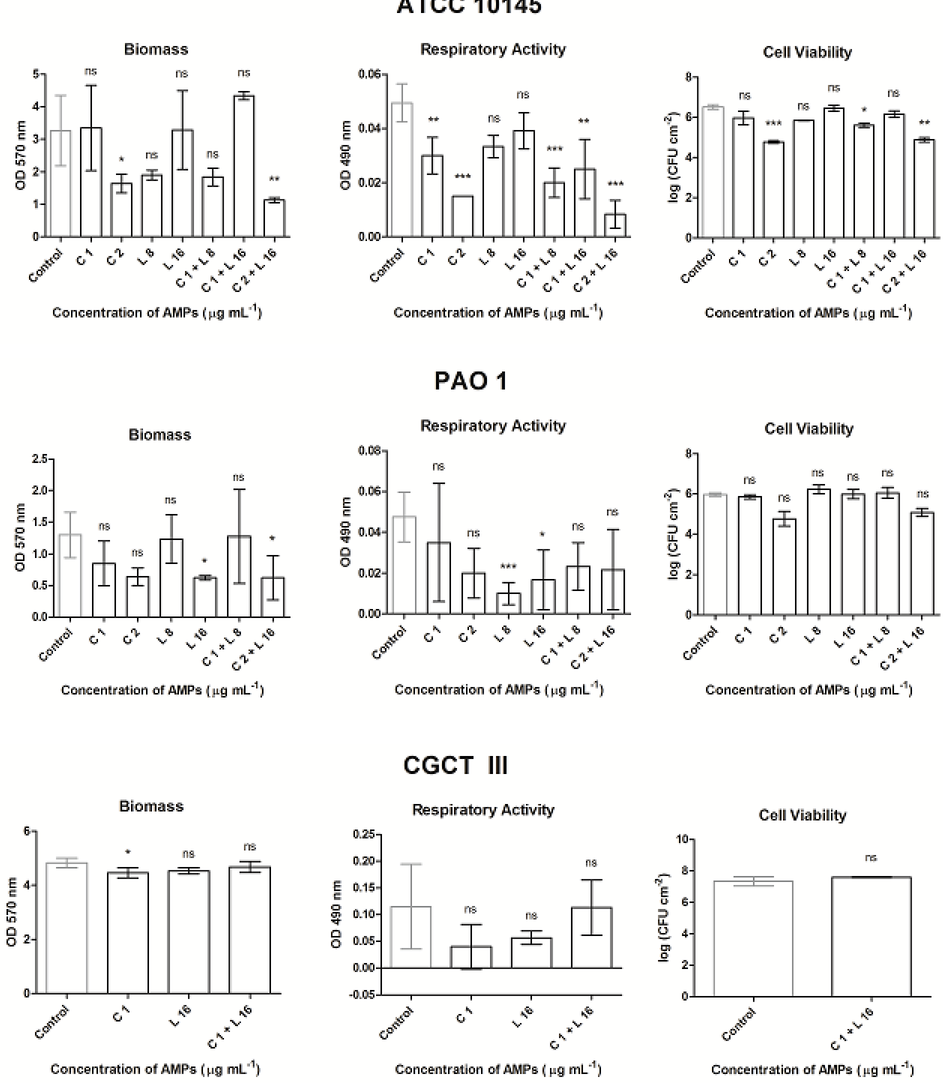


Figure 6-13: Treatment of *P. aeruginosa* ATCC 10145 , *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilms for 2h with the AMPs colistin and lactoferricin B, alone and combined. Bars show the mean value and vertical lines show the SD value. The control experiment (no treatment) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns). "C" stands for colistin and "L" for lactoferricin B.

Colistin alone had better results in *P. aeruginosa* CGCT III, *i.e.* was able to better reduce biomass and respiratory activity, than the combination of colistin and lactoferricin B. This, again, may suggest a form of antagonism between these two AMPs when acting on mature biofilms of this strain.

6.3.4 Therapeutic Approach with Tachyplesin III and Lactoferricin B

The results of the combinations of the AMPs tachyplesin III and lactoferricin B on biofilm eradication are shown in Figure 6–14. Since good outcomes were observed for the inhibition of biofilm growth in the previous section, the concentrations used here were the same and 2-fold higher, to assess if an increase in AMP concentration would cause better biofilm eradication. The time of treatment chosen was 2h, given the good results in the previous section, especially for *P. aeruginosa* ATCC 10145.

Despite the very good inhibition activity of 16 µg mL^{-1} tachyplesin III in the prophylactic approach, this peptide does not show the same results when applied to already established biofilms. Similarly to the treatment with colistin and lactoferricin B, the strain *P. aeruginosa* CGCT III showed no statistically differences for treatments with AMP combinations. Regarding the other two strains, *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO 1:

i) There was a 59% decrease in biomass and 30% decrease in respiratory activity in *P. aeruginosa* ATCC 10145 for 16 µg mL^{-1} of tachyplesin III and lactoferricin B. Interestingly, by doubling the AMPs concentration to 32 µg mL^{-1}, the results were not so good.

ii) *P. aeruginosa* PAO 1 showed no statistically significant differences, aside from biomass decrease by 26% for 32 µg mL^{-1} of tachyplesin III and lactoferricin B.

Overall, in *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO 1, the tendency is to obtain lower values of biomass, respiratory actibity and cell viability from lactoferricin B to tachyplesin III and from these two to their combination. However, in the case of *P. aeruginosa* CGCT

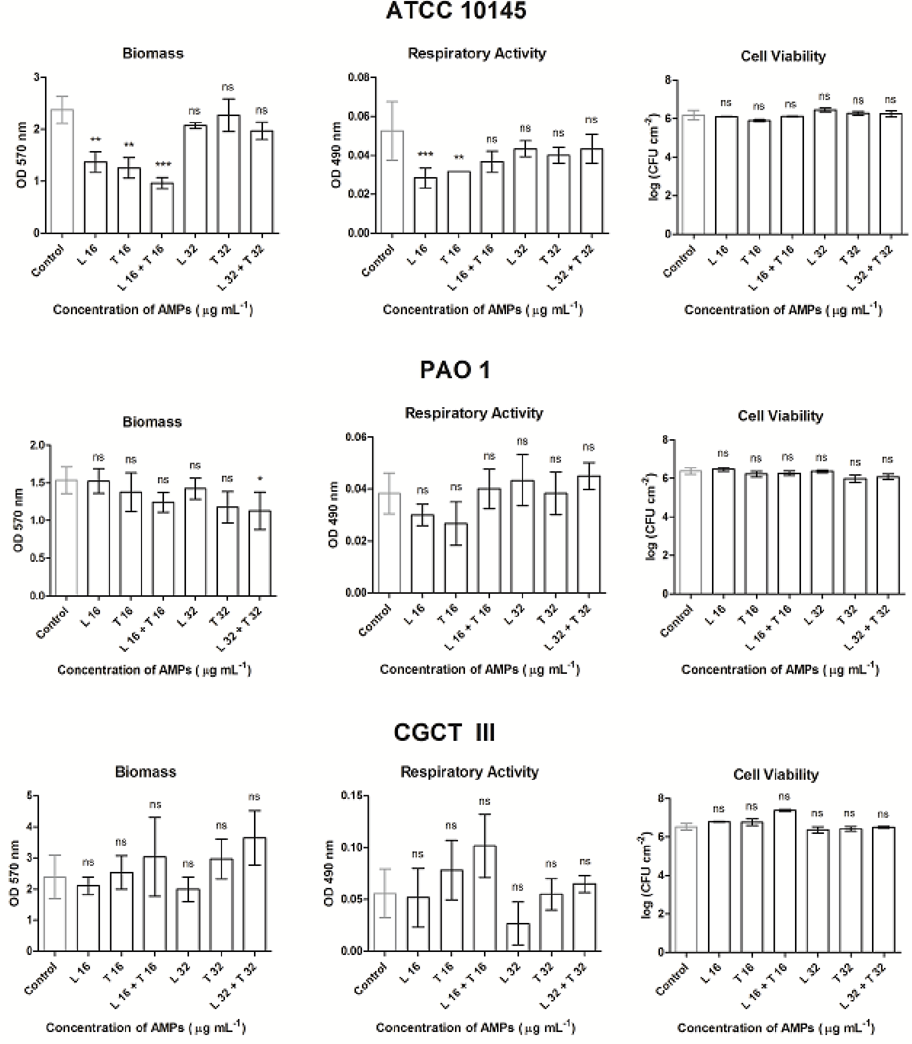


Figure 6-14: Treatment of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III biofilms for 2h with the AMPs tachyplesin III and lactoferricin B, alone and combined. Bars show the mean value and vertical lines show the SD value. The control experiment (no treatment) is in grey. The differences comparing with the control are represented by the *p*-value from the Dunn's test as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns). "T" stands for tachyplesin III and "L" for lactoferricin B.

III, the value augments in this order. If some kind of antagonistic association is being held, it would be interesting to study why is happens and why only in this strain.

7 Conclusions and Future Work

P. aeruginosa biofilms are one of the most important study subjects when it comes to biofilm infections with clinical relevancy. They are related to CF pneumonia and other infections, either biomaterial related or not. These bacteria are resistant to almost all known antibiotics, and pandrug resistant *P. aeruginosa* isolates have been related recently. The emerging of these and other biofilm infections in the nosocomial scenario and their recalcitrance to treatment with traditional antimicrobials has raised the interest of studying other antimicrobial compounds with new mechanisms of action. AMPs have been gaining interest in biofilm control since they have a general unspecific mode of action which retards the development of resistance. However, these types of biofilm control studies lack standardization, which impairs the comparison of results.

In this dissertation, the two main objectives were:

i) Initiate the establishment of a SOP for the growth of *P. aeruginosa* ATCC 10145 biofilms in 96 well microtriter plates by analyzing the protocols' *repeatability* and *ruggedness*.

ii) Application of the AMPs colistin, tachyplesin III and lactoferricin B, alone and in combination, in the control of biofilms of the strains *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO 1 and *P. aeruginosa* CGCT III, applying a prophylactic and a therapeutic approach.

7.1 Establishment of a Standardized Operating Procedure

One of the objectives of this dissertation was to make a starting point to a standard protocol for the growth of biofilms of *P. aeruginosa* ATCC 10145 in 96 well microtiter plates. A set of primary experimental conditions was chosen as first-to-be-standardized, and a number of experiments were designed to characterize the protocol statistically, namely in terms of *repeatability* and *ruggedness*.

Concerning *repeatability*, the calculation of the *Sr* showed that there were significant differences in the data, majorly caused by between experiment variance. By graphical analysis of the data dispersion, this was visible. In order to make this protocol valid, the changing factor(s), yet unknown, must be controlled and future work must address this. The variability may, however, lie on the living organism, and the strain used must be compared with older and other stocks or analysed to see if a mutation is the cause.

In terms of *ruggedness*, all variables, except for the initial cell concentration, had some influence on the final biofilm phenotype, mainly in terms of biomass. This means that the conditions of this protocol must be tightly controlled so that researchers can have comparable results.

Future work relating this topic will address the:

i) Analysis of the intervals of these variables in which results are not compromised. This will make the confidence interval in which the method can be performed.

ii) Also, after *repeatability* and *ruggedness* are established, the *reproducibility* of the method must be analysed by using different operators and different laboratories, both national and international.

7.2 Control of Biofilms with AMP Combinations

The other objective of this dissertation was to study the biofilm behaviour in the presence of the AMPs colistin, tachyplesin III and lactoferricin B. This was done following a prophylactic and a therapeutic approach.

7.2.1 Prophylactic Approach

The prophylactic approach showed good synergistic outcomes in the three strains, using small peptide concentrations:

i) Colistin (1 µg mL^{-1}) with lactoferricin B (8 µg mL^{-1}) resulted in: total inhibition of biofilm formation, in terms of biomass and respiratory activity in *P. aeruginosa* ATCC 10145 and *P. aeruginosa* CGCT III; and caused a almost 4 log reduction in *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO 1 and total reduction in *P. aeruginosa* CGCT III, in terms of cell viability.

ii) The combination of tachyplesin III (16 µg mL^{-1}) and lactoferricin B (16 µg mL^{-1}) attained the best results: biomass and respiratory activity were virtually inexistent and the cell viability lowered for over 4 log for all three strains.

These outcomes suggest that the proposed AMP combinations should be considered as effective antimicrobial agents in *P. aeruginosa* prophylactic treatments. Future work will address the:

i) Evaluation of the effectiveness of these combinations in surface coating, *i.e.* preventing the attachment of *P. aeruginosa* cells by treating the surfaces with the AMPs.
ii) Testing other AMP combinations.
iii) Testing mixed biofilms of *P. aeruginosa* with other clinical relevant species.
iv) Study of the biofilms' phenotype more profoundly, applying microscopic observations, live/dead assays and transcriptomic and proteomic analysis, in order to investigate the AMPs mode of action and synergies.

7.2.2 Therapeutic Approach

The therapeutic approach, although still preliminary, showed that the AMPs were not as active over already established biofilms as they were preventing biofilm growth. Still, promising results were encountered:

i) Colistin (2 µg mL^{-1}) and lactoferricn B (16 µg mL^{-1}) combination after 2h of treatment caused: biomass eradication of 65% in *P. aeruginosa* ATCC 10145 and 52% in *P. aeruginosa* PAO; and respiratory activity reduction by 83% in *P. aeruginosa* ATCC 10145 and 54% in *P. aeruginosa* PAO 1.
ii) Tachyplesin (16 µg mL^{-1}) and lactoferricin B (16 µg mL^{-1}) combination made *P. aeruginosa* ATCC 10145 to suffer a 59% decrease in biomass and 30% decrease in respiratory after 2h of treatment.

Overall, this synergetic evaluation suggests that the global anti-biofilm efficacy here shown is the result of the different bioactivities of the peptides .Further tests will address:

i) Replication and confirmation of these preliminary results.
ii) Testing of different time treatments and AMP concentrations.
iii) Testing other AMP combinations.
iv) Testing mixed biofilms of *P. aeruginosa* with other clinical relevant species.
v) Assessment of the development of resistance of these treated biofilms by testing treatment cycles.
vi) Study of the biofilms' phenotype more profoundly, applying microscopic observations, live/dead assays and transcriptomic and proteomic analysis, in order to investigate the AMPs mode of action and synergies.

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Annex I – Publications

This dissertation originated three publications:

Jorge, P., Lourenço, A. and Pereira, M.O. *New Trends in Peptide-Based Anti-Biofilm Strategies – Recent Achievements and Bioinformatics Approaches*, Biofouling, 2012 (28): 1033–1061.

Jorge, P., Lourenço, A. and Pereira, M.O. *New Prophylactic Strategies Against Pseudomonas aeruginosa Biofilms Based on Antimicrobial Peptide Synergies*, AMP2012 - Third International Symposium on Antimicrobial Peptides, Lille (France), June 2012.

Jorge, P., Pereira, M.O. and Lourenço, A. *Facilitating the Screening of AMP Literature Via Text Mining Strategies*, AMP2012 - Third International Symposium on Antimicrobial Peptides, Lille (France), June 2012.

Annex II – Antimicrobial Peptides

Table A: AMP sources and classes.

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
Mammals	Cathelicidins	• Gene encoded [Tomasinsig *et al.*, 2005] • 12–100 a.a. [Wang *et al.*, 2011] • Cathelin domain (highly conserved N-terminal region) [Bals *et al.*, 2003; Tomasinsig *et al.*, 2005] • Peptides with little similarity besides their precursor protein (hCAP18) [Smet *et al.*, 2005; Lai *et al.*, 2009]	• Expressed in leukocytes (neutrophils, monocytes, NK cells, T and B cells), epithelial cells of the testis, skin, gastrointestinal and respiratory tracts [Bals *et al.*, 2003; Smet *et al.*, 2005]	• Bacteria • Fungi [Cirioni *et al.*, 2006]	• Also produced by invertebrates, fish, birds, frogs and snakes [Cirioni *et al.*, 2006; Lai *et al.*, 2009]
	Defensins	• 3.5-6 kDa [Smet *et al.*, 2005] • 6 conserved cysteine residues, forming 3 disulfide bridges [Smet *et al.*, 2005] • 50 α-defensins and 90 β-defensins [Lai *et al.*, 2009] • 4α neutrophil defensins (HNP-1 to 4), 2α enteric defensins (HD-5 and H-6) and 4β epithelial defensins (hβD-1 to 4) (http://peptidesaustralia.com/peptides-and-proteins-with-antimicrobial-activity/)	• Expressed in monocytes, macrophages, keratinocytes and epithelial cells of the respiratory, digestive, urinary and reproductive systems [Lai *et al.*, 2009] • Stored in the neutrophils' granules and in Paneth cells [Lai *et al.*, 2009]	• Bacteria • Fungi • Protozoans • Viruses [Cederlund *et al.*, 2011]	• Antitumoral • Stimulate cell proliferation • Interferes with signal transduction pathways • Chemo-attract immune cells • Stimulate cytokine and adhesion molecules' expression
	Histatins	• 3-4 kDa • Histidine-rich (7 histidine residues) • Linear structure • [Oppenheim *et al.*, 1988; Smet *et al.*, 2005] • Histatin 5: random coil structure in	• Present in human saliva [Oppenheim *et al.*, 1988; Smet *et al.*, 2005] • Produced and secreted by the submandibular, sublingual and parotid glands [Smet *et al.*,	• Bacteria • Fungi [Oppenheim *et al.*, 1988; Smet *et al.*, 2005]	• Inhibit inflammatory cytokine induction from human fibroblasts • Inhibit host and bacterial enzymes implicated in periodontal disease

		aqueous solvents and α-helix structure in non-aqueous solvents [Raj *et al.*, 1998]	2005]		• Metallopeptide-like properties
Amphibians	Dermaseptins	• α-helical • Amphipathic • Undergo coil-to-helix transition upon binding to lipid bilayers • Signature pattern of a conserved Trp residue at position 3 and an AA(A/G)KAAL(G/N)A consensus motif in the midregion [Nicolas *et al.*, 2009]	• Produced by *Phyllomedusa*, *Pachymedusa*, *Agalichnis* and *Hylomantis* frogs [Nicolas *et al.*, 2009; Zhang *et al.*, 2010] • Synthesized and stored in the granular glands of the skin [Nicolas *et al.*, 2009]	• Bacteria • Yeast • Protozoa • Mollicutes • Enveloped viruses [Nicolas *et al.*, 2009]	• Antimalarial [Nicolas *et al.*, 2009]
	Phylloseptins	• 19–21 a.a. (1.7–2.1 kDa) • Highly conserved N-terminal region and C-terminal amidation • Amphiphilic [Leite *et al.*, 2005; Zhang *et al.*, 2010]	• Present in skin secretions of *Phyllomedusa* and *Hylomantis* frogs [Nicolas *et al.*, 2009]	• Bacteria • Protozoans • [Leite *et al.*, 2005; Zhang *et al.*, 2010]	-
	Magainins	• 23 a.a. [Zairi *et al.*, 2009] • α-helical [Zairi *et al.*, 2009] • Also known as PGS (peptide glycine serine) [Giovannini *et al.*, 1987; Zairi *et al.*, 2009]	• Present in the skin of the African clawed frog *Xenopus laevis* [Zairi *et al.*, 2009; Duclohier, 2010]	• Bacteria • Fungi • Viruses [Zairi *et al.*, 2009; Duclohier, 2010]	• Facilitate wound closure and reduce inflammation [Sai *et al.*, 1995; Duclohier, 2010] • Spermicidal [Reddy *et al.*, 1996] • Antitumoral [Duclohier, 2010] • Act as dimmers via the toroidal model [Duclohier, 2010]
Crustaceans	Tachyplesins	• 17 a.a. • Unique arginine α-amide at the COOH terminal end • [Nakamura *et al.*, 1988] • Disulfide-stabilized β-sheet or β-hairpin conformation • 2 cross-strand disulfide bonds • 6 cationic residues [Doherty *et al.*, 2006]	• Produced by horseshoe crabs' hemocytes [Nakamura *et al.*, 1988; Minardi *et al.*, 2007]	• Bacteria • Fungi • Viruses • [Nakamura *et al.*, 1988; Hancock *et al.*, 1998]	-

	Stylicins	• Anionic • Theoretical pI of 5 • 82 a.a. (8.9 kDa) • Proline-rich N-terminal region • C-terminal portion with 13 cysteine residues [Rolland *et al.*, 2010; Rosa *et al.*, 2010]	• Produced by the penaeid shrimp *Litopenaeus stylirostris* [Rolland *et al.*, 2010]	• Fungi [Rolland *et al.*, 2010]	• Bind LPS [Rolland *et al.*, 2010]
	Crustins	• 50 a.a. • 8 cysteine residues • Signal sequence at the N-terminus • Whey acidic protein (WAP) domain at the C-terminus • Tightly packed structure • 3 types: between the signal sequence and the WAP domain, type I crustins contain a cysteine-rich region, type II a glycine-rich and a cysteine-rich region and type III a proline-arginine-rich domain [Krusong *et al.*, 2012]	• Produced by various crustaceans, including several shrimp species [Krusong *et al.*, 2012]	• Bacteria [Krusong *et al.*, 2012]	-
Plants	Thionins	• ~5 kDa • Cysteine-rich • Basic • 2 types: α/β-thionins and γ-thionins (or plant defensins) [Stec, 2006]	• Produced by monocots (grains) and eudicots, including dicotyledonous plants (different species of mistletoe and *Pyrularia pubera*), and rosids (crambin) [Stec, 2006]	• Bacteria • Fungi [Stec, 2006]	-
	Cyclotides	• 30 a.a. • Amphipathic • Zero charge at neutral pH • Globular proteins • Head-to-tail cyclic backbone structure combined with a cystine knot [Garcia *et al.*, 2010; Smith *et al.*, 2011]	• Produced by large plants [Garcia *et al.*, 2010; Smith *et al.*, 2011]	• Bacteria • Viruses • Insects [Garcia *et al.*, 2010; Smith *et al.*, 2011]	• Ultra-stable to thermal, chemical, and enzymatic degradation [Garcia *et al.*, 2010; Smith *et al.*, 2011]

	Lipid Transfer Proteins (LTPs)	• 2 families: LTP1 (90-95 a.a., 9 kDa) and LTP2 (70 a.a., 7 kDa), with different patterns of disulfide bridges [Kido *et al.*, 2010]	• Produced by higher plants [Kader, 1996]	• Fungi • Bacteria [Kader, 1996; Wang *et al.*, 2004]	• Transfer phospholipids between a donor and an acceptor membrane [Kader, 1996; Kido *et al.*, 2010]
Bacteria	Bacteriocins	• Ribossomally synthesized • Class I bacteriocins: small (18–39 a.a.); called lantibiotics because of their lanthionine or β-methyllanthionine residues, formed during post-translational modification • Class II bacteriocins: very diverse group not subject to extensive posttranslational modification • Class III bacteriolysins: large, heat-labile proteins; catalyze the hydrolysis of bacterial cell walls [Sang *et al.*, 2008]	• Produced by different groups of bacteria and archaea [Gálvez *et al.*, 2007; Sang *et al.*, 2008]	• Bacteria of the same species (narrow spectrum) • Bacteria of other genera (broad spectrum) [Sang *et al.*, 2008]	-
	Gramicidins	• Non-ribosomally synthesized • Prototypical channel formers • Gramicidins A, B, and C: linear penta-decapeptides; β-helix (6.3 a.a. per turn); alternating L- and D-a.a. composition except for position 2 (Gly) • Gramicidin S: backbone-cyclized cationic decapeptide; antiparallel β-sheet [Mogi *et al.*, 2009; Yala *et al.*, 2011]	• Produced by the bacterium *Aneurinibacillus migulanus* (formerly known as *Bacillus brevis*) [Mogi *et al.*, 2009]	• Bacteria [Mogi *et al.*, 2009; Yala *et al.*, 2011]	• Used to study organization, dynamics, and function of membrane-spanning channels [Yala *et al.*, 2011] • Linear gramicidins enter lipid membranes forming a dimer channel that conducts a cation flow [Mogi *et al.*, 2009; Yala *et al.*, 2011]
	Polymyxins	• Non-ribosomally synthesized • Pentabasic decapeptides • Contain a cyclo-heptapeptide ring with a C9 or C10 hydrophobic fatty acid chain through a α-amide linkage [Mogi *et al.*, 2009]	• Produced by Gram-positive bacterium *Bacillus polymyxa* [Mogi *et al.*, 2009]	• Gram-negative bacteria [Martti, 2010]	• Polymyxins B and E (colistin) used in the last resort treatment of Gram-negative bacterial infections • Bind LPS [Martti, 2010]

Fungi	Peptaibols	• 10-20 a.a. • Non-ribosomally synthesized • High proportion of genetically non-coded amino acids (α-aminoisobutyric acids (Aibs) or α-methylalanines and isovaline (Iva)) • C-terminal alcohol [Sang *et al.*, 2008; Duclohier, 2010]	• Produced by the hyphomycetous fungi of the *Trichoderma* genus [Duclohier, 2010]	• Bacteria • Fungi [Sang *et al.*, 2008]	-
Synthetic and Modified	Specifically (or selectively) targeted antimicrobial peptides (STAMPs)	• Targeting peptide domain fused to an antimicrobial, or "killing", peptide domain, both functionally independent [Eckert *et al.*, 2006b; He *et al.*, 2010]	• Chemically synthesized with high-yield in vitro [Eckert *et al.*, 2006b]	• Bacteria [Eckert *et al.*, 2006b; He *et al.*, 2010]	• Increased killing potency, selectivity, and kinetics [Eckert *et al.*, 2006b]
	Synthetic antimicrobial peptidomimetics (SAMPs)	• Extremely short [Flemming *et al.*, 2009]	• Developed through modifications of the cationic antimicrobial peptides (CAPs) [Flemming *et al.*, 2009]	• Same as CAP used [Flemming *et al.*, 2009]	• Improved pharmacokinetic properties [Flemming *et al.*, 2009]
	Hydrophobic end-tagged AMPs	• Hydrophobic amino acid stretches at the C-terminal end [Malmsten *et al.*, 2011]	• Developed through hydrophobic C-terminal tagging of AMPs [Malmsten *et al.*, 2011]	• Bacteria [Malmsten *et al.*, 2011]	• Improved bactericidal potency [Malmsten *et al.*, 2011]
	Adepantins (Automatically designed peptide antibiotics)	• Based on anuran AMPs [Juretić *et al.*, 2009]	• *De novo* peptides, outputs of a Designer algorithm [Juretić *et al.*, 2009]	• Gram-negative bacteria [Juretić *et al.*, 2009]	• Designer algorithm uses an objective construction procedure, based on collected experimental data from anuran AMPs [Juretić *et al.*, 2009]
AMPs mimetics	Multimeric or Dendrimeric Peptides	• Branched polymers with peptides attached centrally to a template or core matrix • Higher local concentration of bioactive units [Giuliani *et al.*, 2011]	• *De novo* molecules with multiple peptide sequences added to an inner core usually of radially branched lysine residues [Bruschi *et al.*, 2010; Giuliani *et al.*, 2011]	• Bacteria [Bruschi *et al.*, 2010; Giuliani *et al.*, 2011] • Viruses [Giuliani *et al.*, 2011]	• Resistant to peptidases and proteases • Increased activity [Giuliani *et al.*, 2011]
	Peptoids or Amptoids	• Oligo N-substituted glycines • Isomers of AMPs with side chains attached to the backbone nitrogen	• *De novo* peptides contructed via a library of peptoid monomers tuned for	• Bacteria • Fungi [Giuliani *et al.*,	• Resistant to proteases • Reduced immunogenicity • Improved bioavailability

		rather than the α-carbon [Giuliani *et al.*, 2011]	hydrophobicity and side-chain charge [Chongsiriwatana *et al.*, 2008; Giuliani *et al.*, 2011]	2011; Kapoor *et al.*, 2011]	• Highly tunable side-chain chemistry • [Chongsiriwatana *et al.*, 2008; Giuliani *et al.*, 2011]
	Oligoacyllysines (OAKs)	• Composed of tandem repeats of acyllysines [Giuliani *et al.*, 2011] • Hydrophobic and cationic [Giuliani *et al.*, 2011]	• *De novo* peptides that mimic the primary structure and function of AMPs [Giuliani *et al.*, 2011]	• Bacteria [Sarig *et al.*, 2010; Giuliani *et al.*, 2011]	• Does not form defined secondary structures due to optimal rotational freedom of the carbon atoms in an acyl chain • Increased vivo efficacy • Less toxic [Giuliani *et al.*, 2011]
	Ceragenins	• Derivatives of bile acids with covalently attached amines • Cationic [Giuliani *et al.*, 2011]	• *De novo* molecules whose design was inspired by the aminosterol squalamine [Giuliani *et al.*, 2011]	• Bacteria [Savage *et al.*, 2002; Giuliani *et al.*, 2011]	• LPS and (lipoteichoic acid) LTA binding activity [Giuliani *et al.*, 2011]
	DL-Amino Acid Diastereomers	• Do not retain α-helical structure of AMPs [Vooturi *et al.*, 2010]	• *De novo* molecules obtained by incorporation of D-a.a. in the peptides [Vooturi *et al.*, 2010]	• Bacteria [Vooturi *et al.*, 2010]	• Increased activity • Resistant to proteases • Not cytotoxic on mammalian cells [Vooturi *et al.*, 2010]

Table B: Recent applications of AMPs on the control of biofilms

AMPs	Microorganisms	Minimal active concentration				Reference
		Pre-treatment		Therapy		
		B	P	B	P	
1010cys	*Pseudomonas aeruginosa*	14.2 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		-		[Gao *et al.*, 2011]
Aurein 2.5	*Bacillus subtilis* *Escherichia coli*	125 µM (MIC)	30 µM (MIC)	-		[Dennison *et al.*, 2009]
BMAP-28	*Staphylococcus aureus*	2 µg mL^{-1} (MIC) 4 µg mL^{-1} (MBC)		4 µg mL^{-1} (MIC) 16 µg mL^{-1} (MBC) 10 µg mL^{-1} (CVC infection in rat)	-	[Cirioni *et al.*, 2006]
C16G2	*Streptococcus mutans*	-	3 µM	25 µM	-	[Eckert *et al.*, 2006b]
Chrysophsin-1	*Streptococcus mutans*	-	4 µg mL^{-1} (MIC) 8 µg mL^{-1} (MBC)	16 µg mL^{-1}	-	[Wang *et al.*, 2012]
F2,5,12W	*Staphylococcus epidermidis*	2.5 µM	10 µM	40 µM	-	[Molhoek *et al.*, 2011]
G10KHc	*Pseudomonas aeruginosa*	-	0.5 – 29 µM (MIC)	100 µg mL^{-1}	-	[Eckert *et al.*, 2006a]
Gramicidin A	*Listeria ivanovii* *Enterococcus faecalis* *Staphylococcus aureus* *Escherichia coli* *Candida albicans*	20 µg mL^{-1} for 2h (cystamine self-assembled monolayers on gold surfaces)	-	-		[Yala *et al.*, 2011]
HBD3	*Staphylococcus aureus Candida albicans*	-	0.2 & 0.4 µg mL^{-1} 25 & 50 µg mL^{-1} (MIC & MBC)	0.2 µg mL^{-1} 25 µg mL^{-1} (on maxillofacial silicone elastomer disks)	-	[Shi *et al.*, 2009]
KSL	*Streptococcus mutans*	62.5 µg mL^{-1} (MIC)		0.25 mg mL^{-1}	-	[Liu *et al.*, 2011]
Lacticin 3147	*Streptococcus mutans*	6.3 µmol L^{-1}	1.9–3.8 µmol L^{-1} (MIC) 38 µmol L^{-1} (MBC)	50 µmol L^{-1}	-	[Dobson *et al.*, 2011]

LL-31	*Burkholderia pseudomallei*	-		20 µM		[Kanthawong *et al.*, 2012]
LL-37	*Burkholderia pseudomallei*	-		20 µM		[Kanthawong *et al.*, 2012]
	Staphylococcus aureus	10 µg mL^{-1}	1.27 µg mL^{-1} (EC_{50})	-		[Dean *et al.*, 2011]
	Candida albicans	20 µg mL^{-1}	-	-	20 µg mL^{-1}	[Tsai *et al.*, 2011]
	Pseudomonas aeruginosa	16 µg mL^{-1} (MIC)	64 µg mL^{-1} (MIC)	4 µg mL^{-1}	-	[Overhage *et al.*, 2008]
Ltx10	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	-	2 µg mL^{-1} 4 µg mL^{-1} (MIC)	50 µg mL^{-1}	-	[Flemming *et al.*, 2009]
Ltx5	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	-	4 µg mL^{-1} 8 µg mL^{-1} (MIC)	50 µg mL^{-1}	-	[Flemming *et al.*, 2009]
Ltx9	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	-	2 µg mL^{-1} 4 µg mL^{-1} (MIC)	50 µg mL^{-1}	-	[Flemming *et al.*, 2009]
Magainin I	*Listeria ivanovii*	0.1 mg mL^{-1} (on SS-SC-Chi-Tere surfaces)	-	-		[Héquet *et al.*, 2011]
	Listeria ivanovii *Bacillus cereus*	-		5% of a polymer brush		[Glinel *et al.*, 2009]
Nisin	*Staphylococcus aureus*	1.25 µg mL^{-1}	10 µg mL^{-1}	-	-	[Qi *et al.*, 2011]
	Listeria ivanovii	0.1 mg mL^{-1} (on SS-SC-Chi-Tere surfaces)	-	-		[Héquet *et al.*, 2011]
Omiganan pentahydrochloride	MSSA MRSA MSSE MRSE	-	2–4 µg mL^{-1} 2–8 µg mL^{-1} 4 µg mL^{-1} 4 µg mL^{-1} (MIC)	1% omigan aqueous gel (on skin colonisation models)	-	[Rubinchik *et al.*, 2009]
Peptoid 1 Peptoid 1-C134mer	*Pseudomonas aeruginosa*	12.5 µM	12.5 µM (MIC)	12.5 µM	-	[Kapoor *et al.*, 2011]
Phylloseptin-1	*Staphylococcus aureus*	5 µM (MBEC)	5 µM (MIC)	-		[Zhang *et al.*, 2010]

Pleurocidin	*Streptococcus mutans* *Streptococcus sanguinis* *Streptococcus sobrinus* *Streptococcus gordonii* *Lactobacillus fermenti*	-	8-16 μg mL^{-1} (MIC) 16 μg mL^{-1} (MBC)	64 μg mL^{-1}	-	[Tao *et al.*, 2011]
PTP-7	*Staphylococcus aureus*	4 μM	2-4 μM (MIC) 4-8 μM (MBC)	20-40 μM	-	[Kharidia *et al.*, 2011]
Tachyplesin III	*Pseudomonas aeruginosa*	4 & 32 μg mL^{-1} (MIC & MBC) 10 μg mL^{-1} (for 30 min. *in vivo* on ureteral stents)	2 & 4 μg mL^{-1} (MIC & MBC)	-		[Minardi *et al.*, 2007]
Tet20	*Pseudomonas aeruginosa*	10.2 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		-		[Gao *et al.*, 2011]
Tet213	*Staphylococcus aureus* *Pseudomonas aeruginosa*	~9 mg cm^{-2}		-		[Kazemzadeh-Narbat *et al.*, 2010]
		0.20 chains nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		-		[Holloway, 1955]
	Pseudomonas aeruginosa	14.5 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		-		[Gao *et al.*, 2011]
Tet26		10.5 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		-		[Gao *et al.*, 2011]
Tn-AFP1	*Candida tropicalis*	32 μg mL^{-1} (MIC)		-		[Mandal *et al.*, 2011]
α-MSH	*MRSA*	-		12 μM	3 μM	[Madhuri *et al.*, 2009]
(RW)4D	*Escherichia coli*	40 μM		-		[Hou *et al.*, 2009]

Note: MSSA - methicillin-sensitive *S. aureus*; MRSA - methicillin-resistant *S. aureus*; MSSE - methicillin-sensitive *S. epidermidis*; MRSE - methicillin-resistant *S. epidermidis*; CVC - central venous catheter; B - biofilm; P - planktonic; MIC - minimal inhibitory concentration ;MBC - minimal bactericidal concentration; MBEC - minimal biofilm eradication concentration; EC50 - half maximal effective concentration; IC50 - fifty percent growth inhibitory concentration. Pre-treatment accounts for the growth of cells in the presence of the AMP and also for the surface treatment, when indicated. Therapy accounts for the application of AMPs to the pre-grown biofilms or cells.

Annex III – Tukey's Tests for *Repeatability*

Table C: Results of the multiple comparisons Tukey's test for the biomass dataset of the *repeatability* testing.

Biomass								
Pairs	**Mean Diff.**	**Summary**	**Pairs**	**Mean Diff.**	**Summary**	**Pairs**	**Mean Diff.**	**Summary**
1 vs 11	-0.81	***	7 vs 16	0.6863	***	14 vs 15	0.5688	***
1 vs 12	-0.625	***	7 vs 17	0.76	***	14 vs 16	0.7113	***
1 vs 16	0.4513	***	8 vs 11	-0.85	***	14 vs 17	0.785	***
1 vs 17	0.525	***	8 vs 12	-0.665	***	16 vs 18	-0.5138	***
2 vs 11	-0.7987	***	8 vs 16	0.4112	***	17 vs 18	-0.5875	***
2 vs 12	-0.6138	***	8 vs 17	0.485	***	4 vs 10	-0.3763	**
2 vs 16	0.4625	***	9 vs 11	-0.61	***	4 vs 16	0.395	**
2 vs 17	0.5363	***	9 vs 12	-0.425	***	5 vs 10	-0.3925	**
3 vs 11	-0.81	***	9 vs 15	0.5088	***	5 vs 16	0.3788	**
3 vs 12	-0.625	***	9 vs 16	0.6512	***	7 vs 12	-0.39	**
3 vs 16	0.4513	***	9 vs 17	0.725	***	8 vs 10	-0.36	**
3 vs 17	0.525	***	10 vs 11	-0.49	***	15 vs 18	-0.3713	**
4 vs 11	-0.8663	***	10 vs 15	0.6288	***	1 vs 10	-0.32	*
4 vs 12	-0.6813	***	10 vs 16	0.7712	***	1 vs 15	0.3088	*
4 vs 17	0.4687	***	10 vs 17	0.845	***	2 vs 10	-0.3087	*
5 vs 11	-0.8825	***	11 vs 14	0.55	***	2 vs 15	0.32	*
5 vs 12	-0.6975	***	11 vs 15	1.119	***	3 vs 10	-0.32	*
5 vs 17	0.4525	***	11 vs 16	1.261	***	5 vs 7	-0.3075	*
6 vs 11	-0.8013	***	11 vs 17	1.335	***	5 vs 14	-0.3325	*
6 vs 12	-0.6163	***	11 vs 18	0.7475	***	6 vs 10	-0.3113	*
6 vs 16	0.46	***	12 vs 15	0.9338	***	6 vs 15	0.3175	*
6 vs 17	0.5338	***	12 vs 16	1.076	***	12 vs 14	0.365	*
7 vs 11	-0.575	***	12 vs 17	1.15	***			
7 vs 15	0.5438	***	12 vs 18	0.5625	***			

Note: .Only the statistically significant results are shown. The mean difference values are in OD 570 nm. The differences are represented by the *p*-value as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

Table D: Results of the multiple comparisons Tukey's test for the respiratory activity dataset of the *repeatability* testing.

Respiratory activity								
Pairs	**Mean Diff.**	**Summary**	**Pairs**	**Mean Diff.**	**Summary**	**Pairs**	**Mean Diff.**	**Summary**
1 vs 7	-0.1146	***	8 vs 10	0.1014	***	5 vs 18	0.08	**
1 vs 8	-0.09768	***	8 vs 11	0.1302	***	9 vs 12	0.0875	**
2 vs 7	-0.09833	***	8 vs 12	0.1477	***	9 vs 18	0.0875	**
4 vs 7	-0.1008	***	8 vs 13	0.1102	***	2 vs 8	-0.08143	*
6 vs 12	0.095	***	8 vs 15	0.1289	***	3 vs 7	-0.08458	*
6 vs 18	0.095	***	8 vs 16	0.1214	***	5 vs 7	-0.08458	*
7 vs 10	0.1183	***	8 vs 17	0.1277	***	6 vs 11	0.0775	*

7 vs 11	0.1471	***	8 vs 18	0.1477	***	6 vs 15	0.07625	*
7 vs 12	0.1646	***	12 vs 14	-0.09375	***	6 vs 17	0.075	*
7 vs 13	0.1271	***	14 vs 18	0.09375	***	7 vs 9	0.07708	*
7 vs 15	0.1458	***	3 vs 12	0.08	**	9 vs 11	0.07	*
7 vs 16	0.1383	***	3 vs 18	0.08	**	11 vs 14	-0.07625	*
7 vs 17	0.1446	***	4 vs 8	-0.08393	**	14 vs 15	0.075	*
7 vs 18	0.1646	***	5 vs 12	0.08	**	14 vs 17	0.07375	*

Note: .Only the statistically significant results are shown. The mean difference values are in OD 490 nm. The differences are represented by the *p*-value as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).

Table E: Results of the multiple comparisons Tukey's test for the cell viability dataset of the repeatability testing.

Cell Viability								
Pairs	**Mean Diff,**	**Summary**	**Pairs**	**Mean Diff,**	**Summary**	**Pairs**	**Mean Diff,**	**Summary**
11 vs 12	-5229000	***	9 vs 17	-3856000	**	6 vs 16	3039000	*
2 vs 6	-3137000	**	11 vs 13	-4161000	**	7 vs 11	3725000	*
2 vs 12	-3987000	**	11 vs 17	-4379000	**	9 vs 13	-3638000	*
2 vs 17	-3137000	**	12 vs 15	3758000	**	10 vs 12	-4052000	*
3 vs 6	-3333000	**	12 vs 16	3889000	**	12 vs 14	3725000	*
3 vs 12	-4183000	**	2 vs 13	-2919000	*	14 vs 17	-2876000	*
3 vs 17	-3333000	**	3 vs 7	-2680000	*	15 vs 17	-2908000	*
6 vs 9	3856000	**	3 vs 13	-3115000	*	16 vs 17	-3039000	*
6 vs 11	4379000	**	6 vs 14	2876000	*			
9 vs 12	-4706000	**	6 vs 15	2908000	*			

Note: .Only the statistically significant results are shown. The mean difference values are in CFU cm^{-2}. The differences are represented by the *p*-value as follows: $p < 0.001$ (extremely significant - ***); $0.001 < p < 0.01$ (very significant - **); $0.01 < p < 0.05$ (significant - *); $p > 0.05$ (not significant – ns).