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Investigation of heteroresistance towards antibiotics by biofilm-associated bacteria

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Resumo

"Investigação de heteroresistência a antibióticos por bactérias associadas a biofilmes"

As infeções hospitalares por bactérias resistentes constituem uma ameaça à saúde humana, causando cerca de 25 mil mortes por ano. A mortalidade associada a estas infeções tem sido ligada à formação de biofilmes e ao desenvolvimento de resistência antimicrobiana, nomeadamente heteroresistência, em que as bactérias podem ser selecionadas durante terapia com antibióticos, levando à falha terapêutica. Assim, é importante entender estes fenómenos desenvolvidos por muitas bactérias. Os objetivos deste trabalho foram: i) verificar e comparar a capacidade de formação de biofilmes por *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* e *Acinetobacter baumannii* em consórcios simples e polimicrobianos; ii) estabelecer e comparar a resposta de células planctónicas e de biofilmes destas bactérias a antibióticos convencionais; iii) verificar a ocorrência de heteroresistência em populações planctónicas e em biofilmes.

A capacidade de formação de biofilme e a viabilidade das populações simples e mistas foi obtida por cristal violeta (CV) e contagem de unidades formadoras de colónias (CFU). *Staphylococcus aureus* apresentou o maior número de células viáveis e maior produção de biomassa. Foi observada uma relação de antagonismo em populações mistas de *S. aureus* e *P. aeruginosa*. Uma diminuição na produção de biomassa por *P. aeruginosa* foi observada quando esta foi co-cultivada com *K. pneumoniae* e *A. baumannii*, sugerindo um mecanismo de proteção desenvolvido por estas bactérias contra *P. aeruginosa*. Os padrões de susceptibilidade das células planctónicas e de biofilme foram determinados pelo teste da microdiluição. As culturas planctónicas de *P. aeruginosa, A. baumanni* e *K. pneumoniae* mostraram um comportamento resistente à colistina, exibindo um valor de concentração mínima inibitória (MIC) duas vezes maior do que os MICs propostos pela EUCAST. Os padrões de susceptibilidade dos biofilmes revelaram um aumento significativo da resistência antimicrobiana em comparação com as células planctónicas. A ocorrência de heteroresistência foi averiguada através de análise de perfil populacional (PAP). Foi detetada heteroresistência nas culturas planctónicas de *K. pneumoniae* e *S. aureus*, para a colistina e a vancomicina, respetivamente. Nenhuns estudos anteriores reportaram populações de *K. pneumoniae* capazes de crescer até 16 mg/L de colistina. Não foram detetadas subpopulações heteroresistentes em células de biofilmes de 24 h. No entanto, os padrões de suscetibilidade dos biofilmes de 48 h e 72 h sugeriram que a capacidade de formar biofilmes e sua virulência podem depender do tempo de incubação de biofilmes, enfatizando a relevância da avaliação da heterosistência em biofilmes mais antigos.

Abstract

"Investigation of heteroresistance towards antibiotics by biofilm-associated bacteria"

Nosocomial infections by resistant bacteria are becoming a health threat causing around 25.000 deaths, per year. The mortality of these infections has been associated to the ability of bacteria in forming biofilms and developing resistance to antimicrobials, namely "heteroresistance", where bacteria can be selected during antibiotic therapy, leading to treatment failure. It is, therefore, of crucial importance to understand this phenomenon developed by several bacteria. The goals of this work were: i) to assess and to compare the ability for biofilm formation of *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae and Acinetobacter baumannii* in single- and mixed-species populations; ii) to assess and to compare the response of planktonic cells and biofilms formed by those species to conventional antibiotics; iii) to inspect about the occurrence of heteroresistance in planktonic and biofilm populations.

The biofilm-forming ability and viability of single and mixed populations were obtained by Cristal Violet (CV) assay and counting plate method expressed in colony forming units (CFU). *Staphylococcus aureus* displayed the highest number of viable cells and the greater biomass production. An antagonist relationship was observed for *S. aureus* and *P. aeruginosa* in mixed-species populations. A decrease in biomass production by *P. aeruginosa* was noted when co-cultured with *K. pneumoniae* and with *A. baumannii,* suggesting a mechanism of protection developed by these bacteria against *P. aeruginosa.* The susceptibility patterns of planktonic and biofilm cells were determined by the microdilution test. Planktonic cultures of *P. aeruginosa, A. baumannii* and *K. pneumoniae* showed a resistant behavior towards the antibiotic colistin, displaying a minimum inhibitory concentration (MIC) value 2-fold higher than the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. Biofilms susceptibility patterns revealed a significant enhance of antimicrobial resistance compared to their planktonic counterparts. The occurrence of heteroresistance was investigated by population analysis profile (PAP). The heteroresistance phenomenon was found in planktonic cultures of *K. pneumoniae* and *S. aureus*, towards colistin and vancomycin, respectively. No previous studies had reported *K. pneumoniae* populations able to grow up to 16 mg/L of colistin. No heteroresistant subpopulations were detected in biofilm-related cells of 24 h-old biofilms. However, the susceptibility patterns of 48 h- and 72 h-old biofilms suggested that the ability to form biofilms and its virulence are dependent of the biofilms incubation time, emphasizing the relevance of evaluating heteroresistance in older/mature biofilms.

General Index

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Acronyms

°C – Celsius degree

µL – Microliter

AMPs - Antimicrobial peptides

CFU - Colony-forming unit

cm^2- square centimeters

CV - Cristal violet

DNA - Deoxyribonucleic acid

eDNA - Extracellular DNA

EPS – Extracelular polymeric substances

ESBLs - Extended-spectrum β-lactamases

EUCAST - European Committee on Antimicrobial Susceptibility Testing

h – Hour

HAIs - Hospital acquired infections

HGT - Horizontal gene transfer

hVISA - Heteroresistant vancomycin-intermediate *S. aureus*

KSA - *Klebsiella* Selective HiCrome™ Agar Base

MBC - Minimum bactericidal concentration

MBEC - Minimum biofilm eliminating concentration

MDR - Multi-drug resistant

MHA - Mueller Hinton Agar

MHB - Mueller Hinton Broth

MIC - Minimum inhibitory concentration

min – Minute

mL - Milliliter

MLST - Multilocus sequence typing

MRSA - Methicillin-resistant *Staphylococcus aureus*

MS - Mass spectrometry

MSA - Mannitol salt agar

nm - Nanometer

OD - Optical Density

ODc - Optical density of the negative control

PAP - Population analysis profile

PAP-AUC – PAP Area under curve

PBPs - Penicillin-binding proteins

PCR – Polymerase Chain Reaction

PFGE - Pulsed-field gel electrophoresis

PIA - *Pseudomonas* Isolation Agar

qPCR – Quantitative PCR

QS - Quorum sensing

RNA - Ribonucleic acid

rpm - Rotations per minute

tRNA - Transfer RNA

TSA - Tryptic Soy Agar

TSB - Tryptic Soy Broth

VISA - Vancomycin-intermediate *S. aureus*

WHO - World Health Organization

CHAPTER 1: Introduction

Nosocomial infections are caused by an infectious agent or toxin acquired by patients under medical care. It is estimated that about 90% of those infections are caused by bacteria (e.g *Streptococcus spp., Acinetobacter spp., Enterococci, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Legionella* and *Enterobacteriaceae* family such as *Klebsiella pneumoniae and Escherichia coli).* Generally, bacterial-related infections often lead to an excessive and improper use of broad-spectrum antibiotics during hospitalization and heath care treatments, which are known to be the main cause for the development of antibiotic resistance (Khan *et al.*, 2015).

Bacteria rapidly evolve and the cases of antibiotic resistant infections are increasing. Reports of the World Health Organization (WHO) estimated that infections caused by resistant bacteria in European Union result in 25 000 deaths and costs that approximate to 1.5 billion, per year. Infections caused by resistant microorganisms are, therefore, of very difficult eradication with antibiotic therapy, moving the world into a post-antibiotic era, where common infections and minor wounds that have been treatable for decades, can become a serious threat or even lead to death (WHO, 2014).

In hospital facilities, there are two major problems that can enhance microbial resistant nosocomial infections: the ability for biofilm formation by bacteria, leading to critical infections and the development of "heteroresistance" by bacterial subpopulations in a susceptible population.

Bacteria can aggregate in a self–produced extracellular matrix and adhere to biotic and inanimate (e.g. medical devices) surfaces forming biofilms, which play a central role in hospital acquired infections (HAIs). It is estimated that up to 65 - 80% of HAIs are associated with biofilm formation. This virulence mechanism developed by several bacteria is a serious concern in medical care settings as biofilms are difficult to eradicate with antibiotic therapy (Bandeira *et al.*, 2017). The biofilm formation is a survival strategy developed by several bacteria, restricting the diffusion of antibiotics, enhancing the resistance towards those agents and leading to different responses to antibiotic therapy in biofilm-related infections (Cerca *et al.*, 2005; Hoiby *et al.*, 2010).

The "heteroresistance" phenomenon is a recent problem emerging in nosocomial infections. It is defined as a heterogeneous resistance to a defined antimicrobial agent within a single clinical isolate, wherein only a sub-population is resistant. The main problem associated to this phenomenon is that it goes unnoticed in routine antibiograms as the fraction of the resistant sub-

population is extremely low. This small fraction can be selected during the presence of the antimicrobial agent and lead to the failure of antimicrobial-based treatments, making more difficult or impossible to treat some bacterial-related infections (El-Halfawy & Valvano, 2013).

In the full age of antibiotic resistance, it becomes crucial to study these mechanisms of virulence developed by bacteria, being increasingly important to understand the role of biofilms in the increase of antimicrobial resistance, and fundamentally, to understand the phenomenon of heteroresistance, in order to reverse, prevent or fight these processes that neglect the treatment of many human-associated infections.

1.2 Motivation, objectives and thesis contribution

Nowadays, nosocomial infections are a serious concern in hospitalized patients. The ability of bacteria to adapt to several conditions and to develop virulence factors, such as growing on biofilms and developing heteroresistance, leads to high medical costs, prolonged hospital stays, and increased mortality (Khan *et al.*, 2015).

Recently, we are perceiving a spread in the development of multi-drug resistant (MDR) strains, in biofilm-related infections and in the isolation of heteroresistant strains in clinical isolates. These compromise the current clinical practices, thereby difficulting the establishment of preventive and effective treatment strategies of those infections (Davies & Davies, 2010).

The most common and serious MDR pathogens that are responsible for a vast number of nosocomial infections are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp* (Davies & Davies., 2010).

Recently, it has been observed a decrease in the incidence of infections caused by *Enterobacteriaceae* family. However, an increasing incidence of extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing strains by *Klebsiella pneumoniae* has been notable, compromising the efficacy of β-lactams and carbapenems. Similarly, the reemergence of coagulase-negative *S. aureus* infections in hospital infections has been observed in the recent years, becoming a serious concern due to the increase of MRSA (methicillin-resistant *Sthaphylococcus aureus*) strains known to display resistance towards β-lactams. Also, *Acinetobacter* spp. and *P. aeruginosa* are known to carry out serious complications in clinical settings (Khan *et al.*, 2015).

In this scope, three main goals were proposed. First, to assess the ability of biofilm formation under *in vitro* conditions of *P. aeruginosa, S. aureus, K. pneumoniae and A. baumannii* in single- and mixed-species populations; Second, to investigate the response of planktonic and biofilm populations towards conventional antibiotics (colistin, vancomycin, rifampicin and imipenem) by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum biofilm eliminating concentration (MBEC); Third, to inspect about the occurrence of heteroresistance in planktonic and in biofilm-associated cells through the Population Analysis Profile (PAP).

This study may have a great impact once antibiotic resistance represents a major global threat in health. A better understanding on how bacteria can develop mechanisms of virulence and finding effective strategies to resist to antibiotic therapy will be helpful in the treatment of these infections. Thus, the present study will allow to realize the ability of four of the major bacteria involved in HAIs to form biofilm, and their susceptibility to different classes of antibiotics, as well as their ability to develop heteroresistant sub-populations comparing to their planktonic counterparts.

1.3 Dissertation outline

This dissertation is composed by five chapters. The present chapter (chapter 1) consists of presenting and framing this research as well as presenting its objectives and its importance. Chapter 2 presents the state of art of this thesis. This chapter describes the role of microorganisms and biofilms in infections as well as strategies for their control; and also presents the principal antibiotic resistance mechanisms developed by several bacteria to overcome current antibiotic therapy, focusing the heteroresistance phenomena. Chapter 3 describes the methodologies performed in the experimental work and Chapter 4 presents the obtained results as well as the discussion of the findings obtained during this work. At the end, Chapter 5 attains the main conclusions obtained from this work and perspectives for future work are also delivered.

CHAPTER 2: Biofilm-associated infections, antibiotic therapy and development of antimicrobial resistance

2.1 Clinically-relevant Microorganisms and related infections

Microorganisms exist on Earth for more than 3.8 billion years, composing about 50% of the living biomass and playing an essential role in the maintenance and sustainability of the ecosystems (Byarugaba, 2009). Microorganisms can be found in nature and even be part the normal flora in the human body, (e.g. on the skin and in the urinary, gastrointestinal, and respiratory tracts). However, non-desirable microorganisms can invade human body through a portal of entry and start their multiplication and spread, leading to several infections. Bacterial infections are becoming the most global concern in human health and the major cause of hospital-acquired infections (HAIs), representing around 90 % of nosocomial infections. Frequently, these infections are treated with antibiotic therapy but due to the emergence of antimicrobial resistance, with some pathogens displaying resistance to multiple classes of antibiotics, the efficacy of antibiotic agents is compromised (Khan *et al.*, 2015).

2.1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic, non-fermenter, gram-negative bacterium existing in the normal microbial human flora (e.g. skin, nasal mucosa, and throat). This bacterium is known to survive on minimal nutritional requirements and to tolerate a variety of physical conditions which allow this organism to persist in hospital settings, causing serious infections, especially among immune-compromised individuals (Khan *et al.*, 2015).

P. aeruginosa contributes to 11 % of all nosocomial infections and it is also the second leading cause of nosocomial pneumonia (14 to 16 %), the third most common cause of urinary tract infections (7 to 11 %), the fourth most frequently isolated pathogen in surgical site infections (8 %), and the seventh leading contributor in bloodstream infections (2 to 6 %). Nearly all *P. aeruginosa* infections are associated with compromised host defenses such as in neutropenia, severe burns and cystic fibrosis, where bacteria adapt to the lung environment and grow as a biofilm, often resulting in a chronic infection (Lister *et al.*, 2009).

Actually, many antibiotics such as cephalosporins, trimethoprim, macrolides, chloramphenicol, tetracyclines and fluoroquinolones have shown to be ineffective in the treatment of *P. aeruginosa* infections (Khan *et al.*, 2015).

2.1.2 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive coccus, non-spore forming, catalase and coagulase positive, nonmotile and facultative anaerobe bacterium, existing as part of the normal human microbiota in the upper respiratory tract, skin and in the gut mucosa (Tong *et al.*, 2015).

S. aureus is the principal microorganism responsible for nosocomial infections, causing a wide range of clinical infections. *S. aureus* infects the superficial and deep tissues, being the main cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Tong *et al.*, 2015).

S. aureus infections are particularly difficult to fight given the development of MRSA (methicillin-resistant *S. aureus*) strains, strongly associated with β-lactams resistance by modification of penicillin-binding proteins, which compromises the treatment of *S. aureus* infections (Khan *et al.*, 2015).

Transmission of *S. aureus* is through infected individuals skin or contact via shared items and surfaces like door handles, benches, towels and taps. It is often found in biofilms formed on medical devices implanted in the body or on human tissue, strongly associated with another pathogen, *Candida albicans*, forming multispecies biofilms (Khan *et al.*, 2015).

2.1.3 *Acinetobacter baumannii*

Acinetobacter baumannii is a gram-negative, non-fermenter bacterium, colonizing the respiratory tract, skin, the urinary system and the gastrointestinal system. In the last decade, the incidence of *A. baumanni* infections have increased in a number of regions around the world.

The ability of *A. baumannii* to survive under a wide range of environmental conditions and to persist for extended periods of time on surfaces make it a frequent cause of outbreaks infections and health-care associated infections, being responsible for serious nosocomial infections (e.g bacteremia, pneumonia, meningitis, urinary tract infections and wound infections) (Maragakis & Perl, 2007; Davies & Davies, 2010; Hakyemez *et al.*, 2013).

Due to its impermeable outer membrane and its environmental exposure to a large reservoir of antibiotic resistance-associated genes, it has been observed an increase of *A. baumannii* multidrug-

resistant (MDR) strains in hospitalized patients, allowing it to escape several antibiotic therapies, which lead to a raising of mortality and morbidity and hospitalization period (Maragakis & Perl, 2007; Davies & Davies, 2010; Hakyemez *et al.*, 2013).

2.1.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a gram-negative, lactose fermenting and facultative anaerobic bacterium, member of *Enterobacteriaceae* family that can be found in the normal flora of the mouth, skin and intestines.

K. pneumoniae infections, in healthcare settings, can be spread through person-to-person contact and commonly occur among sick patients whose care requires ventilators or intravenous catheters and patients who are taking long courses of certain antibiotics. *K. pneumoniae* can cause serious nosocomial infections, including pneumonia, bloodstream infections, wound or surgical site infections, and meningitis (Chung, 2006).

The ability of this microorganism to form biofilms in medical devices such as urinary catheter is considered an important mechanism in nosocomial acquired and persistent infections (Khan *et al.*, 2015).

The treatment of K. pneumoniae-associated infections is being compromised with the increasing incidence of extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing strains which confers resistance to β-lactams and particularly to carbapenems antibiotics that are the last line of defense against resistant gram-negative infections (Chung, 2006).

2.2 Antibiotic therapy to treat bacterial infections

The treatment of bacterial-associated infections is frequently accomplished with antibiotic therapy. The selection of antibiotic is based on type of infection and the mechanism of action of the drug. The antibiotics can be classified according to their mechanism of action as inhibitors of the cell wall synthesis, inhibitors of the cytoplasmic membrane synthesis, inhibitors of the protein synthesis, inhibitors of the nucleic acid function or synthesis and inhibitors of the cell metabolism. **Table 1** summarizes the classes of antibiotics and their respective action spectrum.

Table 1: List of antibiotics by family and their action spectrum (*Adapted from Byarugaba, 2009*)

Mechanism of action	Class	Examples of antibiotics		Action spectrun
Inhibitors of cell wall synthesis	β-Lactamics	Penicillins	Benzylpenicillin	Spirochetes and cocci
			Phenoxymethylpenicillin	Gonococcus
			Amoxicillin and Ampicillin	*Haemophilus influenzae*, *Escherichia coli*, *Proteus mirabilis*
			Flucloxacillin	Staphylococci producing penicillinase
			Piperacillin, Azlocillin and Mezlocillin	Gram negative bacteria
			Carboxypenicillin	*Pseudomonas spp..*
		Cephalosporins	First generation	Gram positive bacteria
			Second generation	Gram negative and gram positive bacteria
			Third generation	Gram negative bacteria
			Fourth generation	Gram negative bacteria
		Carbapenems	Imipenem and Meropenem	Gram negative and gram positive bacteria
		Monobactam	Aztreonam	Gram negative, aerobic bacteria
	Glycopeptides	Vancomycin and Teicoplanina		Gram positive bacteria
Inhibition of cytoplasmic membrane synthesis		Colistin		*Pseudomona aeruginosa*, *Enterobacter spp.*, *Escherichia coli*, *Klebsiella spp.*, *Salmonella spp.*,
		Polymyxin E		*Pasteurella sp.*, *Bordetella sp.* *Shigella sp.*
Inhibition of protein synthesis	Acting in 30S ribosomal subunit	Aminoglycosides		Gram negative aerobic bacteria and *Staphylococcus aureus*
		Tetracyclines		*Vibrio cholerae, Mycobacterium leprae, Brucella spp., Rickettsiaceae, Chlamydia spp., Mycoplasma spp.*
	Acting in 50S ribosomal subunit	Chloramphenicol		*Salmonella typhi*
		Macrolides		*Chlamydia spp., Mycoplasma spp., Legionella pneumophila, Haemophilus influenzae, Haemophilus ducreyi, Campylobacter spp.,Moraxella catarrhalis*
		Clindamycin		Gram positive anaerobic bacteria
Inhibitors of Nucleic Acid Synthesis		Fluoroquinolones		*Escherichia coli, Salmonella spp., Shigella spp., Enterobacter spp., Campylobacter spp., Neisseria spp.*
		Rifampicin		*Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria meningitidis, Brucella spp.*

The cell wall inhibitors include β-lactams and glycopeptides. Beta-lactams enclose penicillin derivatives, cephalosporins, monobactams, carbapenems and beta-lactamase inhibitors. Their mechanism of action involves their bond to penicillin-binding proteins (PBPs), which are transpeptidases anchored in the cell membrane and involved in the cross-linking of the bacterial cell wall in the final stages of peptidoglycan synthesis. The antibiotic binds to PBPs rendering the enzymes unable to perform their role in cell wall synthesis, leading to bacterial death by osmotic instability or autolysis. Generally, beta-lactamase inhibitors, such as cluvanic acid are used to amplify the penicillin spectrum in beta lactamase producers (Byarugaba, 2009).

Glycopeptides, such as vancomycin and teicoplanin, allow the inhibition of the late stage of cell wall peptidoglycan synthesis by steric hindrance. They form stable complexes through hydrogen bindings to L-aa-D-Ala-D-Ala groups in wall intermediates, which unable the formation of the backbone glycan from the simple wall subunits as they are extruded through the cytoplasmic membrane. These events prevent the transpeptidation reaction responsible for imparting rigidity to the cell wall (Baptista, 2013).

Inhibitors of cytoplasmic membrane synthesis such as colistin binds to the phospholipids of cell membrane, altering their structure and disrupting it, leading to an increase of cell permeability which can break the osmotic balance causing the leakage of cellular components, inhibition of cell respiration and an exceeding uptake of water yielding to cellular death (Andersson, 2005).

The mechanism of action of the inhibitors of protein synthesis depends on the subunit of the ribosome where they act. The major families of antibiotics acting on the 30S subunit of the ribosome are aminoglycosides and tetracyclines. Aminoglycosides bind to a specific protein (12S) in the ribosomal 30s subunit, leading to the inhibition of protein synthesis either by interference on the initiation complex or by RNA codon misreading, which leads to the incorporation of different amino acids, resulting in a non-functional protein. Tetracyclines inhibit protein synthesis through their reversible binding to the 30s subunit, which blocks the access of the transfer RNA (tRNA) to the site of action in the ribosomal complex, preventing the addition of amino-acids to the forming peptides. The antibiotics acting on the 50S subunit of the ribosome include chloramphenicol, macrolides and clindamycin. Chloramphenicol prevents attachment of the aminoacyl end of the transfer RNA to the 50s ribosomal subunit receptor site, leading to inhibition of peptide binding. Macrolides bind reversibly to the ribosomal 50S subunit, inhibiting the translocation step.

Clindamycin binds to the ribosomal 50s subunit, making protein synthesis impossible (Byarugaba, 2009)

Antibiotics that interfere with nucleic acid synthesis are fluoroquinolones and rifampicin. The mechanism of action of fluoroquinolones is based on their binding to DNA gyrase and topoisomerase IV which lead the cell unable to perform DNA replication. Rifampicin binds to subunit β of RNA polymerase, changing its conformation which lead to an inhibition of RNA synthesis (Baptista, 2013).

2.3. Biofilms

Microorganisms can exist either in their planktonic form, as free-floating cells, or colonizing biotic/abiotic surfaces, protected by a self-produced polymeric matrix. These microbial communities can encompass one or even more microbial species, forming single or polymicrobial biofilms, respectively. Biofilms are usually a form of living of bacterial species, however, fungi are often found in these living structures. Biofilm can form and develop spontaneously, playing an important role in several human-associated infections (e.g. wound infections, vaginitis, upper respiratory tract infections, otitis media, and endocarditis). Biofilms can also be formed on medical devices like catheters, acting as a reservoir for living cells, capable of cross-contamination, which allows the contamination to other surfaces (Lewandowski & Beyenal., 2014; Dijkshoorn *et al.*, 2007).

Biofilms act as protective barrier from hostile environments (e.g. iron deprivation, osmotic stress, metal toxicity, and antibiotic exposure) and is considered a virulence factor once microorganisms that form the biofilm establish relationships differing from their suspended (planktonic) counterparts (Moghadam *et al.*, 2014; Harrison *et al.*, 2007; Nadell *et al.*, 2015).

Biofilms play a huge contribution in the severity of acute and chronic infectious diseases, presenting recalcitrance towards antibiotic treatment, and ultimately leading to high morbidity/mortality rates and to highly expensive treatments (Bjarnsholt, 2013; Donlan & Costerton, 2002).

2.3.1 Biofilm composition

Biofilms are organized surface-associated communities of bacterial cells that are accumulated in a self-produced hydrated matrix of extracellular polymeric substances (EPS) composed of

material synthesized by the sessile cells in the community, which can be formed in biotic or abiotic surfaces (Moghadam *et al.*, 2014; Costerton, 2007).

Biofilms play an important role as a reservoir of genetic diversity material, which favours the adaptation, evolution, and survival of bacteria in adverse environments. Living in a biofilm community carries several advantages such as availability of nutrients and proximity to other bacteria which facilitate plasmid and gene horizontal transfer. The extrapolymeric matrix of biofilms also allows protection against antimicrobials and increases the binding of water molecules which reduces the possibility of dehydration (Limoli *et al.*, 2014; Høiby *et al.*, 2010).

In a biofilm, it is possible to discern the cells and the biofilm polymeric matrix, which represents 15 and 85% of the composition of the biofilm, respectively (Sutherland, 2001). The biofilm polymeric matrix consists in macromolecular products secreted by replicating adherent bacteria that include EPS, proteins, minerals, extracellular DNA, and anionic/cationic molecules (Flemming *et al.*, 2007). The EPS (e.g. poly-N-acetyl-Dglucosamine, acetan, alginate, cellulose, gellan, pullulan, and xanthan) are the main macromolecular constituents of the EPS matrix and serve as biofilm scaffolding for different microbial species (Öner, 2013; Vu *et al.*, 2009). Extracellular DNA play a major role in stabilizing biofilm throughout ionic interactions, representing a relevant mechanism for horizontal gene transfer, known to be responsible for antimicrobial resistance (Montanaro et al., 2011). Water channels are also present within the biofilm polymeric matrix, providing structural support for biofilm architecture, helping in cell-to-cell interaction and surface adhesion and facilitating oxygen transport to cells (Flemming *et al.*, 2007; De Beer *et al.*, 1994).

In general, microbial cells residing in a biofilm remain metabolically active and viable and may change their location/distribution and phenotype in the biofilm for the purpose of better survival (Irie & Parsek, 2008).

2.3.2 Biofilm formation

Biofilm formation encompasses three main steps: the attachment of cells (reversible or irreversible), followed by proliferation into small microbial clusters and excretion of extracellular polymeric substances (O'Toole *et al.*, 2000).

First, bacteria start to adhere to the surface, by non-specific mechanisms such as physical-chemical attraction forces and/or specific receptor-ligand adhesion mechanisms. Then, colonisation starts with the production of extracellular metabolites and up-regulation of virulence factors (Otto, 2006; Azeredo & Oliveira, 2003). The young biofilms proliferate and continue to

mature by cell division and recruitment of other bacterial species to the consortium, leading to increased thickness of the biofilm. In the mature biofilms, portions of cells slough out of the main biofilm structure, being able to colonize other surfaces and develop new biofilms (**Figure 1**).

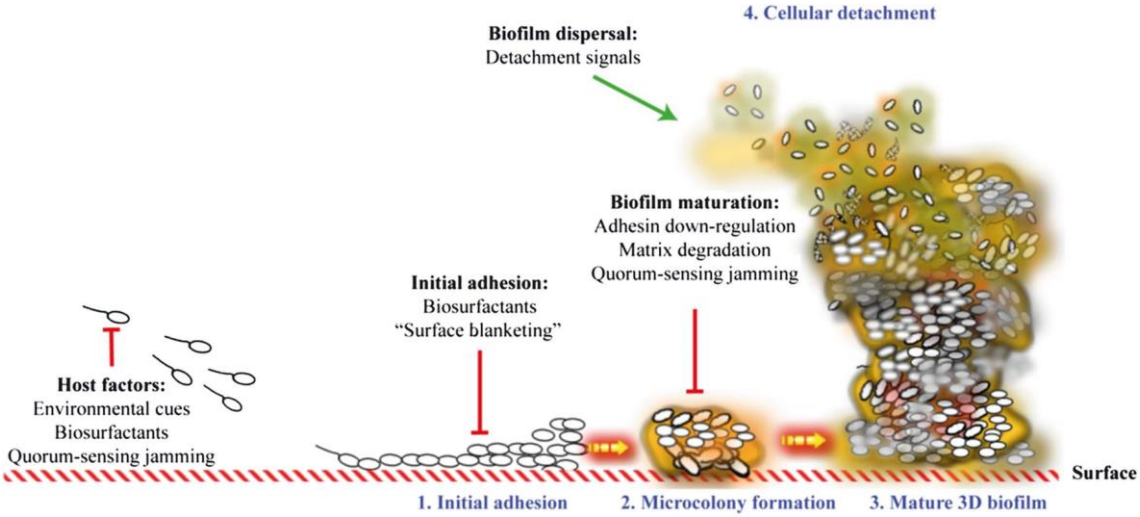

Figure 1: Steps of biofilm formation: 1. Planktonic bacteria encounter a submerged surface to attach; 2. Attached cells start to colonize the surface by the production of the EPS matrix ; 3. EPS production allows the development of a complex, three-dimensional structure by the emerging biofilm community; 4. Biofilms can propagate through the detachment of small or large clumps of cells, or by a type of "seeding dispersal" that releases individual cells which can attach to another surface or to a biofilm downstream of the original community (adapted from Rendueles & Ghigo, 2012).

2.3.3 Biofilms control

The antibiotic therapy is not suitable to eradicate biofilms, as high concentrations are required to kill microorganisms encased in the biofilms and also because the biofilm polymeric matrix often restricts the diffusion of several antimicrobial substances in biofilms and the agents cannot be delivered continuously (Stewart *et al.*, 2000; Donlan & Costerton, 2002; Davison *et al.*, 2010; Tkachenko & Karas, 2012). Besides that, bacteria within biofilms are able to tolerate high levels of antibiotics compared to planktonic bacteria. Earlier studies have showed that biofilm bacteria are 10-fold higher resistant comparatively with respective planktonic bacteria (Cerca *et al.*, 2005) and the antibiotic concentration needed to eradicate the biofilm is often above the peak serum concentration of the antibiotic (Monzón *et al.,* 2002), suggesting that antibiotic therapy is often ineffective in the treatment of biofilm infections.

Due to the failures of antibiotic therapy towards bacterial biofilms, alternative approaches to treat biofilms include the use of bismuth thiols (BTs), silver nanoparticles, QS inhibitors, bacteriophages, antimicrobial peptides (AMPs) and biofilm-dispersing enzymes as alternatives to conventional antibacterial therapies.

Bismuth thiols (BTs) have antimicrobial and antibiofilm activity against several bacterial species and are being suggested as great alternative to antibiotics that inhibit biofilm formation (Domenico *et al.*, 1997; Domenico *et al.*, 2001). Also, encapsulated metal ions such as silver and selenium nanoparticles were able to inhibit biofilm in some studies although the mechanisms are not yet fully understood. (Gurunathan *et al.*, 2014; Palanisamy *et al.*, 2014; Shakibaie *et al.*, 2015). Thiazolidine-2,4-dione (TZD) derivatives, which belongs to anti-diabetic drug family seems to have anti-biofilm and anti-adhesion activity and could be incorporated into food packaging where they successfully interfere with bacterial and fungal biofilms (Feldman *et al.*, 2014; Srebnik *et al.*, 2011). Bacteriophages are highly specific self-replicating agents that can also be used against biofilms, infecting and rapidly lysing bacteria cells (Atterbury et al., 2007; Jassim & Limoges., 2014; Jones et al., 2012). Cationic antimicrobial peptides (CAMPs), positively charged hydrophobic amino acid molecules, are being increasingly considered an attractive and potential, presenting low mammalian cell toxicity and being able to disrupt biofilm bacteria and destabilize the biofilm matrix by binding to the eDNA (Pompilio *et al.*, 2011; Pompilio *et al.*, .2012; Wimley, 2010).

The combination of antibiotics and biofilm matrix-degrading enzymes (e.g polysaccharide-hydrolyzing enzymes, DNAse, and proteases), which are able to inhibit or reduce the cell-to-cell and cell-to-surface associations during the biofilms formation making the cells sensitive to killing by antibiotic is a promising, highly effective strategy to treat established biofilm-related infections (Kaplan *et al.*, 2012).

QS is a signaling system responsible for the regulation of genes involved in biofilm physiology and virulence (Uroz *et al.*, 2009). The use of QS inhibitors, which quench the action of QS molecules, could be an alternative in the control and impairment of biofilm formation (Antunes *et al.*, 2010; Fuqua & Greenberg, 2002).

2.4 Mechanisms of antimicrobial resistance

Due to the widespread use of antibiotics in medicine, veterinary medicine and agriculture, microorganisms have developed mechanisms that enabled them to respond to the selective pressure exerted by various environments and competitive challenges, allowing the development of antimicrobial resistance (Byarugaba, 2009; Richard D. Smith & Joanna Coast, 2002). This phenomenon - known as resistance - allows microorganisms resisting the action of an antimicrobial agent. This impairs an effective control of biofilm-related infections, contributing for the high

morbidity, mortality, and healthcare-associated costs, making the treatment of patients more difficult and expensive (WHO, 2014).

Currently, there are several therapeutic agents used in the treatment of biofilm-related infections, which are being compromised by the development of microbial resistance.

The resistance can be described as "intrinsic resistance", whereby microorganisms naturally do not have target sites for the drugs, or have low permeability to the antimicrobial agent, and as "acquired resistance" whereby susceptible microorganisms are capable of developing mechanisms to overcome the effects of the drug (Byarugaba, 2009). Mechanisms of acquired resistance can enclose the presence of an enzyme that inactivates the agent, a mutation in the antimicrobial agent's target, a post-transcriptional or post-translational modification of the therapeutic agent, a reduced uptake of the antimicrobial agent, an active efflux of the agent and an expression or suppression of a gene **(Figure 2)** (Byarugaba, 2009).

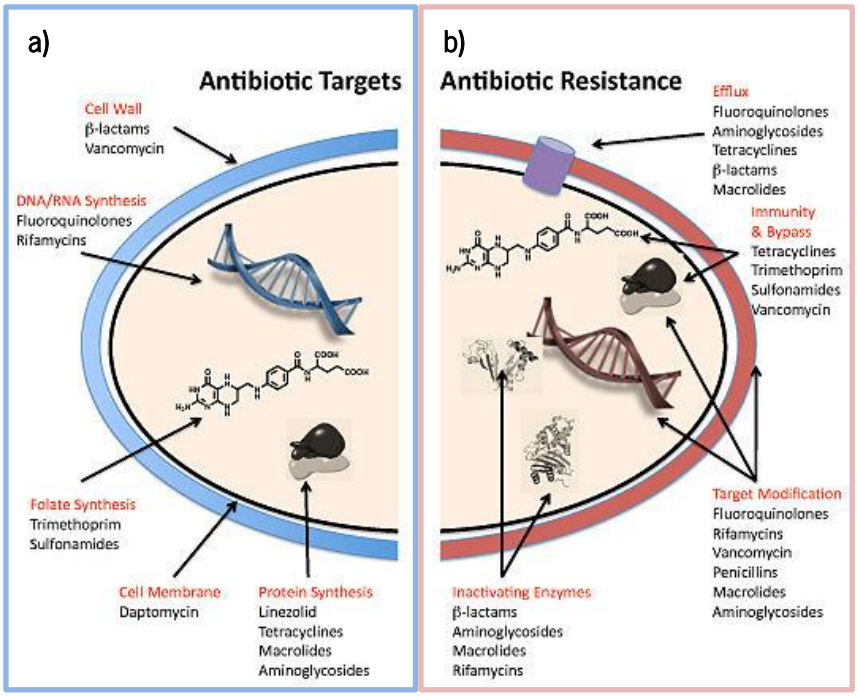

Figure 2: Principal cell targets of antibiotics and resistance mechanisms developed by bacteria towards some antibiotics: a) antibiotic targets - an antibiotic can inhibit the cell wall, DRNA/RNA synthesis, folate synthesis, cell membrane and protein synthesis; **b)** the main mechanisms of resistance developed by the bacteria include efflux pumps that compromises the cell wall targets, target modifications which stop the DNA/RNA and protein synthesis, and the production of inactivating enzymes (e.g β- lactamases, aminoglycosides modifying enzymes) that enable the antibiotic action (*adapted from: http://www.wikiwand.com/en/Antimicrobial_resistance).*

The antimicrobial resistance mechanism by enzymatic modification or destruction of the antibiotic is achieved when the microorganism has the ability to produce enzymes able to destruct or inactivate a particular antibiotic. For instance, β-lactamase is capable of splitting the β-lactam

ring comprising the nucleus of penicillin, cephalosporin, carbapenem and monobactam antibiotics. Aminoglycoside-modifying enzymes are another example of bacterial coded enzymes that are able to alter the structure of aminoglycoside antibiotics by adenylation, acetylation or phosphorylation, leading to antibiotic inactivation or impairing the ability of the antibiotic to penetrate the cell (DeBellis & Zdanawicz, 2000).

The decreased uptake of antimicrobial agent is also an acquired resistance mechanism that can occur by genetic mutations capable of decreasing the membrane permeability, genetic mutations, which alter the porin channels, reducing the ability of antibiotics to get inside the cell, and by lower rate of electron transport chain activity, that compromises the transport of the drug into the cell due to insufficient electrochemical gradients (DeBellis & Zdanawicz, 2000).

Another mechanism of acquired resistance involves active drug efflux pumps, which pump the antimicrobial agent out of the cell or the cellular membrane, maintaining the intracellular drug concentration low and ribosomes free from the agent (Byarugaba, 2009).

Genetic mutations that can lead to the alteration of the antimicrobial target aim at preventing the binding of the drug to their target. Currently, mutations in PBPs, DNA gyrase, ribosomal proteins and RNA polymerase which are the binding targets of β-lactam, fluoroquinolone, aminoglycoside antibiotics and rifampicin, respectively, have currently being identified, making impossible for them to act as substrates for their specific drugs (DeBellis & Zdanawicz, 2000).

2.4.1 Mechanisms of antibiotic resistance in biofilms

Several studies have suggested that microbial residing in biofilms have enhanced drug resistance and tolerance to antimicrobial agents comparatively to their planktonic counterparts, mainly due to their genetic diversification and their EPS matrix that acts as a physical barrier to antimicrobial substances (Cerca *et al.*, 2005, Hoiby *et al.,* 2010).

The mechanisms of antibiotic tolerance within biofilms include, for instance, the binding of the EPS molecules to charged antimicrobial agents, leading to their inactivation; the sequestration of cationic antibiotics by EPS allow the development of tolerance to these molecules; limited diffusion of antibiotics, which may limit the transport of antimicrobial agents into biofilm cells or mitigate their antibacterial activity against biofilm bacteria (Mah &. O'Toole, 2001; Hoiby *et al.*, 2010; Colvin *et al.*, 2011).

The biofilm heterogeneous bacterial populations display different growth rates, which also play a crucial role in antibiotic tolerance by biofilm-encased bacteria, once most antibiotics usually target

actively replicating cells, whereas the inactive or slower growing cells will be less affected by the antibiotic (Mulcahy *et al.*, 2008; Hall-Stoodley *et al.*, 2004).

The mechanisms of antibiotic resistance in biofilms include (i) phenotypic changes in bacteria, resulting in resistance occurring within the biofilm environment, (ii) inactivation of the antibiotics by extracellular polymers or modifying enzymes, and (iii) nutrient limitation resulting in slow growth rate (Gilbert *et al.*, 1997) (**Figure 3**).

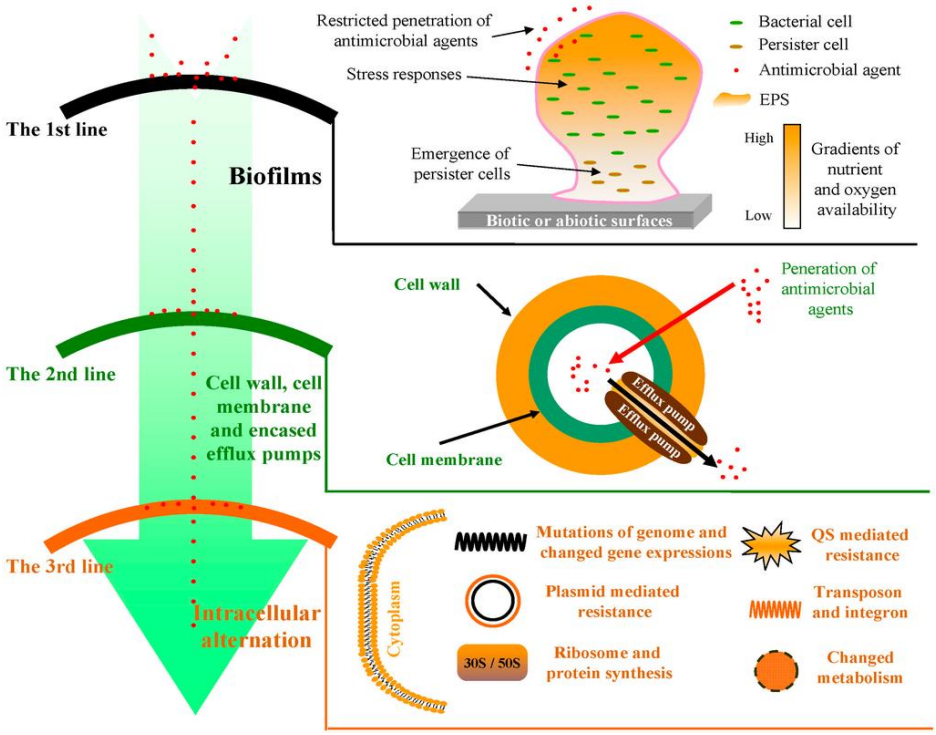

Figure 3: Simplified diagram summarizing the mechanisms of the three defense lines of resistance to antimicrobial agents for bacterial biofilms. Bacterial biofilms limit the penetration of antimicrobial agents. The cell wall, cell membrane and the encased efflux pumps limit the absorbance or elevate the excretion of antimicrobial agents. When antimicrobial agents enter in the bacterial cells may occur the alteration of target sites, regulation of gene expression and production of certain enzymes (*Zhou et al., 2015*).

The polymicrobial nature of biofilms and the close proximity between the biofilm-residing microbial species may facilitate the emergence and dissemination of antibiotic resistance genes through horizontal gene transfer (HGT) and integrative conjugative elements. The biofilm matrix is extremely hydrated, providing conditions for the transfer of extracellular DNA and natural transformation (Olsen, 2015; Ceri *et al.*, 2010; Poole, 2011).

The overexpression of efflux pumps is also implicated in antibiotic resistance and may promote biofilm antimicrobial resistance in several bacterial species (Zhang & Mah, 2008).

2.4.2 Methods to investigate antimicrobial resistance

The effectiveness of antimicrobial agents against infectious microorganisms is performed by *in vitro* tests that include two main methodologies: microorganism kinetics assays and determination of the minimal inhibitory concentration (MIC) of a particular antibiotic against a microbial strain. These tests supply a characterization of the interactions between the antimicrobial substance and the microorganism.

The MIC value corresponds to the minimum concentration of an antibiotic that prevent the further growth of the organism *in vitro.* Currently, the degree of drug effectiveness is based on MIC value and can be characterized as "susceptible," "intermediate," or "resistant". According to *ISO 20776-1:2006* the characterization terms are the follows:

- Susceptible (S): A bacterial strain is susceptible to a given antibiotic when it is inhibited *in vitro* by a concentration of this drug that is associated with a high likelihood of therapeutic success.
- Intermediate (i): The sensitivity of a bacterial strain to a given antibiotic is intermediate when it is inhibited *in vitro* by a concentration of this drug that is associated with an uncertain therapeutic effect.
- Resistant (R): A bacterial strain is resistant to a given antibiotic when it is inhibited *in vitro* by a concentration of this drug that is associated with a high likelihood of therapeutic failure.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has released standardized breakpoints across Europe where are stipulated the MIC values of several antibiotic drugs for particular species of microorganisms. These breakpoints can be accessed directly via the web site http://www.eucast.org/.

2.4.3 Microbial Heteroresistance

One particular type of microbial resistance that is increasingly becoming a health concern is the phenomenon of heteroresistance, which describes a population-wide variation in antibiotic resistance (El-Halfawy & Valvano, 2013). In other words, it describes a microbial subpopulation within a large population of antimicrobial-susceptible microorganisms developing lower susceptibility to a certain antibiotic (Saravolatz *et al.*, 2014). Similar to resistance, heteroresistance can also be intrinsic or can be acquired, as described in the above sections. This type of resistance

compromises the treatment of infections with effective therapeutic agents, becoming essential the development of methods for inspecting heteroresistance.

2.4.3.1 Methods for evaluating microbial heteroresistance

The most common methods used for measuring heteroresistance are population analysis profiling (PAP), disc diffusion and E-test assays. Although, there are other methodologies that can be applied to characterize heteroresistance (e.g. agar plates in a linear gradient of antibiotic concentrations allowing determination of the antibiotic susceptibility of clinical isolates and the identification of antibiotic-resistant cells within bacterial populations, flow cytometry using a fluorescent penicillin derivative to assess heteroresistance by comparing to isolates with known heteroresistance and also measuring the bacterial regrowth at later time points in time-kill assays after an initial significant growth reduction and determination of increased MIC values of the same strain on extending the incubation time) (Liu *et al.,* 2011; Jarzembowski *et al.*, 2009; Chambers *et al.*, 1985).

The population analysis profiling (PAP) method, despite the lack of a standard protocol and being time-consuming, is considered the most used process to measure antimicrobial heteroresistance. In this method, the bacterial population is exposed to a gradient of antibiotic concentrations, and the occurrence of bacterial growth is checked for each antibiotic concentration. PAP is usually accomplished with 2-fold antibiotic increments and using spread plate techniques for colony-forming unit (CFU) counting to perform MIC determination (El-Halfawy & Valvano, 2013; Søgaard, 1985; Søgaard & Gahrn-Hansen, 1986).

Disc diffusion and E-test assays are widely used to detect heteroresistance for *in vitro* susceptibility testing. Both methodologies consist in inoculating the microorganism in an appropriate medium using agar plate, in which the antimicrobial discs/strips are applied. The inoculated plates must be incubated at appropriate temperature for the growth of the organism (**Figure 4**). In the disc diffusion assay, the diameter of the inhibition zone is proportional to the MIC value, according to EUCAST standard breakpoint tables. The E-test assay allows the direct reading of the MIC value through the reading scale in the antimicrobial strip (EUCAST, 2015).

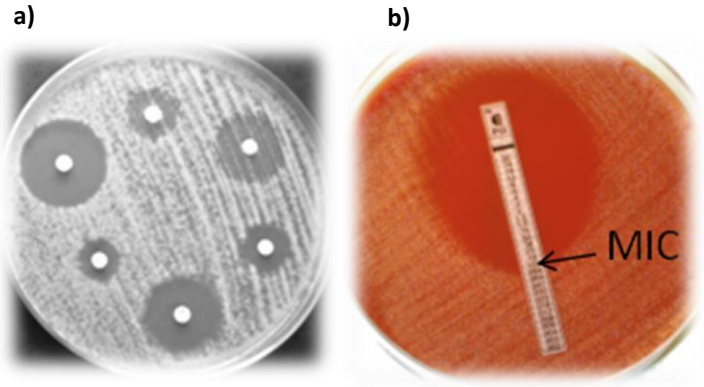

Figure 4: Conventional tests used to investigate microbial heteroresistance: a) disk diffusion assay -the zone of inhibition is measured and MIC is accessed based on standard tables that have the relation between the diameter zone and the MIC value; **b) E-test** - the MIC value corresponds to the concentration marked on the antibiotic strip that is visible no growth (adapted from: *https://en.wikipedia.org/wiki/Etest#/media/File:E-test_Ngono.jpg; http://jcm.asm.org/content/44/10/3616/F1.large.jpg)*

2.4.4.2 Reported cases of Microbial Heteroresistance – a review

An interesting work conducted by El-Halfawy & Valvano (2015) has recently reviewed some of the existing heteroresistance in microbial species reports until June, 2014. Since other cases of heteroresistance have arisen in the meantime, a review of these new cases has been made. Therefore, the review included studies reported in the literature since June (2014) - up to July (2017)

An analysis on the number of reports investigating heteroresistance was undertaken in order to evaluate its emergence over the last years **(Figure 5)**. A significant increase in the number of reports evaluating microbial heteroresistance has been displayed, especially in the last two years in which a significant number of studies were published. For instance, in 2015, at least 14 articles reporting heteroresistance in microbial species were found, which substantially corresponds to the number of articles in the period between 2012 and 2014 and 2009 and 2011 (El-Halfawy & Valvano, 2015). Similarly, at least 13 articles in 2016 and until July 2017 reporting this type of resistance were evidenced, which significantly remarks that the phenomenon of heteroresistance associated to bacterial and fungal species is becoming widely established, which may have a great impact on the treatment of infectious diseases with antibiotics.

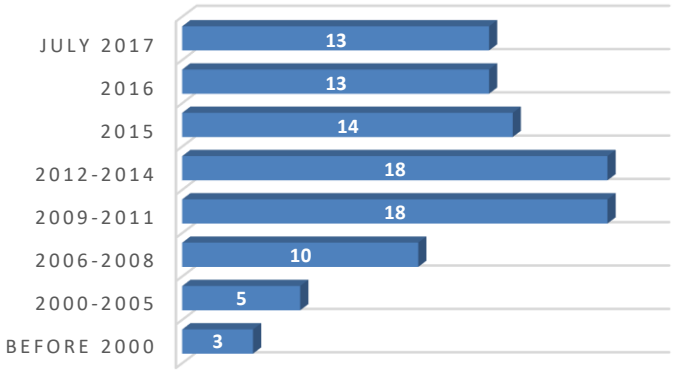

Figure 5: Number of papers per year reporting heteroresistance

Those studies have shown that a variety of bacterial and fungal species can develop heteroresistance to one or more antibiotic. So far, 15 microbial species were found to develop heteroresistance, with *S. aureus* as the most reported bacteria (30 %), followed by *A. baumannii* (15 %) and *Mycobacterium tuberculosiss* (10 %) (**Figure 6**). But not only bacteria can develop heteroresistance. A few studies have demonstrated that yeast species, such as *Candida* spp., *Cryptococcus gatti and Cryptococcus neoformans*, can also develop heteroresistance to at least one or more antibiotic (Claudino *et al.*, 2009; Huang *et al.*, 2010).

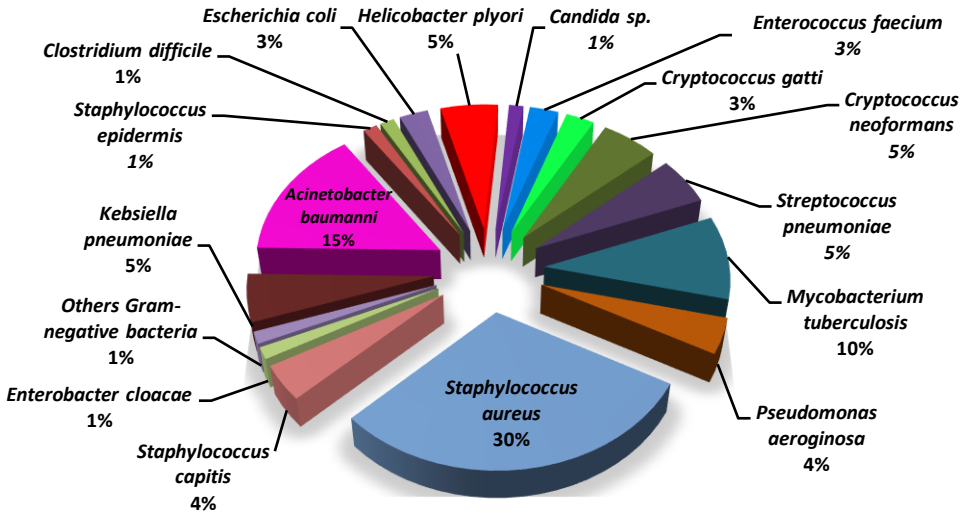

Figure 6: Distribution of different bacterial from reported cases of heteroresistance since June 2014 to July 2017

Antibiotics acting as cell wall inhibitors (e.g. beta-lactams and glycopeptides) are reported as the most frequent agents in heteroresistance cases, representing 29 % and 28 % of the studies, respectively (**Figure 7**). This can be explained by the increase of reported mutations at the level of antibiotic targets such as PBPs and by the presence of enzymes capable of inactivating

antimicrobial agents such as β-lactamases and aminoglycoside-modifying enzymes. The azoles family seems to be the overarching target of heteroresistance investigations (11 %) against yeasts, mainly due to the intrinsic capacity of certain yeasts such as *Cryptococcus* and *Candida* species to escape to azoles therapy.

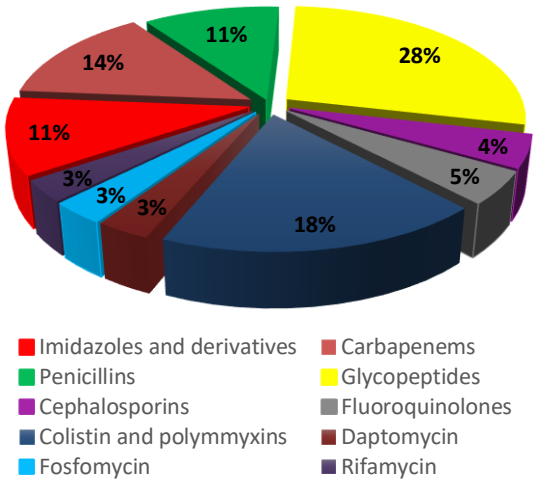

Figure 7: Distribution of different antibiotic from reported cases of heteroresistance since June 2014 to July 2017

Intrinsic heteroresistance

Intrinsic heteroresistance occurs without pre-exposure to the antibiotic but may also be acquired or induced after initial exposure to antibiotics (e.g repeated exposure of homogeneously sensitive bacteria result in mixed populations resembling intrinsically heteroresistant strains). Intrinsic heteroresistance to azoles by *Cryptococcus gattii and Cryptococcus neoformans* have been reported (Varma & Kwon-Chung, 2010; Ferreira *et al.*, 2015; Yamazumi *et al.*, 2003 and Sionov *et al.*, 2009). Some reports also showed that heteroresistance of *Staphylococcus capitis to vancomycin* was intrinsic (*D'mello et al., 2008)*

Varma & Kwon-Chung (2010) showed that heteroresistance of *Cryptococcus gattii* to azoles was an intrinsic mechanism and it was associated with the strain virulence, wherein heteroresistant populations have shown to be more resistant to xenobiotics and significantly more virulent. Ferreira *et al.* (2015) also demonstrated intrinsic heteroresistance of *C. gatti* and found that heteroresistant strains increased virulence through structural and metabolic changes in the cells (**Table 2**).

Yamazumi *et al.* (2003) and Sionov *et al.* (2009) found evidences of intrinsic heteroresistance of *Cryptococcus neoformans* to fluconazole that could be developed by selection from heteroresistant clones and induction by exposure to drug therapy (**Table 2**).

Staphylococcus capitis heteroresistance to vancomycin was suggested by D'mello *et al*. (2008) to be an intrinsic property of *S. capitis.* Van Der Zwet *et al.* (2002) demonstrated that it resulted from the spread of a single heteroresistant strain. Bianco *et al*. (2014) suggested the intravenously clindamycin administration as an alternative to vancomycin treatment in *S. capitis* infections (**Table 2**).

Acquired heteroresistance

Acquired heteroresistance bacteria can be mediated by (1) enzymatic modifications of antibiotics through the production of enzymes, such as β-lactamases and aminoglycoside-modifying; (2) antibiotic target modifications by mutations in penicillin-binding proteins (PBPs), DNA gyrase, ribosomal proteins and RNA polymerase; (3) membrane impermeability to the drug caused by mutations that alter the membrane permeability and the porin channels or by a lower rate of electron transport chain activity; (4) active drug efflux pumps; (5) expression or suppression of a gene; and (6) presence of antibiotic resistance genes through horizontal gene transfer and integrative conjugative elements (DeBellis & Zdanawicz, 2000).

The pre-exposure to some antibiotics also can be a main cause in the development of heteroresistance by selection and induction processes. There are some reports showing that heteroresistance of some microbial species *such as Candida* spp, *A. baumannii* and *Enterobacter cloacae* resulted from selection and induction during exposure to the antibiotics. Claudino *et al*. (2009) reported that exposure to fluconazole by *Candida* species leads to the occurrence of colonization or infection by resistant populations, selected or induced during the treatment, suggesting that the heteroresistance to fluconazole and amphotericin B may occur by selection and induction processes; Rodriguez *et al*. (2014) and Moosavian *et al*. (2015) showed that heteroresistance to colistin in *A. baumannii* populations was a consequence of colistin consumption and Hawley *et al*. (2007) and Moosavian *et al.* (2015), verified that it resulted from the selection during exposure to the antibiotic; Napier *et al*. (2014) reported that colistin heteroresistance by *Enterobacter cloacae* was significantly increased upon treatment, suggesting that pretreatment with colistin induces an increased frequency of antibiotic resistance (**Table 2**).

The production of β-lactamases and aminoglycoside-modifying enzymes is a reported mechanism developed by many bacteria species to different antibiotics. These mechanism is deeply reviewed by El-Halfawy & Valvano (2016). However, recently, new reports have emerged evidencing this mechanism as responsible for the development of this type of resistance. Lee *et al*.

(2010) demonstrated that heteroresistance of *A. baumannii* to imipenem was related to mutations in *Acinetobacter*-derived cephalosporinase (ADC-29) and an insertion sequence in the promoter region of a class C β-lactamase gene - *blaADC-29*. Ma *et al.* (2016) and Sun *et al.* (2015) revealed heteroresistance to cefepime and carbapenem by *Escherichia coli*, which were highly connected to bacterial extended spectrum β-lactamase (ESBL) production. Also, Hawley *et al.* (2007) and Moosavian *et al.* (2015) verified the presence of the *blaOXA-23*-like gene in colistin heteroresistant *A. baumannii* strains, which is a gene involved in the production of a specific β-lactamase, but its role was not well understood.

Antibiotic target modifications have recently been reported for *S. aureus*, *Streptococcus pneumoniae* and *Helicobacter pylori*. Espedido *et al.* (2014) showed that *S. aureus* heteroresistance to ceftaroline was related to a *mecA* mutation, which is a gene encoding an alternative PBP, *PBP2A*. Sorg & Veening (2015) have shown that one of the heteroresistance mechanisms developed by *Streptococcus pneumoniae* to different antibiotics was related with mutations in the PBPs. Engel *et al.* (2013, 2014) related the existence of a low-affinity penicillin-binding protein by *S. pneumoniae* heteroresistance to penicillin and fosfomycin; Jarabo *et al.* (2015) found mutations in 23S *rRNA* and *gyrA* gene in clarithromycin and levofloxacin *H. pylori* heteroresistant strains that lead to therapeutic failure (**Table 2**).

Expression or suppression of resistance genes as well as mutations in proteins and regulatory genes are the most mechanisms described to explain the heteroresistance to different antibiotics by several bacteria (e.g. *S. aureus*, *H. pylori*, *K. pneumoniae*, *M. tuberculosis*, *S. pneumoniae)*. Cases of *S. aureus* heteroresistance towards vancomycin of are deeply revised by El-Halfawy & Valvano (2016). However, this type of resistance was recently related with *agr* dysfunction and *sel, sec* and *tst* superantigen genes, which increased the mortality and virulence of MRSA infections (Rose *et al.*, 2014; Park *et al.*, 2014). *Staphyloccocus aureus* heteroresistance to daptomycin was recently reported by Capone *et al.* (2016), associating the phenomenon with mutations and/or up-regulation of genes involved in cell wall turnover and cell membrane perturbation, namely in *rpoB* and *mprF* genes. Multiple evolutionary pathways involving *yycH* and *mprF* were suggested by Chen *et al.* (2015) to mediate cross-resistance to glycopeptides and daptomycin during persistent MRSA bacteraemia under antibiotic selective pressure. *H. pylori* resistance to metronidazole showed to be related with the inactivation of *RdxA* and mutations in other nitroreductase genes (Matteo *et al.* (2006). Heteroresistance to colistin has also been found for *K. pneumoniae* (Jayol *et al.* (2015), associating it to a single nucleotide substitution in protein *PhoP* leading to colistin resistance. Other

mutations/regulation of resistance genes like *katG, rpsL, embB, rpoB*, were suggested to be involved in *M. tuberculosis* to isoniazid, streptomycin, ethambutol and rifampin heteroresistance by Rinder *et al.* (2001), Kumar *et al.* (2013); Mekonnenet *et al*. (2015). Mutations in many codons of quinolone resistance-determining region of *gyrA* were suggested by Zhang *et al*. (2012) to play role in fluoroquinolone heteroresistance by *M. tuberculosis*. *S. pneumoniae* heteroresistance to different antibiotics were also related with continued gene expression activity, decreased epigenetic susceptibility and to the occurrence of mutations inside *rpoB* (Sorg & Veening, 2015)

Active drug efflux pumps were reported by Mei *et al.* (2015) that related high expression in the *MexAB* of *P. aerugionsa* efflux pump to imipenem heteroresistance and horizontal gene transfer was suggested by Silveira *et al*. (2015) to explain vancomycin heteroresistance in *S. aureus* (**Table 2**).

Only a few studies have reported cases of heteroresistance in biofilms. Silva *et al.* (2016) demonstrated that *K. pneumoniae* manifested heteroresistance to colistin only when grown in biofilm arrangements, a similar phenomenon reported by Gazzola & Cocconcelli (2008), who showed that the disruption of *AtlE*, a protein involved in biofilm formation abolished heteroresistance of *Staphylococcus epidermidis* to vancomycin.

As seen, the cases of heteroresistance are increasingly rising and there are already a high number of bacteria able to develop this type of resistance, which compromise the efficacy of a great number of antibiotics against microbial infections. Further studies are needed to better understand the mechanisms underlying these phenomena. Furthermore, as observed, bacteria can develop a set of heteroresistance mechanisms, making fundamental the development of methods that allow the proper study of heteroresistance.

Table 2: Reported cases of microbial heteroresistance between June (2014) to July (2017)

Microorganism	Antibiotic	Sample	Methods	Comments	Reference
Acinetobacter baumannii	Colistin	Clinical multidrug-resistant isolates subcultured onto horse-blood agar plates	Broth microdilution, PAP, time-kill kinetics	23% of isolates were heteroresistant, with subpopulations growing at doses >2 mg/L; heteroresistance is not an intrinsic property of *A. baumannii*	Yau, W. *et al.*, 2008
		Clinical samples	Agar dilution method, PAP, MALDI-TOF mass spectrometry (MS) system, PCR	Percentage of resistance to carbapenems increased from 70 to 98.4% during the period studied, owing to the expression of *OXA-carbapenemases* in all the isolates; it was observed a steady rise in the MIC50 of imipenem-resistant isolates, as well as an increase in the presence of *blaOXA-23-like* gene; all isolates harbored the *tet (B)* gene; it was observed a rise of heteroresistance to colistin from 46.4% in 2004 to 95% in 2012; selection of colistin subpopulations is independent of the degenerate oligonucleotide primers-PCR (DO-PCR) types; colistin-resistant isolates were unstable and susceptible to associations of colistin plus rifampicin or meropenem;	Rodriguez, C. H. *et al.*, 2014
		Isolates from cerebrospinal fluid of a 20-year-old man who had a gunshot trauma to the abdomen	Multiplex PCR, REP-PCR, E-test, disk diffusion test,	Both isolates were *blaOXA-51*-like positive; at the beginning of colistin treatment, isolate 1 was susceptible to colistin with MIC 0.25 μg/mL but some colonies were grown within the zone of inhibition that indicate heteroresistant subpopulations; after treatment with colistin, isolate 2 was recovered with colistin MIC >256 μg/mL that was completely resistant to colistin, suggesting that a colistin resistant subpopulation was selected; the first isolate was susceptible to polymyxin B, but the second isolate was completely resistant without any previous exposure to this antibiotic, suggesting a phenomenon of cross-resistance between colistin and polymyxin B; the two isolates were sensitive to ampicillin-sulbactam *in vitro* but the patient didn't responded to the treatment	Moosavian, M. *et al.*, 2015
	Different antimicrobial agentes	Clinical isolates from blood, skin swabs and tracheal aspirates	Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE), broth microdilution, population analysis profiling	All isolates were resistant to imipenem, cefepime, ciprofloxacin and piperacillin/tazobactam; all colistin-susceptible isolates showed heteroresistance to colistin, and four were persisters; Heteroresistance, persistence or evasion of the innate immune response may explain the failure of antimicrobial treatments in this patient	Choi, H.J. *et al.* 2017
	Meropenem	Clinical isolates from blood, urine, sputum and cerebrospinal fluid	Agar dilution, PFGE, PCR, PAP, Thigh Infection Model	Colonies of clinical isolates that grew in the highest meropenem concentration, exhibited stable meropenem heteroresistance; PCR was positive only for the intrinsic blaOXA-51-like carbapenemase; the mice infected by one persister and two heteroresistant subpopulations survived 24 h without treatment, suggesting a relatively higher virulence of the clinical isolates compared with the heterogeneously grown subpopulations; meropenem dosing schemes exhibited very poor therapeutic responses; it was suggested a fitness cost conferred by mutations related to the expression of the heterogeneous mode of growth against meropenem.	Neou, E. *et al.*, 2016

Candida species	Amphotericin B and fluconazole	*C. albicans, C. tropicalis, C. parapsilosis, C. glabrata* clinical isolates	Etest, YEPD inoculation, selection, induction, PCR, agarose gel electrophoresis	Phenotype of heteroresistance to fluconazole and amphotericin B can be produced by two methodologies: selection and induction, exposure to fluconazole leads to the occurrence of colonization or infection by resistant populations, selected or induced during the treatment.	Claudino, A.L.R. *et al.*, 2009
Cryptococcus gattii	Fluconazole	Clinical and environmental strains of *C. gattii*	PCR, E-test, spot test analysis of serial dilutions	The strains which expressed a higher level of heteroresistance to fluconazole were more resistant to xenobiotics and significantly more virulent, heteroresistance of *C. gattii* to azoles is an intrinsic mechanism and it is associated with the strains virulence.	Varma, A. &. Kwon-Chung, K. J., 2010
		Isolates from a cat	Multilocus sequence typing, qPCR, YPD broth	Reversion to wild-type susceptibility was observed when maintained in antifungal-free media, confirming the *in vivo* development of heteroresistance. The in vivo development of heteroresistance to fluconazole in the patient with *C. gattii* was secondary to overexpression of the efflux pump PDR11 and the drug target ERG11.	Sykes, J. E .*et al.* 2017
	Itraconazole	clinical strains isolated from cerebrospinal fluid and one strain from the environment of *C. gattii*	Microdilution, spot tests on Sabouraud's dextrose agar, time-kill kinetic, PFGE, Phagocytosis assay	Heteroresistance to itraconazole was intrinsic in all strains, reduced the capsule size and the cell diameter, induced molecular heterogeneity at the chromosomal level, changed the negatively charged cells, reduced ergosterol content, and improved the antioxidant system. Heteroresistance to itraconazole is intrinsic and increases the virulence of *C. gattii.*	Ferreira, G. F. *et al.*, 2015
Cryptococcus neoformans	Fluconazole and Voriconazole	Clinical isolates from non-AIDS patient and AIDS patient who had suffered recurrent episodes of cryptococcal meningitis	E-test, agar dilution, Sabouraud modified antibiotic medium	Resistance was innate and unrelated to drug exposure, clones highly resistant to fluconazole (100 mg/ml) and moderately resistant to voriconazole (1 mg/ml) always produced a homogeneous population of resistant cells, the pattern of heteroresistance was not affected by the pH or osmolarity of the medium, the resistance appeared to be suppressed at 35°C and was completely abolished at 40°C.	Mondon, P. *et al.*, 1999
	Fluconazole	Clinical isolates of *C. neoformans*	Broth microdilution, E-tests, agar dilution, CFU, electrophoresis	Fluconazole-heteroresistant phenotype of *C. neoformans* exists in a significant proportion of clinical isolates, fluconazole resistance can be developed by selection from heteroresistant clones and induction by exposure to fluconazole.	Yamazumi, T. *et al.*, 2003
		Clinical and environmental strains of *C. neoformans (suspended cells)*	E-test, spot tests on YPD agar, CFU, gas chromatography - mass spectrometry (GC-MS), PCR	The ABC transporter AFR1, known to efflux FLC, was unrelated to the heteroresistance mechanism; heteroresistance is intrinsic and contributes to relapse of cryptococcosis during azole maintenance therapy.	Sionov, E. *et al.*, 2009
		Strains of clinical isolates suspended in sterile saline	Broth dilution, plating on YPD plates, quantitative PCR	Fluconazole-resistant clones with Chr1 disomy emerge in the brains of mice undergoing prolonged fluconazole therapy; overexpression of Hsp90 did not affect the heteroresistance levels	Sionov, E. *et al.*, 2013

Enterobacter cloacae	Colistisn	Strain (colR/S) of *Enterobacter cloacae* from a bronchoalveolar lavage specimen from a kidney transplant patient.	Etest, agar plating, MH broth dillution	The colistin-heteroresistant *E. cloacae* strains tested have large resistant subpopulations, whose frequency is significantly increased upon colistin treatment. Pre-treatment with colistin induces an increased frequency of colistin resistance as well as cross-resistance to the host antimicrobial lysozyme.	Napier, B. A. *et al.*, 2014
Escherichia coli	Cefepime	Ckinical isolates from blood and other sterile body fluids	Broth microdilution, disk diffusion, E-test, PAP, PFGE	21.9 % of isolates harbored phenotypic heteroresistance to cefepime traits; distinct *E. coli* coloni could grow in the inhibitory halo around cefepime disks, hinting the intrinsic potential of this subpopulation to survive under the antibiotic pressure; cephalosporins, nitroimidazoles and combined use of antibiotics were significantly associated with acquisition of resistance; extended spectrum beta-lactamase (ESBL) production was also significantly associated with the FEP-PHR case group	Ma, W. *et al.*, 2016
	Carbapenem	Clinical isolates of invasive *Escherichia coli*	Vitek2, broth microdilution, disk diffusion, PAP, CFU, PFGE	Presence of subpopulations with higher carbapenem resistance with MICs ranging from 2.0–128.0 mg/L; male gender, invasive intervention, antibiotic use and bacterial extended spectrum β-lactamase (ESBL) production contributed to invasive infections by carbapenem-heteroresistant *E. coli* (CHEC); the production of ESBL was identified as the common independent risk factor for heteroresistance to both ertapenem and imipenem; clonal diversity among the CHEC isolates was revealed; two successive *E. coli* strains isolated from the same patient indicated that carbapenem resistance evolved from heteroresistance	Sun, J. D. *et al.*, 2015
Helicobacter pylori	Metronidazole	Clinical *H. pylori*-positive isolates from antral biopsies and upper endoscopy cultured on Blood Agar	Agar dilution, DNA extraction and ethanol precipitation, PCR, sequencing, agarose electrophoresis	76 % of heteroresisance isolates showed deletion of one nucleotide in position 54 of poly (A) tract or in position 215 of poly (T) tract of the *RdxA* gene leading to the occurrence of a stop codon, inactivation of *frxA* is associated with high-level MTZ resistance, RdxA inactivation, or mutations in other nitroreductase genes, could play an additional role in metronidazole-resistant isolates with moderate or low-level MIC	Matteo, M. J. *et al.*, 2006
	Clarithromycin (CLA) and levofloxacin (LF)	Antrum and gastric corpus samples from patients who had undergone an upper gastrointestinal endoscopy	Genotype HelicoDR test, PCR, reverse hybridization,	Heteroresistant to CLA was 51.38% and to LFR was 50%; mutations of the 23S rRNA gene defining CLA resistance and mutations of the gene *gyrA* defining LF resistance; the detected mutations on CLA were A2143G, A2142G and A2142C; gyrA mutation of LF resistance were at positions 91 and 87; the risk of CLA resistance is associated with the female sex and the risk of LF resistance increased with age; therapeutic failure were correlated with the phenotypic CLA resistance and A2143G mutation and not with the heteroresistant status.	Jarabo, J. M. N. *et al.*, 2015

Haemophilus influenzae	Imipenem	Clinical samples	Gene sequencing, competition assay with Bocillin-FL, broth dilution and viable cell counting, whole-genome sequencing	All imipenem-heteroresistant isolates harbored amino acid substitutions in the ftsI gene, which encodes PBP3; Different amino acid substitutions and insertions were noted in *OmpP2*, suggesting a relationship with imipenem heteroresistance; combination with the altered PBP3, the slowed drug influx and its enhanced efflux due to the loss of regulation led to the development of imipenem heteroresistance.	Cherkaoui, A. *et al.,* 2017
Klebsiella pneumoniae	Colistin	Multidrug-resistant *Klebsiella pneumoniae* isolate exhibiting heteroresistance to colistin	Etest, broth microdilution, PCR, sequencing, growth kinetics, CFU, quantitative real-time PCR (qRT-PCR)	The colistin-resistant subpopulation harbored a single amino acid change (*Asp191Tyr*) in protein PhoP; a partial deletion of the phoP gene disrupted the reading frame of phoP, leading to a longer and inactive protein being responsible for a reversion in colistin susceptibility.	Jayol, A. *et al.*, 2015
		K. pneumoniae isolated from a urinary sample	microtiter plate test, Colony Forming Units method, microdilution method, plating in Muller Hinton Agar, PAP, observation of colony morphology	Existence of a sub-population resistant to colistin within a *K. pneumoniae* strain that seemed to be exclusively associated with biofilms; small colonies variants (SCV) represent the sub-population responsible for heteroresistance to colistin.	Silva, A. *et al.*, 2016
Mycobacterium tuberculosis	Isoniazid, streptomycin and ethambutol	Sputum clinical samples	DNA isolation, PCR-RFLP (restriction fragment length polymorphism analysis of polymerase chain reaction products), agarose gel electrophoresis, cloning, sequencing	*katG, rpsL* and *embB* genotypes involved in resistance were tested and the results showed that more than one genotype are present in most of the original samples.	Rinder, H. *et al.*, 2001
		Clinical isolates	DNA extraction, Mycobacterial Interspersed Repetitive Unit- Variable Number Tandem Repeat (MIRU-VNTR) genotyping, Culture and DST, clinical (chest X-ray) and microbiological diagnostic procedures (microscopy and culture of clinical sample)	66 TB patients, 53% (35/66) showed mixed infection; among the 66 TB patients, 24.24% showed heteroresistance; In mixed samples 45.71% showed different resistance patterns; there was a strong significant association between heteroresistance and treatment failure; mixed infections, have a negative impact on treatment of TB patients especially when co-infecting *M. tuberculosis* strains display heteroresistance.	Kargarpour, K.M. *et al.*, 2017
	Rifampin and isoniazid	Sputum specimens of suspected drug resistant TB (DR-TB) patients	Bactec MGIT 960 culture and DST, GenoType MTBDRplus (LPA), sequencing	High frequency of mutations in MDR-TB suspected cases: S531L, 530-533, H526Y and D516V for rifampicin resistance, S315T1 and 315 in *katG* gene and C15T and -15/116 inhA gene for INH resistance. A high rate of heteroresistance pattern was observed in *rpoB* gene (28.8%) and katG gene (9.8%).	Kumar, P. *et al.*, 2013
		Clinical TB positive isolates from sputum, peritoneal fluid, tissue, lymph node aspirate, and pus specimens	Ziehl–Neelsen (ZN) method, DNA extraction, PCR, line probe assay (LPA),	Mutations on *rpoB* and *KatG* genes. The *Ser531Leu (rpoB)* and *Ser315Thr1(KatG)* substitutions were the highest gene mutations for rifampicin and isoniazid, respectively.	Mekonnen, D. *et al.*, 2015
	Fluoroquinolone	clinical specimens	absolute concentration method, PCR, sequence analysis	The predominant mutations observed occurred on codons 90, 91, and 94, which are located in the quinolone resistance-determining region of *gyrA* (5, 8, 10, 15) the improper use of FQ was suggested to be the the major cause of FQ resistance.	Zhang, X. *et al.*, 2012

	Rifampin	Clinical Isolates	DNA extract and genotyping, PCR, sequencing	Manu2 was the predominant lineage in the cases of mixed infections, and this might be the main reason for heteroresistance and a possible mechanism for isolates without any mutation in the *rpoB* gene to become rifampin resistant.	Zheng, C *et al.*, 2015
Pseudomonas aeruginosa	Imipenem	Clinical isolates	KB method and VITEK method, double-disk synergy test (DDST), Quantitative RT-PCR	None imipinem-heteroresistant *P. aeruginosa* strains were found to produce metallo-β-lactamase; heteroresistance was related to high expression in the *MexAB* of PA efflux pump.	Mei, S. *et al.*, 2015
	Fosfomycin	Clinical isolates from sputum and blood; *P. Aeruginosa ATCC 27853* (*in vitro)*	Agar dilution on Mueller–Hinton agar, PAPs, ProtoCOL colony counter, time–kill studies, PAE,	Heteroresistance was found for the all isolates; saturation of the rate of killing with increasing fosfomycin concentrations was observed for both the reference strain and clinical isolates, indicating that bacterial killing of *P. aeruginosa* by fosfomycin was time dependent; regrowth occurred rapidly at the lower inoculum following initial modest bacterial killing even with concentrations well above those that are clinically achievable following intravenous therapy which could be explained by the presence of pre-existing fosfomycin-resistant subpopulations; most concentrations of fosfomycin resulted in complete replacement of fosfomycin-susceptible colonies by fosfomycin-resistant colonies; monotherapy with fosfomycin may be problematic for the treatment of infections caused by *P. aeruginosa.*	Walsh, C. C *et al.*, 2015
Salmonella enterica serovar Typhimurium	Tigecycline	Bacterial strains and plasmids	Broth microdilution, DNA sequencing, Population analysis profiling (PAP), real-time PCR	Heteroresistant isolates had elevated expression of *acrB, ramA, and oqxB*. DNA sequencing identified numerous mutations in RamR that have been shown to lead to *ramA* overexpression; heteroresistance to tigecycline was manifested in a plasmid-bearing strain and was associated with overexpression of the *AcrAB-TolC* and *OqxAB* efflux pumps.	Chen, Y. *et al.*, 2017
Staphylococcus aureus	Vancomycin	Clinical isolates from patients with MRSA bloodstream infections who were treated with vancomycin	Etest, broth microdilution, PFGE, Fisher's exact test, classification and regression tree (CART) analysis, Poisson regression analyses	Reduced vancomycin susceptibility phenotypes contribute to deleterious outcomes in concert and underscore their combined importance in predicting the risk of failure for MRSA bloodstream infections; treatment failure was more common among healthcare-associated *versus* community-associated MRSA isolates.	Lodise, T. P *et al.*, 2014
		Clinical isolates	β-hemolysin assay, CellTiter (Promega), DNA sequencing, PFGE, multilocus sequence typing, PCR, broth microdilution, Etest	Reduced susceptibility to vancomycin has been linked to clinical failure of vancomycin treatment and agr dysfunction; poor concordance between vancomycin heteroresistance and high MICs; the high vancomycin MIC (≥1.5 μg/mL) was associated with low cytotoxic activity;	Rose, H. R. *et al.*, 2014
		Methicillin-resistant *S. aureus* bacteremia (MR-SAB) from blood cultures	Agglutination test, oxacillin agar screen method, Etest, multiplex PCR, PAP-AUC	Vancomycin heteroresistance decreased over time in *SCCmec types II* and *IV* only in patients 51+ years, probably because of the small numbers of heterogeneous vancomycin-intermediate *S. aureus* (hVISA)	Khatib, R. *et al.*, 2015

				in the younger age groups; isolate exposure to vancomycin at lower concentrations may facilitate the generation of less susceptible progeny; older subjects have higher incidence of isolates with V-MIC≥2 mg/L and hVISA phenotypes, likely related to a higher exposure to healthcare settings and/or prior treatment with vancomycin among older patients.	
		Clinical isolates from blood and/or respiratory tract from adult's patients with MRSA pneumonia	PAP/AUC, broth microdilution, laser colony counter, multiplex PCR, δ-hemolysin assay	Mortality was significantly higher in heterogeneous vancomycin-intermediate *S. aureus* (hVISA) patients and subsequent vancomycin treatment failure comparing to vancomycin-susceptible *S. aureus* (VSSA) patients.	Claeys, K.C. *et al.*, 2016
		MRSA clinical isolates from blood, cerebrospinal fluid, ascites, and pleural effusion	Broth microdilution method, E-test, PAP-AUC, cassette chromosome mec (SCCmec) typing, multilocus sequence typing (MLST)	The heteroresistance vancomycin-intermediate susceptibility (hVISA) prevalence increased significantly in last years; hVISA and VISA isolates were less susceptible to ciprofloxacin, clindamycin, daptomycin, gentamicin, rifampin, and trimethoprim/sulfamethoxazole, and have *SCCmec II* or III element. A twofold increase in either vancomycin or teicoplanin MIC doubled the probability of being hVISA.	Huang, S. H. *et al.*, 2015
		12 clinical isolates of vancomycin heteroresistance *S. aureus*	Disk diffusion, PAP-AUC, D test, multiplex PCR, PCR, PFGE	The heteroresistance to vancomycin had heterogeneous genomic patterns consistent to little or no epidemiological relationship among the isolates; phenotypic instability and often prolonged vancomycin therapy for selection, clonal spread is not as common as for other resistance mechanisms disseminated through horizontal gene transfer; two isolates exhibiting inducible clindamycin resistance contained the *ermA* gene and all other isolates showed constitutive clindamycin resistance; among the 12 isolates, only one had *SCCmec type IV* and the others had *SCCmec type II*, which is associated with hVISA.	Silveira, A. C. O *et al.*, 2015
	Vancomycin and methicillin	Clinical isolates from hospital-acquired pneumonia (HAP) patients *(in vitro)*	Broth microdilution, PAP-AUC	Telavancin activity was unaffected by the presence of resistance phenotypes, including MRSA and heteroresistant vancomycin-intermediate *S. aureus* (hVISA); telavancin may be a useful therapeutic option for the treatment of hospital acquired pneumonia due to MRSA.	Krause, K. M. *et al.*, 2012
	Methicillin	MRSA bacteraemia isolates	Etest, PAP-AUC, d-haemolysin activity assay, MLST, multiplex PCR,	*Sel, sec* and *tst* superantigen genes were associated with higher mortality; community aquired-MRSA strains lacking staphylococcal superantigens may be less virulent; strain-specific virulence factors, rather than vancomycin susceptibility, may contribute to the outcome of MRSA bacteraemia.	Park, K. H. *et al.*, 2014
	Daptomycine	Daptomycin-susceptible (DAP-S) and daptomycin-resistant (DAP-R) MRSA strains isolated from the blood cultures *(In vitro)*	Glycopeptide resistance detection, PAP/AUC, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), staphylococcal cassette chromosome mec (SCCmec) typing, agr typing, retro-	All daptomycin-resistant (DAP-R) strains showed hVISA or DAP-R traits, including mutations and/or up-regulation of genes involved in cell wall turnover and cell membrane perturbation, namely in *rpoB* and *mprF* genes; daptomycin resistance arose during glycopeptide therapy; the use of daptomycin as first-line therapy at optimal dosage combined with	Capone, A. *et al.*, 2016

			transcription, real-time qPCR, sequencing, SNPs analysis	other agents, such as beta-lactams should be considered for treating MRSA infections.	
		MRSA isolate from a bloodstream patient with Down's syndrome and tetralogy of Fal	Next-generation sequencing, Nesoni 0.58, WGS comparison, PCR sequencing, Etest, PAP-AUC, qRT-PCR	Six non-synonymous mutations and three evolutionary pathways were identified during the development of vancomycin-intermediate *Staphylococcus aureus* (VISA) and a daptomycin-resistant *S. aureus* (DRSA) phenotype; one of the pathways involved two steps of evolution: an initial 1 bp insertion into *yych* and a subsequent gain-in-function point mutation in *mprf* that was correlated with heteroresistance to daptomycin/vancomycin and full development of the VISA/DRSA phenotype; the other patway involved an 11 bp deletion mutation in *yych* and point mutations at two genes, correlated with the development of the VISA phenotype and heteroresistance to daptomycin; mutation in *mprf* and a 5 bp deletion mutation in *yych* were identified in the third pathway and corresponded to conversion into the full VISA/DRSA phenotype; these finding suggests that multiple evolutionary pathways involving *yycH* and *mprF* can proceed simultaneously and may mediate cross-resistance to glycopeptides and daptomycin during persistent MRSA bacteraemia under antibiotic selective pressure.	Chen, C. J. *et al.*, 2015
	Ceftaroline	MRSA, vancomycin-intermediate *S. aureus* (VISA), daptomycin-nonsusceptible *S. aureus* (DNSSA), linezolid-nonsusceptible *S. aureus* (LNSSA), and heteroresistant VISA (hVISA) isolates.	Broth microdilution, PFGE, multiplex PCR. PAP	21% isolates tested were ceftaroline-heteroresistant *S. aureus* (CHSA); CHSA occurred among strains with reduced susceptibilities to vancomycin, daptomycin, and linezolid; heteroresistant strains were mainly *SCCmec type II*; heteroresistant strains showed an unstable phenotype.	Saravolatz, S. N *et al.*, 2014
		MRSA bacteraemia isolates	Broth microdilution, MIC Evaluator (MICE) strips, PAP	All isolates were susceptible to ceftaroline, except one that displayed heteroresistance that may be related to a mecA mutation; ceftaroline resistance in *S. aureus* is a rare phenomenon; ceftaroline remains active and displays enhanced activity following vancomycin failure, suggesting that is a salvage therapy for the treatment of MRSA bloodstream infections.	Espedido, B. A. *et al.*, 2014
Staphylococcus capitis	Vancomycin	Bloodstream Isolates	E-test, PAP, CFUs, broth microdilution	All heteroresistant strains were identical suggesting the spread of a single heteroresistant *S. capitis* strain had occurred over a period of 3 years; the combination of vancomycin and meropenem could have enhanced the level of vancomycin resistance in *S. capitis* and might have contributed to the therapeutic failure.	Van Der Zwet, W. C. *et al.*, 2002
		Case report: 66-year-old man with severe pain in his back irradiated to the right hip, with severe limitation of motion	Blood culture, Vitek2, matrix-assisted laser desorption ionization-time of flight (MALDI-	Therapy with high level of vancomycin led to development of spondylodiscitis, being the first report of spondylodiscitis by vancomycin heteroresistant *S. capitis*; vancomycin treatment was	Bianco, C. *et al.*, 2014

		and no response to non-steroidal anti-inflammatory drugs and corticosteroids, associated with occasional fever spikes.	TOF) mass spectrometry, sequencing of 16S rDNA, PAP, macro E-test	replaced by linezolid and, then, linezolid was replaced for intravenously clindamycin administration, which showed a good clinical response.	
Streptococcus pneumoniae	Bacteriostatic and bactericidal antibiotics	Different strains of *Streptococcus pneumoniae*	Columbia agar supplemented with sheep blood plating, cellular metabolic activity assays, standard dilution, broth microdilution, microtiter plate reader assays, time-lapse microscopy, sequencing	Cells treated with bacteriostatic agents show continued gene expression activity; nine point mutations inside *rpoB* were found; a mutated penicillin-binding protein (PBP2x G601V)49 was found and showed significantly more resistant cells; the presence of a fraction of cells with decreased epigenetic susceptibility, can potentiate the ability of a population to develop genotypic resistance; mutated strains showed again heteroresistance development at an increased concentration window compared to the wil-type strain, demonstrating that resistance can build up in an iterative process via heteroresisters and suggesting that heteroresistance potentiates the accumulation of genotypic resistance.	Sorg, R. A. & Veening, J. W., 2015

CHAPTER 3: Materials and methods

3.1. Bacterial strains, preservation and growth conditions

The work was conducted using the strains *S. aureus ATCC 25923*, *P. aeruginosa PA14*, *K. pneumoniae ATCC 11296* and *A. baumannii ATCC 19606* available in Centre of Biological Engineering. These strains were stored in broth medium supplemented with 20 % (v/v) of glycerol within cryopreservation vials at - 80°C. Small samples taken from these were then spread onto Tryptic Soy Agar (TSA) plates, which grown overnight at 37°C. TSA plates were subsequently stored at 4 °C for a month (first culture). Before each experiment, several colonies from TSA plates were transferred to erlenmeyers containing Tryptic Soy Broth (TSB) and grown overnight at 37°C and 120 rpm (liquid culture).

3.2. Culture Media

Throughout this work, different culture media were used: TSB (TSB, 30 g/L, *LiofilChem)* was used for the planktonic growth of the microorganisms and for biofilm formation; TSA (or Tryptic Soy Broth, 30 g/L, *LiofilChem*) supplemented with agar, 12 g/L (*LiofilChem*) was used as solid culture medium and for Colony Forming Unit (CFU) assay; MHB (Mueller Hinton Broth, 21 g/L, *LiofilChem*) for the susceptibility assays, as recommended by EUCAST, for MIC determination; MHA (or Mueller Hinton Broth, 21 g/L, *LiofilChem*) supplemented with agar, 12 g/L (*LiofilChem*) for determination of minimum bactericidal concentration (MBC) and minimum biofilm eliminating concentration (MBEC). MHA supplemented with several antibiotic concentrations was used for PAP assays.

Selective agar media were also used in the experimental work: PIA (*Pseudomonas* Isolation Agar, 45.03 g/L, *LiofilChem*) supplemented with 2 % (v/v) glycerol for the identification of *P. aeruginosa* in mixed-species populations; MSA (Mannitol salt agar, 111g/L, *LiofilChem*) for the detection of *S. aureus;* and KSA (*Klebsiella* Selective HiCrome™ Agar Base, 40.8 g/L, *Sigma*), supplemented with *Klebsiella* Selective Supplement (Carbenicilin, 50 mg/L, *Sigma*) for detection of *K. pneumoniae* colonies.

3.3. Calibration curves (OD vs CFU)

Bacteria from pure liquid inocula were harvested by centrifugation for 10 min at 9000 xg and then washed twice in 1500 µL of TSB. Successive 10-fold dilutions of the suspended inoculum were made in TSB. For each diluted cell suspension, 200 µL were used to read the optical density (OD) at 640 nm and other 200 µL were transferred to a microtiter plate, where successive dilutions were performed in TSB. Subsequently, 10 µL drops of each dilution were plated onto TSA, following overnight incubation at 37°C. After incubation, CFU were counted and the calibration curves [OD at 640 nm vs CFU/mL] were conducted (**Figure A1 – annexes**). These curves were used for the adjustment of cell concentration in each experiment.

3.4. Biofilm formation

3.4.1 Single-species biofilms

Biofilms were formed in 96-well flat bottom microtiter plates (*Orange Scientific*). To prepare the biofilm inocula, bacteria from the pure liquid cultures were harvested by centrifugation for 10 min at 9000 xg and then washed with TSB. The OD at 640 nm was measured and then diluted to adjust the cell concentration to 1×10^7 CFU/mL, according to the calibration curves previously determined. To promote biofilm formation, 200 µL of the biofilm inoculum at 1×10^7 CFU/mL were transferred to each well of the microtiter plate and these were incubated for 24 h, at 37°C and 120 rpm.

3.4.2 Mixed-species biofilms

The formation of mixed-species biofilms followed a similar procedure to that described for single-species biofilms, however combining the following cultures (at 1×10^7 CFU/mL) in 1:1 proportions: *P. aeruginosa and S. aureus, P. aeruginosa and K. pneumoniae, P. aeruginosa and A. baumannii.* Afterwards, 200 µL of the mixed-species bacterial suspensions (1×10^7 CFU/mL) were transferred to each well of the microtiter plate, following an incubation of 24 h at 37°C and 120 rpm.

3.5. Biofilm analysis

After 24 h of incubation, the content of the plates (cell suspension) was discarded and the wells (containing the adhered cells) washed once with sterile deionized water to remove nonadherent or weakly adherent cells.

3.5.1 Biofilm adhered biomass

The amount of biomass was accessed using the Crystal Violet (CV) assay. CV assay is a staining assay adopted for biofilm biomass quantification. This allows to quantify the biofilm biomass by binding the CV dye to the negative charged molecules and polysaccharides of the EPS biofilm matrix. Afterwards, a solvent (e.g. acetic acid or ethanol) is used to elute the CV dye allowing the quantification of solubilized dye by the measurement of optical absorbance at 570 nm (Pantanella *et al.,* 2013).

Briefly, 200 µL of methanol 100 % (v/v) was added to each well to allow the fixation of the cells. After 15 minutes, methanol was discarded and 200 µL of CV at 1 % (v/v) was added to each well, for 5 minutes. Afterwards, the excess of CV dye was discarded and the wells were washed with 200 µL of distilled water. The plates were allowed to air dry for 15 to 20 min and 200 µL of acetic acid at 33 % (v/v) was added to each well and the OD measured at 570 nm.

3.5.1.1 Classification of strains according to their biomass production

The ability for biomass production determined for *P. aeruginosa*, *S. aureus*, *K. pneumoniae* and *A. baumannii* was determined and classified into four categories, according to that described by Christensen *et al.* (1985). For that, the critical OD (ODc) was calculated from the arithmetic mean of the absorbance measured from the negative control (containing only TSB medium) with three times addition of standard deviation (SD), as follows:

$$ODc = (Mean+3\times SD)$$

The classification of each species regarding their ability for biomass production was as follows: no biofilm producer (OD_{570nm} strain ≤ ODc), weak biofilm producer (ODc ≤ OD_{570nm} strain ≤ 2×ODc), moderate biofilm producer (2×ODc ≤ OD_{570nm} strain ≤ 4×ODc), and strong biofilm producer (4×ODc < OD_{570nm} strain).

3.5.2 Biofilm - cells cultivability

After the rinse step, 200 µL of sterile deionized water was added to each well. To detach biofilm-cells from the surface of the wells, the plates followed to an ultrasonic cleaning bath for 10 minutes. Then, the content of 5 wells (total volume of 1 mL) was recovered to an Eppendorf tube, homogenized using the vortex and 200 µL of the biofilm suspension were transferred to a new microtiter plate. Serial 1:10 dilutions were performed in TSB. Posteriorly, 10 µL drops of each

dilution was plated onto TSA and onto selective growth solid medium, and plates followed an overnight incubation at 37°C. After incubation for 24 h, CFU were counted and values were expressed as CFU/cm^2.

3.6. Susceptibility testing

The antimicrobial agents used throughout this work were: colistin (*Sigma*), vancomycin (*AppliChem*), rifampicin (*AppliChem*) and imipenem (*Sigma*). From each antibiotic, stock aliquots were prepared at 1000 mg/L and stored according to the manufacturer´s instructions.

3.6.1 Planktonic susceptibility

The MICs and MBCs obtained for the planktonic cultures were determined according to the microdilution test, using 96-well round bottom microtiter plates (*Orange Scientific*) and cation adjusted MHB as medium, according to EUCAST guidelines (EUCAST, 2015) .

The range of antibiotic concentrations tested were 0.25 - 16 mg/L for colistin and vancomycin and 0.0625 - 32 mg/L for rifampicin and imipenem. Briefly, the stock solutions of the antibiotics were diluted in MHB to prepare 2-fold of the desired final concentrations. Then, 100 µL of these antibiotic solutions were transferred to the microtiter plates and 100 µL of cell suspensions at 1×10^6 CFU/mL (achieved by the calibration curves) were added to each well, reaching a final concentration of 5×10^5 CFU/mL. For the positive control (bacterial suspension free of antibiotic), 100 µL of bacterial suspension were diluted in 100 µL of MHB per well. For the negative control, only 200 µL of MHB were added to each well. The plates were incubated for 24 h, at 37 °C and 120 rpm. After incubation, the OD at 640nm was measured and the MIC was recorded as the lowest concentration where no visible cell growth was detected. For the MBC, 10 µL of each well were plated onto MHA, which followed an overnight incubation at 37 °C. The MBC was recorded as the lowest antimicrobial concentration that yielded no colony growth.

3.6.2 Biofilm susceptibility

A similar procedure to that described previously was used to determinate the susceptibilities from the biofilm-cells towards the antibiotics. After 24 h of incubation, the plates were washed twice with sterile deionized water and followed an ultrasonic cleaning bath for 10 minutes to detach biofilm cells form the surface of the wells. Then, 10 µL of each well were plated onto MHA and incubated at 37°C for 24 h. The MBEC was recorded as the lowest antimicrobial concentration that yielded no colony growth.

3.7. Population analysis profile (PAP)

This assay was performed for the bacterial cultures with suspected cases of heteroresistance in the susceptibility testing.

3.7.1 Planktonic cells

MHA was properly prepared and sterilized at 121°C, 15 min. The medium was allowed to cool to about 50 °C, with stirring and then supplemented with antibiotic before and spread onto Petri plates. MHA plates supplemented with 1, 2, 4, 8 and 16 mg/L of each antibiotic were prepared. Bacteria from the liquid cultures were harvested by centrifugation for 5 min at 9000 xg and then washed in a saline solution (NaCl at 0,9 % (w/v)). Afterwards, serial dilutions from planktonic cultures were spread (50 µL) onto MHA supplemented with increasing concentrations (1, 2, 4, 8 and 16 mg/L) of each antibiotic. The plates were incubated for 48 h at 37°C and CFU counted. The observation of subpopulations capable of surviving at antibiotic concentrations higher than the MIC value was considered heteroresistant.

3.7.2 Biofilm cells

The PAP was also analyzed for the single-species biofilms and followed a similar procedure to that for planktonic cultures. Biofilms were formed in 24-wells microtiter plates (*Orange Scientific*), by adding 1 mL of bacterial suspension at 1×10^7 CFU/mL (achieved by the respective calibration curve) to each well. Once formed the biofilms, these were scraped using a pipette tip and biofilm cells were res-suspended in NaCl and gently vortexed for 1 min to disrupt possible cell aggregates. The solution was adjusted to 1 x 10^8 CFU/m L and serial dilutions were prepared in NaCl, which were properly spread onto MHA plates with increasing antibiotic concentrations. The plates were incubated for 48 h at 37 °C and CFU were counted.

MHA plates containing colistin concentrations of ¼ × MIC, ½ × MIC, 1 × MIC, 2× MIC, 4× MIC and 8× MIC were prepared to perform PAP of *P. aeruginosa* and *K. pneumoniae* biofilms.

3.7.3 Calculation of resistant fractions

The frequency of resistant sub-populations was calculated by dividing the number of CFU/mL counted on MHA antibiotic-containing plates by the number of CFU per mL counted from the same bacterial inoculum plated onto antibiotic-free plates (**Table A2 - annexes**), as follows:

$$\frac{CFU/ml(antibiotic\ suplemmented\ plate)}{CFU/ml(\text{antibiotic} - \text{free plate})}$$

3.8. Statistical analysis

In order to evaluate the influence of the studied parameters, a statistical analysis was conducted. Three independent assays were, at least, performed all experiments. Biofilm viability and biofilm biomass were statistically analyzed by a model of Kruskal-wallis of *GraphPad Prism* software. Differences between means were considered statistically significant for *p*-values ≤ 0.05

CHAPTER 4: Results and discussion

4.1. Biomass and cell cultivability of single and mixed-species biofilms

4.1.1 Single-species biofilms

Biofilms are becoming a potential concern for public health, mainly in nosocomial settings, being increasingly important to deeply study these consortia (Macia *et al.,* 2014).

In order to access and compare the *in vitro* biofilm formation by *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, CV assay was conducted to analyze the adhered biomass, as well as the culturability of the biofilm-associated cells as assessed through CFU counting (**Figure 8)**.

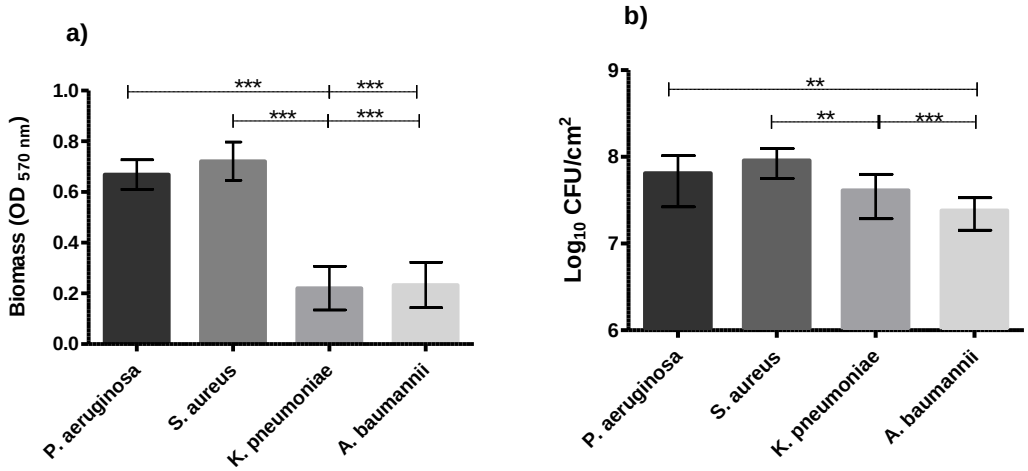

Figure 8: Analysis of single-species biofilms formed by *P. aeruginosa, S. aureus, K. pneumoniae* and *A. baumannii* in terms of: adhered biomass (a) and cell culturability (b). Biomass was assessed by the CV assay, which is proportional to the OD at 570nm; viable cells in biofilms were counted by the plate counting method and is expressed as CFU/cm^2. The means ± SDs for four independent experiments are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ for statistical significant differences (one-way ANOVA, Tukey´s multiple comparison of columns)

In respect to the biomass production (**Figure 8a**), *S. aureus* presented the greatest amount of biofilm adhered biomass, showing the highest OD at 570 nm value (0.72 ± 0.08), followed by *P. aeruginosa* (0.67 ± 0.06), *A. baumannii* (0.23 ± 0.09) and *K. pneumoniae* (0.22 ± 0.09. Results showed that *K. pneumoniae* and *A. baumannii* presented significant lower ability to form biofilm under *in vitro* conditions compared with *P. aeruginosa* and *S. aureus* ($p \leq 0.001$). No significant differences were observed for biomass formed by S*. aureus or P. aeruginosa* and for *K. pneumoniae* or *A. baumannii.*

Regarding the cell cultivability **(Figure 8b)**, *S. aureus* showed the highest CFU counts, followed by *P. aeruginosa*, with no significant difference in the number of biofilm-associated cells. *A.*

baumannii was the species with the lowest CFU counts, presenting no significant differences in the number of viable cells compared with *K. pneumoniae*. *A. baumannii* and *K. pneumoniae* showed statistically significantly less biofilm-associated cells than *P. aeruginosa* ($p \leq 0.01$) and *S. aureus* ($p \leq 0.001$ and $p \leq 0.01$, respectively).

For a better comparative analysis of the previous results, the different species were classified as: no biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer, according to their ability of biofilm production (Christensen *et al.*, 1985). Accordingly, *S. aureus* was considered a strong biofilm producer. Contrariwise, *K. pneumoniae* and *A. baumannii* were weak biofilm producers, whereas *P. aeruginosa* showed to be a moderate biofilm producer.

Several reports have showed the great ability of *S. aureus* to develop *in vitro* biofilms. Stepanovic et al (1999) tested staphylococci strains for their biofilm formation ability and found that 86 % of *S. aureus* strains presented moderate and/or strong adherence capacities. Park et al. (2012) studied the effect of interaction of *S. aureus* ATCC 25923 with other bacterial species in biofilm formation and observed an enhanced biomass production by *S. aureus*. Hou *et al.* (2012) studied the biofilm forming ability of bacteria involved in ocular infections and also verified that *S. aureus* strains could present a great adhesion ability to microliter plates in *in vitro* biofilms.

P. aeruginosa showed to be a moderate biofilm producer. A similar behaviour for this species was found by Lopes *et al.* (2012) and Magalhães *et al.* (2017) who verified a great ability of *P. aeruginosa* to accumulate biomass in the wells of microtiter plates. Particularly, Magalhães *et al.* (2017) had verified values for *P. aeruginosa* biomass very similar to those observed in this study.

A. baumannii was suggested as weak biofilm producer. Indeed, this weak ability for biofilm formation by *A. baumannii* has been already been reported by Varposhti *et al.* (2014), who verified that the tested *A. baumannii* strains did not strongly produce biofilm under *in vtro* conditions.

Benincasa *et al.* (2016) had reported, in an earlier study, that *K. pneumonie* possess very poor adhesive properties, which can explain its low ability to develop biofilms in microtiter plates.

4.1.2 Mixed-species biofilms

Mixed-species biofilms are rarely found in nature. Usually, bacterial biofilms are found as multispecies communities, which differ vastly in their composition, structure, and antimicrobial resistance from mono-species biofilm systems. It is essential inspect into these heterogeneous populations for a better understanding of the interactions occurring amongst the residing microbial species and even with those occurring with the surrounding environment (Magalhães *et al.,* 2017)

Therefore, the following mixed-species populations: *P. aeruginosa* + *S. aureus*; *P. aeruginosa* + *K. pneumoniae* and *P. aeruginosa* + *A. baumannii were analyzed,* due their clinical relevance in nosocomial infections, like wound infections and respiratory infections. *P. aeruginosa and S. aureus* are the main bacteria involved in the early and critical stages of cystic fibrosis (CF), a disease involving the production of thick and sticky mucus leading to a serious and frequent pulmonary infections. *K. pneumoniae* is found together with *P. aeruginosa* in biofilm-mediated chronic wound infections as well as urinary and respiratory tract infections. *P. aeruginosa* and *A. baumannii* are among the most important causative agents in nosocomial respiratory diseases (Dowd *et al.,* 2008; Childers *et al.,* 2013).

The dual-species biofilms analysis was conducted as in single-species biofilm, using CV assay for biomass quantification and CFU counting for the determining the cultivability of the biofilm-associated cells **(Figure 9)**.

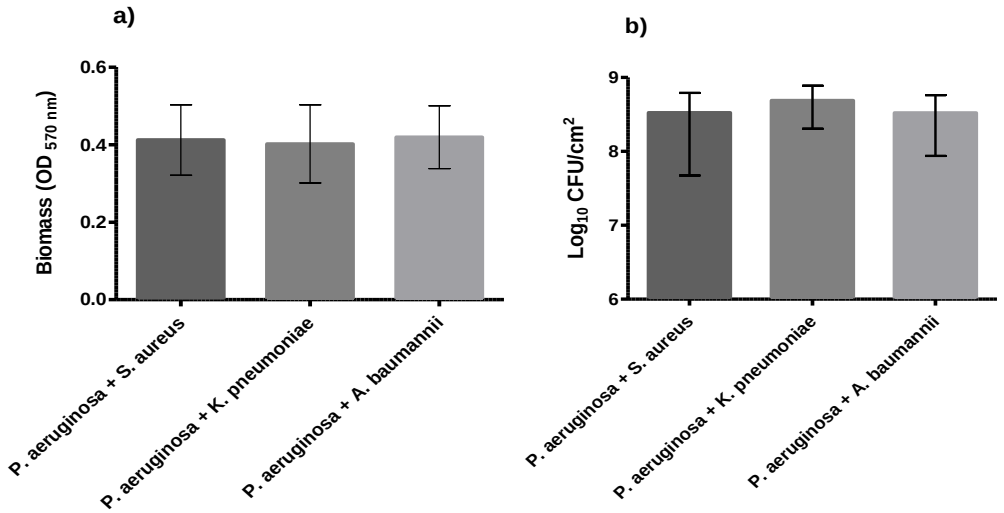

Figure 9: Analysis of single-species biofilms formed by *mixed-species* in terms of: adhered biomass (a) and cell culturability (b). Biomass was assessed by the CV assay, which is proportional to the OD at 570nm; viable cells in biofilms were counted by the plate counting method and is expressed as CFU/cm^2. The means ± SDs for four independent experiments are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ for statistical significant differences (one-way ANOVA, Tukey´s multiple comparison of columns)

As observed, mixed-species biofilms of *P. aeruginosa* co-cultured with *A. baumannii* had the capacity to produce the highest total biomass amount (OD at 570 nm of 0.42 ± 0.08). However, no significant differences were observed in biomass production ability when compared with the other dual-species consortia (**Figure 9a**).

Regarding the culturability data, the mixed population composed of *P. aeruginosa* and *K. pneumoniae* presented the highest number of viable biofilm-associated cells. No significant differences were observed between all tested dual-species biofilms (**Figure 9b**).

Comparing dual-species with single-species biofilms (see **Figure 8**), it was noticed that dual-species biofilms formed by *P. aeruginosa* with any of the other species reached significantly higher biofilm-associated cells comparing to biofilms of *P. aeruginosa* alone ($p \leq 0.01$) (**Figures 8b and 9b**). These results are not completely in accordance with that described in the literature. Lopes *et al.* (2012), Varposhti *et al.* (2014) and Childers *et al.* (2013) had previously studied mixed populations of *P. aeruginosa* with other species and observed that the total number of mixed-biofilm cells presented no significant differences comparing with biofilm cells produced by *P. aeruginosa* alone.

In the case of total amount of biomass, biofilms of *P. aeruginosa with S. aureus* were markedly reduced for biomass production comparing to both single-species biofilms. Contrariwise, when *P. aeruginosa* was co-cultured with *K. pneumoniae* or *A. baumannii,* an increase in the amount of biomass was observed compared to *K. pneumoniae* and *A. baumannii* growing alone, but a reduction compared with *P. aeruginosa* adhered biomass (**Figures 8a and 9a).**

The interactions that occur in multispecies biofilms may encourage the coexistence – synergism relation - or confer advantage to one species, inhibiting the growth of other species – antagonism relation (Harrison, 2007).

P. aeruginosa is particularly known to be an antagonistic organism capable of using a number of effectors (e.g LasA, LasB, N-homoserine lactone, pyocyanin, cis-2-decenoic acid, and rhamnolipids) in order to gain a competitive advantage over other organisms, in both sessile and planktonic cultures. Effectors like phenazine and *LasB* were already demonstrated to be effective against *Candida albicans* and *S. aureus*, respectively (Park *et al.,* 2012).

The reduction in biomass production in mixed biofilms of *P. aeruginosa* with *S. aureus* comparing to the respective single-species biofilms suggested an antagonism relationship between both species. Several studies have already demonstrated this interaction between *P. aeruginosa* and *S. aureus* in CF context, where the competitive ability of *P. aeruginosa* increased *in vitr*o and it can lyse the cells of *S. aureus*, using the iron released from the bacterial lysis to support its own growth (Park *et al.*, 2012; Mashburn *et al.*, 2005). Other evidences have also supported the antagonism between both species. Apparently, *S. aureus* is susceptible to *P. aeruginosa* exoproducts, such as pyocyanin, hydrogencyanide or alkyl-hydroxyquinoline *N*-oxides (HQNO),

which suppress its aerobic metabolism and growth (Hoffman *et al.*, 2006; Biswas *et al.*, 2009). In this way, it is suggested that a reciprocal interference may be occurring between *P. aeruginosa* and *S. aureus,* leading to a decrease on the EPS production by the whole microbial community, resulting in a marked reduction in the total amount of biomass.

Biofilms composed of *P. aeruginosa* and *K. pneumoniae* showed a reduction in biomass when compared with *P. aeruginosa* single biofilms. However, a considerable increase in amount of biomass production in comparison with *K. pneumoniae* alone was also observed. These results suggested that *K. pneumoniae* may be able to protect itself from the *P. aeruginosa* effectors, leading to a reduction in biomass production by *P. aeruginosa*. In fact, Skillman *et al.* (1998) proposed that *K. pneumoniae* formed biofilms more successfully in a mixture than in isolation. Similar results had already been reported by Stewart *et al.* (1997), who showed evidences that *K. pneumoniae* were able to form a stable biofilm with *P. aeruginosa* and suggested a potential mechanism developed by *K. pneumoniae* that allow it to fight *P. aeruginosa* effectors and lead to the disruption of biofilms composed of this bacterium.

The same behavior was observed for mixed-population of *P. aeruginosa* and *A. baumannii*. Varposhti *et al.* (2014) found a marked increase in biofilm formation by *P. aeruginosa* and *A. baumannii* when they were co-cultured with *Stenotrophomonas maltophilia* and perceived that synergistic relationship was dependent of the three species together. It is thus possible that, in this case, such as *K. pneumoniae*, *A. baumannii* has the ability to protect itself from *P. aeruginosa,* leading to a reduction in biomass production by *P. aeruginosa.*

4.2. *In vitro* susceptibility of planktonic and biofilm populations

In a first experimental stage, the MIC, MBC and MBEC values to colistin and vancomycin were determined in a concentration range of 0 - 16 mg/L and to rifampicin and imipenem was used a concentration range of 0 - 32 mg/L.

4.2.1 Planktonic cells susceptibility

Results obtained from *in vitro* susceptibilities of *P. aeruginosa, S. aureus, K. pneumoniae* and *A. baumannii* towards colistin, vancomycin, rifampicin and imipenem are summarized in **Table 3.**

Table 3: *In vitro* susceptibility patterns of single-species planktonic and biofilm populations towards colistin, vancomycin, rifampicin and imipenem

* Visible turbidity (indicating cell growth) in the wells of different concentrations (among replicates), suggesting heteroresistance

** Visible turbidity (indicating cell growth) in the wells of different concentrations (among independent assays),

(R) resistant; (I) intermediate; (S) sensitive, according to the EUCAST breakpoints (Table A1 in Annexes)

		Colistin	Vancomycin	Rifampicin	Imipenem
P. aeruginosa	MIC (mg/L)	4 **(R)**	>16	>16	2-4 **(S)**
	MBC (mg/L)	8	>16	>32	8-16*
	MBEC (mg/L)	8-16*	>16	>32	>32
S. aureus	MIC (mg/L)	>16	4 **(R)**	0,125-0,25** **(I)**	0,0625-0,125** **(S)**
	MBC (mg/L)	>16	8-16*	0,5	0,125
	MBEC (mg/L)	>16	>16	2	0,5-2**
K. pneumoniae	MIC (mg/L)	4 **(R)**	>16	4	0,5 **(I)**
	MBC (mg/L)	8-16*	>16	8	1
	MBEC (mg/L)	>16	>16	16-32**	8
A. baumannii	MIC (mg/L)	8 **(R)**	>16	4-8	2-4 (I)
	MBC (mg/L)	16 - >16**	>16	16-32*	8-16**
	MBEC (mg/L)	≥ 16	>16	> 32	≥ 32

Comparing all the antibiotics, imipenem was the only one in which no resistant behavior was observed by all the tested strains. On the other hand, with the exception of *S. aureus*, which is intrinsically resistant to colistin, all populations showed a resistant behavior against colistin, presenting values 2- or 4-fold times superior than the one established as sensitive by EUCAST.

For all the antibiotics tested, the MBC values were higher than MIC data. These results are strongly linked to the bacteriostatic and bactericidal effects of a given antimicrobial agent. The MIC value demonstrates the lowest level of antimicrobial agent that inhibits the growth of a given organism, whereas MBC value determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. Usually, antibacterial agents regarded as bactericidal if the MBC is no more than four times the MIC. In all cases, the MBC value did not exceed four times the MIC value, suggesting a potential bactericidal effect of the tested antibiotics against planktonic bacteria (Sanfilippo, 1979).

In the course of susceptibility assays, it was observed an inconsistent behavior for *P. aeruginosa* and *K. pneumoniae* exposed to colistin, as well as in *S. aureus* and *A. baumannii* towards to vancomycin and rifampicin, respectively (**Table 3**). The response of these populations was found heterogeneous between replicates, with no visible turbidity observed in some wells, suggesting bacterial growth inhibition, whereas a high turbidity was noticed for other wells in the same condition (antibiotic concentration). This behavior led to suspect for heteroresistance in these populations towards those antibiotics, which was investigated later by PAP.

4.2.2. Single biofilms susceptibility

In general, biofilms showed a significant lower susceptibility pattern towards most antibiotics comparatively with their planktonic counterparts (MBECs in general, higher than MIC data). As such, the MIC values obtained to inhibit the planktonic populations had no effect when used against biofilm cultures. Furthermore, in some cases, any concentration within the tested range showed to be able to eradicate the biofilm cells (MBECs > 16 for colistin and vancomycin and > 32 for rifampicin and imipenem).

Only imipenem and rifampicin were able to kill cells from *S. aureus* and *K. pneumoniae* biofilms at relatively low concentrations, however with MBEC values significantly greater compared with respective MIC data.

It is therefore suggested that it is required higher antibiotic concentrations to kill biofilm cells than planktonic cells, suggesting that biofilms are notoriously difficult to eradicate, being necessary high doses of antimicrobials to eliminate them. These findings were consistent with previous reports that showed that biofilm population exhibited enhanced resistance to antibiotics compared to planktonic populations, tolerating up to 100-1000 times higher concentrations of antibiotics (Macia *et al.*, 2014; Harrison, 2007; Lopes *el al.*, 2012; Magalhães *et al.*, 2017).

4.2.3. Mixed-species biofilms susceptibility

For all mixed-species cultures, antibiotic concentration tested range from 0 to 64 mg/L. Results showing MIC, MBC and MBEC data obtained against mixed-species populations are summarized on **Table 4.**

Table 4: *In vitro* susceptibility patterns of mixed-species planktonic and biofilm populations towards colistin, vancomycin, rifampicin and imipenem

** Visible turbidity (indicating cell growth) in the wells of different concentrations (among independent assays),

		Colistin	Vancomycin	Rifampicin	Imipinem
P. aeruginosa + S. aureus	MIC (mg/L)	32-64**	>64	>64	2-4**
	MBC (mg/L)	>64	>64	>64	32-64**
	MBEC (mg/L)	>64	>64	>64	>64
	PIA	8	>64	>64	8-16
	MS Agar	64	8	0,5	0,25-0,5**
P. aeruginosa + A. baumannii	MIC (mg/L)	8	>64	>64	2-4
	MBC (mg/L)	16	>64	>64	8
	MBEC (mg/L)	>64	>64	>64	>64
	PIA	8	>64	>64	8-16**
	Mackonkey agar	8	>64	>64	16

P. aeruginosa + *K. pneumoniae*	MIC (mg/L)	4	>64	>64	2-4
	MBC (mg/L)	64	>64	>64	8-16
	MBEC (mg/L)	>64	>64	>64	>64
	PIA	8-16**	>64	>64	16
	KP selective agar	8-16**	64	8-16**	1

In general, the antibiotic concentration required to inhibit the growth of mixed population cells was similar to that needed to inhibit the less susceptible microorganism alone. For instance, the MIC value obtained for imipenem against mixed-species populations of *P. aeruginosa* and *S. aureus* showed to be 2 - 4 mg/L, which corresponded to the imipenem MIC value towards *P. aeruginosa* single-species culture (whereas imipenem MIC was found to be 0,0625 - 0,125 mg/L against *S. aureus*). This behavior had already been reported by Lopes *et al.* (2012), who demonstrated that mixed planktonic populations required equal or even higher antibiotic concentrations than those applied to inhibit the planktonic growth of single populations.

Regarding MBEC data, unlike the single-species biofilms, no achievable concentration within the tested antibiotic range could be determined for the dual-species biofilms (**Table 4**), showing that higher doses of antibiotic are needed to eradicate mixed-species biofilms. These results are in agreement with Lopes *et al.* (2012) and Magalhães *et al.* (2017), who suggested that in mixed biofilms may occur changes in the metabolic activity of the consortium that may lead to changes in susceptibility patterns of the population.

In order to investigate which of the species within the consortium was most easily eliminated by the antibiotic agent, the MBEC of mixed biofilm cells was also determined by platting the cells onto selective culture media. To isolate *P. aeruginosa*, *Pseudomonas* Isolation Agar (PIA) was used; to isolate *S. aureus*, Mannitol Salt Agar (MSA) was used; and to isolate *K. pneumoniae* it was used the HiCrome *Klebsiella* Selective Agar (**Table 4**).

All antibiotics tested displayed MBECs > 64 mg/L when *P. aeruginosa* was co-cultured with *S. aureus*. The exposure of the population to vancomycin and rifampicin showed that *P. aeruginosa* was able to growth at 64 mg/L in selective medium, whereas *S. aureus* showed to be more susceptible. The MBEC value of mixed population was the same as for *P. aeruginosa* (MBEC> 64 mg/L), which is in accordance with Lopes *et al.* (2012), who verified that MBEC of mixed biofilms was generally equal to the concentration needed to kill the more resistant single biofilm of the encompassed species. The decrease in the MBEC value of *S. aureus* towards vancomycin and rifampicin when it was co-cultured with *P. aeruginos*a, suggested that *P. aeruginosa* may be capable to increase the susceptibility of *S. aureus* to these antibiotics. As previously discussed, the

interactions established in mixed populations can alter the metabolic activity of certain bacteria, leading to modifications in their patterns of susceptibility. (Lopes *et al.,* 2012 and Magalhães *et al.,* 2017). So, this increase in *S. aureus* susceptibility may derive from the antagonistic relation between *S. aureus* and *P. aeruginosa*. Other evidences have also supported the antagonism between *both species.* Apparently*, S. aureus* is susceptible to *P. aeruginosa* exoproducts which suppress its aerobic metabolism and growth (Hoffman *et al*., 2006; Biswas *et al*., 2009).

On the other hand, the results obtained by selective media of *S. aureus* and *P. aeruginosa* towards imipenem showed an atypical behavior. Both populations showed a decrease in MBEC value when cultured in selective medium compared with when cultured in MHA (MBEC value > 64 mg/L), suggesting the occurrence of bacterial contamination or the possible existence of differences in growth of bacteria in selective solid media and non-selective media. A similar behavior was also observed towards colistin and imipinem to cultures of *P. aeruginosa* with *A. baumannii* and for *P. aeruginosa* co-cultured with *K. pneumoniae*.

All antibiotics displayed MBECs >64 mg/L for mixed-species cultures encompassing *P. aeruginosa* and *A. baumannii.* The analyses of MBEC values towards rifampicin and vancomycin showed that the concentrations tested were not sufficient to kill these biofilms. Even in selective medium, it was possible to observe growth of *A. baumannii* and *P. aeruginosa* cells.

Biofilms composed of *P. aeruginosa* and *K. pneumoniae* also showed low susceptibility to all antibiotics, with MBECs higher than 64 mg/L. *P. aeruginosa* was able to growth at 64 mg/L of vancomycin and rifampicin in selective medium, unlike *K. pneumoniae* which displayed a more susceptible profile. As expected, the MBEC of the mixed-species population was equal to the MBEC obtained for the most resistant population in single-species (*P. aeruginosa*). When the mixed population was exposed to rifampicin, a decrease of MBEC value for *K. pneumoniae* obtained in selective medium was observed, suggesting that the interactions between *P. aeruginosa* and *K. pneumoniae* led to an increase in its susceptibility.

4.2.4. Biofilms susceptibility of bacteria that displayed heterogeneous responses between replicates in *in vitro* susceptibility assays

For the populations suspected to be heteroresistant in previous assays (*P. aeruginosa* and *K. pneumoniae* towards colistin, *S. aureus* against vancomycin and *A. baumannii* towards rifampicin), it was re-assessed their biofilms *in vitro* susceptibility (MBEC), by increasing the range of concentrations of antibiotics up to 1024 mg/L. The results are summarized in **Table 5.**

Table 5: *In vitro* susceptibility patterns of single biofilms that suggested heteroresistance in previous assays
(-) not detected

	Colistin (MBEC - mg/L)	Vancomycin (MBEC - mg/L)	Rifampicin (MBEC - mg/L)
K. pneumoniae	32-64	-	-
S. aureus	-	>1024	-
A. baumannii	-	-	>1024

It was found that concentrations of 32 and 64 mg/L of colistin were able to completely eradicate *K. pneumoniae* biofilms, which is in accordance with Silva *et al.* (2016), who verified that only high concentrations of colistin (32 and 64 μg/mL) resulted in a considerable reduction of biofilm-cells viability in *K. pneumoniae* biofilm-related cells. Contrariwise, a concentration of 1024 mg/L of rifampicin and vancomycin was not enough to eliminate the biofilms of *A. baumannii* and *S. aureus*, respectively.

Once again, these findings emphasize that biofilms require higher doses of antibiotic than planktonic cells in order to completely eliminate them, which is in accordance with several studies that proved that biofilms can tolerate up to 100 - 1000 times higher concentrations of antibiotics than planktonic cells (Macia *et al.,* 2014). In here, even abnormal doses of antibiotic (1024 mg/L) were unable to kill biofilm-cells, showing the difficulty on treating biofilm-related infections in clinical settings.

4.3. Population analysis profile (PAP)

Population analysis profile is one of the most used methods for detection of heteroresistance among diverse microorganisms, allowing the detection and quantification of resistant sub-populations in an isolate (Silva *et al.,* 2016).

4.3.1 Planktonic cells

For temporal reasons, the inspection of heteroresistance by PAP was only conducted for the cases where was observed a heterogeneous behavior (different growth) between replicates in *in vitro* susceptibility assays: *S. aureus* towards vancomycin, *K. pneumoniae* and *P. aeruginosa* towards colistin and *A. baumannii* towards rifampicin. In order to investigate the suspicious of heteroresistance, the growth of those populations was followed onto MHA plates supplemented

with 1, 2, 4, 8 and 16 mg/L of antibiotic, that represents ¼ × MIC, ½ × MIC, 1 × MIC, 2× MIC and 4xMIC. The results are available in **Figure 10.**

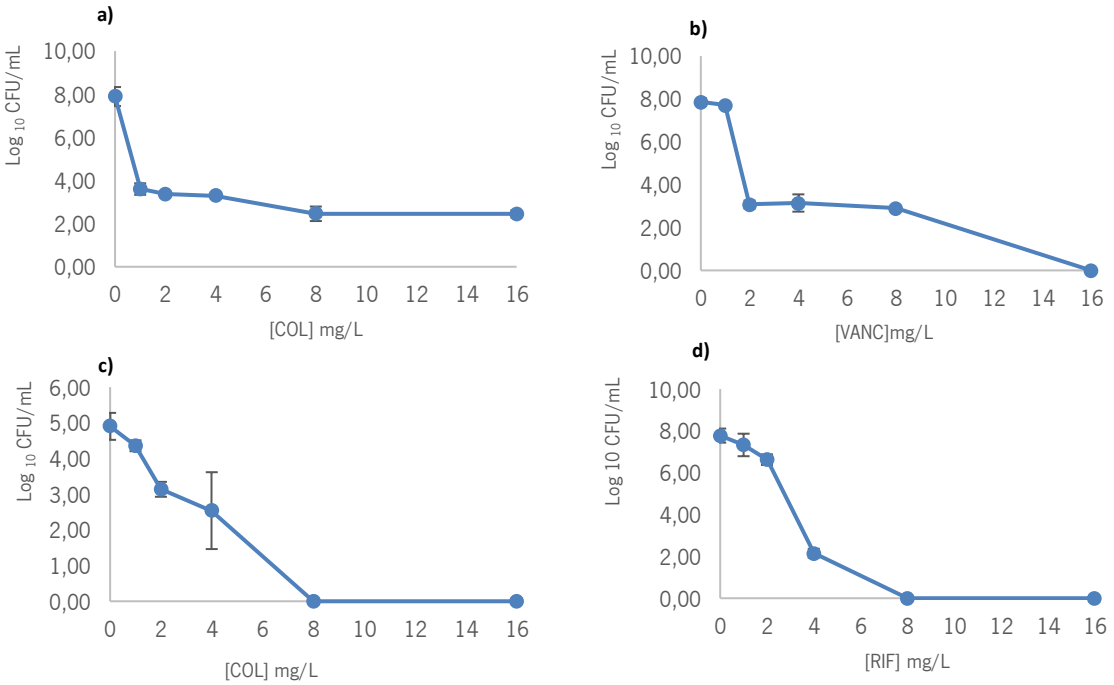

Figure 10: Population analysis profile of planktonic bacteria suggesting heteroresistance in susceptibility assays - a) *K. pneumoniae* towards colistin; b) *S. aureus* towards vancomycin; c) *P. aeruginosa* towards colistin; d) *A. baumannii* towards rifampicin. The bacterial growth was followed by CFU counting onto MHA plates supplemented with 1, 2, 4, 8 and 16 mg/L of antibiotic. Results are expressed as mean ± SD.

In general, CFU declined severely with the increase of antibiotic concentration (**Figure 10**).

Planktonic cultures of *K. pneumoniae* revealed low fractions of the population able to grow in the presence of 8 mg/L and 16 mg/L (**Figure 10a**). The observation of sub-populations able to survive in concentrations of colistin superior to the MIC value (4 mg/L) led to confirm the phenomenon of heteroresistance in planktonic cells of *K. pneumoniae* towards colistin. In fact, similar results had been reported by Meletis *et al.* (2011) and Poudyal *et al.* (2008), who verified that some strains and isolates of *K. pneumoniae* yielded heteroresistant subpopulations that grew in up to 8 mg/L colistin. This is the first report showing *K. pneumoniae* planktonic cells capable of growing at 16 mg/L of colistin.

The frequency of resistant sub-populations was calculated as described in **section 3.7.3** and is available in **Table A2 – Annexes**. The proportion of *K. pneumoniae* resistant subpopulations was $3.59\times10^{-6} \pm 2.58\times10^{-2}$ and $3.03\times10^{-6} \pm 1.11\times10^{-2}$ for population that growth on 8 mg/L and 16

mg/L, respectively. These results are in accordance with Meletis *et al.* (2011), who verified that the proportion of resistant sub-populations was of the order of 1.5×10^{-5} to 3.2×10^{-7} for isolates which were not exposed to colistin.

This resistance was maintained for these sub-populations once there was non-affectation of MIC value (16 mg/L) before and after ten daily sub-cultures on antibiotic free solid medium.

Heteroresistance was also detected in planktonic cells of *S. aureus* towards vancomycin, where the PAP analysis allowed to detect sub-populations able to growth in MHA plates supplemented with 8 mg/L vancomycin (**Figure 10b**). Since the MIC value of vancomycin against *S. aureus* in *in vitro* susceptibility assays was found to be 4 mg/L, PAP clearly evidences the presence of heteroresistant populations in *S. aureus* planktonic cultures. The resistant fraction of the *S. aureus* population, growing at 8 mg/L of vancomycin was calculated as previously described. The proportion of these resistant populations was $1.04\times10^{-5}\pm 7.37\times10^{-1}$ (**Table A2 – Annexes**). *S. aureus* heteroresistance against vancomycin were already resported by other authors (e.g. Silveira *et al.,* 2015; Cui *et al.,* 2010; van Hal *et al.,* 2011; Krause *et al.,* 2012). Silveira *et al.* (2015) had also studied the susceptibility of clinical isolates of *S. aureus,* and found sub-populations able to growth at 8 mg/L of vancomycin.

Antibiotics from the glycopeptides group, like vancomycin, have long been used in the treatment of serious infections. However, the recently reported emergence of *S. aureus* with intermediate resistance to vancomycin and *S. aureus* with heteroresistance to vancomycin already led to therapeutic failures when glycopeptides are used to treat *S. aureus*-related infections (Wong *et al.,* 2016). The vancomycin heteroresistance by *S. aureus* evidences that the treatment of infections with vancomycin may be compromised.

No evidence of heteroresistance was found for planktonic cultures of *P. aeruginosa* and *A. baumannii* towards colistin and rifampicin (**Figure 10c and 10d**), respectively, as no growth was visible onto MHA supplemented with concentrations above the MIC value recorded in the *in vitro* susceptibility assays (4 mg/L and 4-8 mg/L were the MICs obatined for colistin and rifampicin against P. aeruginosa and *A. baumannii*, respectively).

According to Hermes *et al.* (2013), who evaluated the heteroresistance of carbapenem-susceptible and –resistant *P. aeruginosa* to polymyxin B, the heteroresistance to colistin by *P. aeruginosa* is a very uncommon phenomenon. So, it was expected no heteroresistance to colistin by *P. aeruginosa* populations.

As mentioned before, the study of heteroresistance through PAP was performed only for the bacteria that showed turbidity for some subpopulations, for concentrations above the MIC in their susceptibility patterns.

Although it was not observed a turbidity above the MIC when *P. aeruginosa* cells were exposed to imipenem, Lister *et al.* (2009) and Mei *et al.* (2015) have already reported and related the heteroresistance phenomenon of *P. aeruginosa* cells towards imipenem with the overexpression of *Mex-Opr* type efflux pump and /or the repression of porin *orD*.

Although it was not observed, in this study, heteroresistance for *A. baumannii* towards imipenem or colistin, this phenomenon has been reported by other authors. Hernan *et al.* (2009), Yau *et al.* (2008), Li *et al.* (2006), Hawley *et al*. (2007) and Moosavian *et al.* (2015) reported colistin-heteroresistance in many *A. baumannii* isolates. Many authors (Ikonomidis *et al.*, 2009, Lee *et al.*, 2010; Neou *et al.*, 2016), have associated the carbapenem-heteroresistance of *A. baumannii* to the overexpression of a class C β-lactamase gene.

The colistin-heteroresistance of *K. pneumoniae* was confirmed by PAP in this study. However, it was not observed a suspected heteroresistant behavior in susceptibility assays when *K. pneumoniae* cells were exposed to imipenem. Heteroresistance of *K. pneumoniae* to β-lactams has been well reported due the ability of these microorganism to produce β-lactamase and metallo-β-lactamase, which breaks the β-lactam ring, enabling the β-lactams antibiotics (Morosini *et al.*, 2010; Pournaras *et al.,* 2010).

It would be interesting a further inspection of heteroresistance, through PAP, for these cases of heteroresistance already reported in the literature.

4.3.2 Biofilm cells

Regarding biofilms, PAP was conducted only for *P. aeruginosa* and *K. pneumoniae* populations, since no eradication was achieved for the remaining populations that led to suspect for heteroresistance, even with high antibiotic concentrations (MBEC >1024 mg/L). For PAP construction, the antibiotic range tested was ¼ × MIC, ½ × MIC, 1 × MIC, 2× MIC, 4 x MIC and 8 x MIC (**Figure 11).**

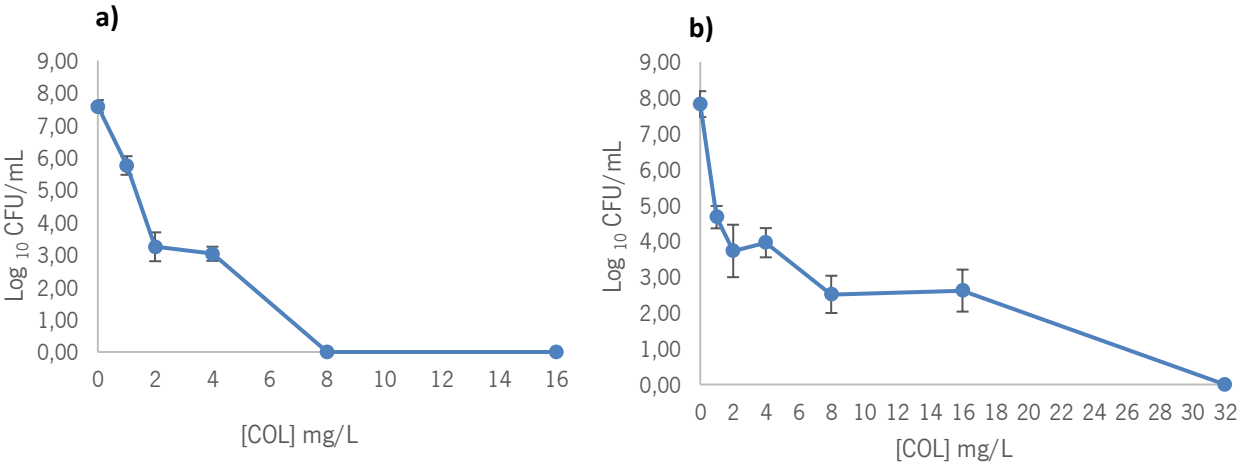

Figure 11: Population analysis profile of *P. aeruginosa* and *K. pneumoniae* biofilms towards colistin - a) *P. aeruginosa*; **b)** *K. pneumoniae.* The bacterial growth was followed by CFU counting onto MHA plates supplemented with 1, 2, 4, 8 16, 32 and 64 mg/L of antibiotic. Results are expressed as mean ± SD.

Biofilms typically showed lower susceptibility to antibiotics compared to planktonic cells, i.e. with the increase in the concentration of the antimicrobial agent, the number of cells decreases slightly. These results emphasize the previous results, with biofilms being notoriously difficult to eradicate with antibiotics and exhibiting enhanced antimicrobial resistance compared to planktonic populations, being necessary higher doses of antimicrobials to reduce the viability of biofilm-related cells.

No colonies capable of growing at concentrations above 4 mg/L were found when *P. aeruginosa* biofilms was exposed to colistin. As for planktonic populations, where no growth above the MIC (4 mg/L) was observed, also biofilms could not grow under MHA plates supplemented with colistin concentrations above the MBEC (8 mg/L) values. So, the PAP results showed no evidence of *P. aeruginosa* heteroresistance towards colistin both in planktonic and biofilm cells. As described before, Hermes *et al.*, 2013 had already shown that heteroresistance to colistin by *P. aeruginosa* was a rare phenomenon.

Regarding the PAP for *K. pneumoniae* biofilms, data showed bacterial grow up to 16 mg/L of colistin. As observed in planktonic cultures, it was never detected growth in plates containing concentrations above 16 mg/L of colistin. The absence of colonies capable of growing in 32 and 64 mg/L of colistin (MBEC), evidenced the absence of heteroresistant sub-populations in *K. pneumoniae* biofilms. It was expected to found heteroresistance in *K. pneumoniae* biofilms since resistant sub-populations were found in planktonic suspensions of this bacterium. However, some studies suggested that incubation time in biofilm formation may be related to the ability of biofilm

cells to develop antimicrobial resistance (Oliveira *et al.*, 2011; Siegel, 2008). The 24-hour incubation time may have been insufficient to allow the detection of heteroresistance in biofilm-related cells.

No heteroresistance was detected in *K. pneumoniae* and *P. aeruginosa* biofilm cells, in these conditions (24 h incubation time, 37°C, and 120 rpm). Pournaras *et al.* (2007) suggested that heteroresistant populations may had a prolonged lag phase comparatively to their native populations. Also, the biofilm formation is regulated by several loci and can proceed through several pathways so its virulence and antimicrobial resistance may differ according to the time of formation of biofilms (Siegel, 2008). So, in order to evaluate the effect of the incubation time in the susceptibility and resistance behavior of biofilm-related cells, the *in vitro* colistin susceptibility of *K. pneumoniae* and *P. aeruginosa* biofilms-related cells in biofilms of 48 h and 72 h was assessed (Table 6).

Table 6: *In vitro* susceptibility patterns of 48 h and 72 h biofilms of *P. aeruginosa* and *K. pneumoniae* towards colistin. These MBEC values represents the colistin susceptibility considering two independent experiments.

* Visible turbidity (indicating cell growth) in the wells of different concentrations (among replicates), suggesting heteroresistance

	Klebsiella pneumoniae		*Pseudomonas aeruginosa*	
Biofilm age	48h	72h	48h	72h
MBEC (mg/L)	64*128*	128	>128* (128*)	>128

Comparing MBEC values of 24 h-old biofilms (32-64 mg/L for *K. pneumoniae*; 16 mg/L for *P. aeruginosa*) with MBEC values obtained for 48 h and 72 h biofilms, a clear decrease in biofilms susceptibility towards colistin was noted, requiring equal or even 2-fold higher concentration to eliminate *K pneumoniae* cells and a concentration 8-fold higher colistin dose to eliminate *P. aeruginosa* cells in older biofilms.

Through the analysis of susceptibility patterns of 48 h and 72 h biofilms to colistin, a correlation between the incubation time of the biofilm and its susceptibility to antimicrobials can be retrieved. In general, older (mature) biofilms present decreased susceptibility towards antibiotics, which require higher doses to eliminate biofilm-related cells. This can suggest that the increase of the incubation time for biofilm formation may lead to the increase in biofilm-forming ability, which consequently may enhance the virulence of the population within the biofilm, leading to a decrease in susceptibility of biofilm cells to the antibiotics. Actually, similar results were reported by Oliveira *et al.* (2011), who verified that the biofilm-forming ability increased significantly with time incubation

and found a strong correlation between biofilm production at 72 h and antimicrobial resistance to many drugs.

As in previous susceptibility assays, was found different responses of these strains between replicates, i.e. at the same antibiotic concentration some replicates showed complete inhibition of growth while in other replicates the growth was quite noticeable. Once again, this behavior suggests the occurrence of the heteroresistance phenomenon.

For temporal issues, it was not possible to conclude about the occurrence of heteroresistance in older biofilms. However, these findings in these susceptibility assays suggest promising results in the investigation of heteroresistance in older biofilm-related cells.

CHAPTER 5: Conclusions and perspectives for future work

5.1 Conclusions

Pseudomonas aeruginosa PA14, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 11296 and *Acinetobacter baumannii* ATCC 19606 strains were able to produce biofilms under *in vitro* conditions. Amongst, *S. aureus* showed to be the strongest biofilm producer, displaying greater cell viability and an augmented capacity of biomass production. Contrariwise, *K. pneumoniae* and *A. baumannii* appeared to be weak biofilm producers.

Mixed-species cultures suggested a reciprocal interference between *S. aureus* and *P. aeruginosa*. When *P. aeruginosa* was co-cultured with *K. pneumoniae* and *A. baumannii*, a significant reduction in biomass production by this organism was noticed, suggesting a mechanism of protection by *K. pneumoniae* and *A. baumannii* against *P. aeruginosa*.

The susceptibility of planktonic populations showed a resistant behavior for *K. pneumoniae, P. aeruginosa and A. baumannii* against colistin, since the MIC value was 2-fold superior than the one established as sensitive by EUCAST. Imipenem was the only antibiotic tested in which no resistant behavior was observed by all the planktonic cultures.

The MIC value determined in the planktonic assays had no effect when used against biofilms, which proved that biofilms have a much lower susceptibility pattern comparatively with their planktonic counterparts. Equal or even higher antibiotic concentrations than those applied to inhibit the planktonic growth of single populations were required to inhibit mixed-species cultures.

Heteroresistance was found in planktonic cultures of *K. pneumoniae* and *S. aureus*, towards colistin and vancomycin, respectively. It was the first time that was observed *K. pneumoniae* populations able to grow up to 16mg/L of colistin, which leads to questioning the therapeutic efficacy of colistin in the treatment of this kind of infections.

No heteroresistance was detected in 24h-old biofilms. However, the susceptibility patterns of 48h and 72h-old biofilms suggested that the ability to form biofilms and its virulence are dependent of the biofilms incubation time, which may indicate that heteroresistance could be detected only in later stages of biofilm formation.

5.2 Perspectives for future work

Multi-species biofilm communities are environments in which complex exchanges between bacteria occur. Bacterial biofilm plays a central and not fully understood role in antimicrobial resistance comparing to their planktonic counterparts.

It would be interesting to re-assess the susceptibility of mixed populations in order to investigate which species within the mixed consortium is most easily eliminated by the different antibiotics.

It would be interesting to study the occurrence of heteroresistance for heteroresistance cases that have been already reported (e.g *A. baumannii* towards colistin and imipenem, *K. pneumoniae* and *P. aeruginosa* towards imipenem).

A further analysis of biomass production and viability of 48 h and 72 h biofilms will be important to find a solid correlation between biofilm-forming ability and biofilm age.

Posteriorly, it would also be crucial to re-assess the susceptibility of 48 h and 72 h biofilms of *P. aeruginosa* to colistin using a broader range of colistin concentrations.

According to these results, it would be interesting to evaluate the occurrence of heteroresistance in older biofilms (48 h and 72 h) of *P. aeruginosa* and *K. pneumoniae*, in order to try to find a relation between the maturity of biofilms and the development of heteroresistance

A later genetic analysis, through qPCR, of the heteroresistant colonies would be interesting, in order to evaluate the expression of efflux bombs, the presence of resistance genes and the occurrence of mutations.

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Annexes

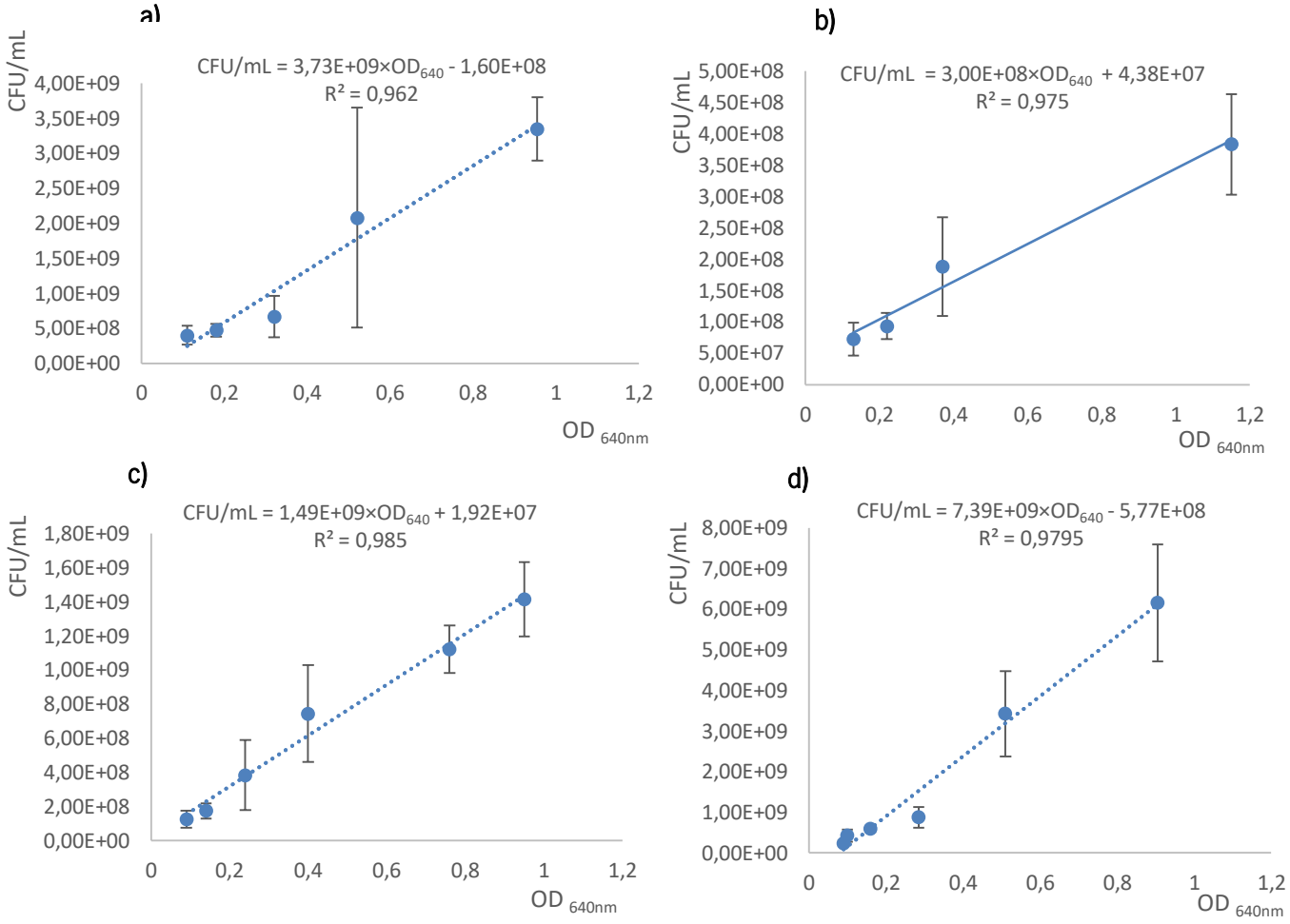

Figure A1 - Calibration curves (OD_{640} versus CFU/mL) obtained for *K. pneumoniae, A. baumannii, S. aureus* and *P. aeruginosa* - a) *Klebsiella pneumoniae ATCC 11296* ; b) *Acinetobacter baumannii ATCC 19606*; c) *Staphylococcus aureus* ATCC *25923*; d) *Pseudomonas aeruginosa PA14.* The results are expressed as the means ± SD, considering four independent experiments performed in duplicate.

Table A1 – EUCAST clinical breakpoints for *Pseudomonas spp.*, *Staphylococcus spp.*, *Enterobacteriaceae* and *Acinetobacter spp.* towards colistin, vancomycin, rifampicin and imipenem

MICs are expressed in mg/L. (S) sensitive; (R) resistant; (-) not detected

	Pseudomonas spp.	*Staphylococcus spp.*	*Enterobacteriaceae*	*Acinetobacter spp.*
Colistin	S≤2 R> 2	-	S≤ 2 R>2	S≤ 2 R>2
Vancomycin	-	S≤ 2 R>2	-	-
Rifampicin	-	S ≤ 0.06 R>0.5	-	-
Imipinem	S≤4 R>8	R>4	S≤ 2 R> 8	S≤ 2 R> 8

Table A2: Resistant fractions of the population in planktonic cells based on the population analysis profile. The results are expressed as mean ± SD

Colistin concentration (mg/L)	Resistant fractions of *Klebsiella pneumoniae*
1	$4{,}78\times10^{-5} \pm 3{,}22\times10^{3}$
2	$2{,}41\times10^{-5} \pm 5{,}56\times10^{2}$
4	$1{,}99\times10^{-5} \pm 3{,}25\times10^{2}$
8	$3{,}59\times10^{-6} \pm 2{,}58\times10^{2}$
16	$3{,}03\times10^{-6} \pm 1{,}11\times10^{2}$
Vancomycin concentration (mg/L)	Resistant fractions of *S. aureus*
1	$6{,}69\times10^{-1} \pm 9{,}75\times10^{6}$
2	$1{,}66\times10^{-5} \pm 5{,}67\times10^{2}$
4	$2{,}42\times10^{-5} \pm 1{,}43\times10^{3}$
8	$1{,}04\times10^{-5} \pm 7{,}37\times10^{1}$

Note: At a concentration of 0 mg/L of colistin, it was counted $9{,}90\times10^{7} \pm 8{,}63\times10^{7}$ CFU/mL of *K. pneumoniae* cells. At a concentration of 0 mg/L of vancomycin it was counted $7{,}72\times10^{7} \pm 3{,}08\times10^{7}$ CFU/mL of *S. aureus* cells.