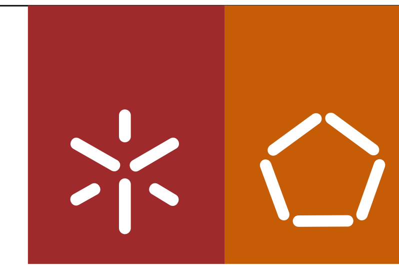

Universidade do Minho
Escola de Engenharia

João Manuel Novais de Castro Macedo

Strategies to combat infections of *Acinetobacter baumannii* biofilms

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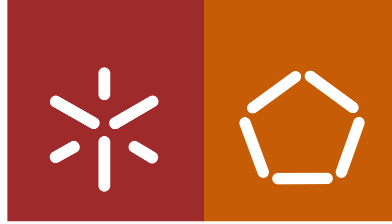

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Strategies to combat infections of *Acinetobacter baumannii* biofilms

Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica
Ramo de Engenharia Clínica

Trabalho efetuado sob orientação da
Doutora Ana Rita Martins Costa
e da
Doutora Sanna Maria Sillankorva

outubro de 2015

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, 30/10/2015

Assinatura:

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ABSTRACT

Acinetobacter baumannii has been emerging as a serious nosocomial pathogen in Portugal and worldwide, being implicated in many opportunistic infections such as ventilator-associated pneumonia, bacteremia, meningitis and urinary tract infections. The development of resistance by this microorganism to most classes of antibiotics, and its high mortality rate (19-54 %) makes *A. baumannii* outbreaks frightening for both patients and healthcare providers.

With this in perspective, the main objective of this work was to find strategies to combat *A. baumannii* infections, particularly those involving biofilms, using antibiotics and bacteriophages (phages).

Three antibiotics (Ampicillin, Kanamycin and Ciprofloxacin) were tested, with the determination of the minimum inhibitory concentration (MIC) and minimum biofilm eradication concentrations (MBEC) against *A. baumannii*. The phage *Aba1* was also evaluated at different multiplicities of infection (1 and 10) and periods of time (4 h and 24 h) on biofilms. Then, a recent therapy – the Phage-Antibiotic combination – was applied, to evaluate possible synergic effects with the antibiotics. Additionally, a new strategy was evaluated to fight *A. baumannii* biofilms, consisting on the stepwise addition of antibiotics and/or phages.

In this work, Ciprofloxacin was the most effective of the antibiotics tested against *A. baumannii* biofilms, according to the MBEC values obtained. Furthermore, the addition of phage *Aba1* did not have any effect on the activity of this antibiotic, contrarily to the synergic effect obtained with Ampicillin or Kanamycin, opening new possibilities for the application of these antibiotics. The novel treatment strategies here evaluated had its best results with Kanamycin. In biofilms, the addition of Kanamycin at 0h, 12h and 24h resulted in a 5 log reduction of the biofilm cells, and the use of phage at 0h followed by Kanamycin at 12h and 24h resulted in a 3 log reduction. Similar results were obtained for planktonic cells.

The promising results of the novel strategy here presented should be further explored for the combat of *A. baumannii* infections. Indeed, with the impressive capacity of *A. baumannii* to acquire resistance to new antibiotics, it is crucial to develop innovative strategies for the efficient application of old antibiotics.

RESUMO

Acinetobacter baumannii é um cocobacilos Gram-negativo que emergiu como um sério agente patogénico nosocomial em Portugal e em todo o mundo, estando envolvido em infeções oportunistas tais como pneumonia associada a ventiladores, bacteremia, meningite e infeções do trato urinário. O desenvolvimento de resistência por este microrganismo à maioria das classes de antibióticos, e a elevada taxa de mortalidade (19-54 %) torna os seus surtos intimidantes tanto para pacientes como para prestadores de cuidados de saúde.

Com isto em perspetiva, o objetivo principal do presente trabalho consistiu na procura de estratégias para combater infeções de *A. baumannii*, particularmente as que envolvem biofilmes, usando antibióticos e bacteriófagos (fagos).

Foram testados três antibióticos, com a determinação da concentração mínima inibitória (MIC) e a concentração mínima de erradicação de biofilmes (MBEC) em *A. baumannii*; e a ação do fago *Aba1* a diferentes multiplicidades de infeção (1 e 10) e tempos de ação (4h e 24h). Foi ainda aplicada uma terapia recente, de combinação Fago-Antibiótico, para avaliar possíveis efeitos sinérgicos do fago *Aba1* com os antibióticos. Adicionalmente, foi testada uma nova estratégia para o combate de biofilmes de *A. baumannii*, através da adição faseada de antibióticos e/ou fagos.

Neste estudo a Ciprofloxacina demonstrou ser o antibiótico mais eficaz contra biofilmes de *A. baumannii*. A adição de fago não teve qualquer efeito na ação deste antibiótico, contrariamente ao efeito sinérgico obtido com Ampicilina ou Canamicina, abrindo nova possibilidades de aplicação para estes antibióticos. A nova estratégia de tratamento aqui testada obteve os seus melhores resultados com a Canamicina. Em biofilmes, a adição de Canamicina às 0 h, 10 h e 24 h resultou numa redução de 5 log nas células viáveis, e o uso de fago às 0h seguido de Canamicina às 10 h e 24 h resultou numa redução de 3 log. Resultados similares foram obtidos para células planctónicas.

Os resultados promissores da estratégia apresentada deverão ser explorados para o combate de infeções de *A. baumannii*. Com a impressionante capacidade da *A. baumannii* para adquirir resistência a novos antibióticos, é crucial o desenvolvimento de estratégias inovadoras para a aplicação eficaz de antibióticos ultrapassados.

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ABBREVIATIONS

Amp – Ampicillin

CDC – Center for Disease Control and Prevention

CEB – Center of Biological Engineering

CFU – Colony Forming Unit

Cipro – Ciprofloxacin

CV – Crystal Violet

ICU – Intensive Care Unit

Kana – Kanamycin

MBEC – Minimum Biofilm Eradication Concentration

MDR – Multi Drug Resistant

MIC – Minimum Inhibitory Concentration

MOI – Multiplicity of Infection

NNIS – National Nosocomial Infection Surveillance

OD – Optical Density

PBPs – Penicillin-Binding Proteins

PNAG – Poly-β-(1,6)-*N*-acetylglucosamine

PFU – Plaque Forming Units

SEM – Scanning Electron Microscopy

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

US – United States

1. INTRODUCTION

1.1 The clinical impact of *Acinetobacter baumannii*

In the last two decades, the Gram-negative coccobacillus *Acinetobacter baumannii*, a normal inhabitant of the human skin flora, intestinal tract, and respiratory system, has been stealthily emerging as a serious nosocomial pathogen in both community and healthcare environment, in Portugal [1], [2] and worldwide [3]. Epidemiological studies of *A. baumannii* infections demonstrate a preference of this organism for hospital environments, particularly for patients in intensive care units (ICU), being responsible for many opportunistic nosocomial infections such as ventilator-associated pneumonia, bacteremia, meningitis, urinary tract and skin and soft tissue infections [2], [3].

This clinical impact results from a combination of factors that include an intrinsic hardiness of this bacterium that allows persistence in surfaces of medical equipment, the physical contact required for adequate patient care, and the high concentration of immune compromised patients in these environments [4]. Adding to this is the changing epidemiology of this bacterium, in particular the capacity to rapidly adapt to stressful conditions and acquire multidrug resistance mechanisms [1]. Indeed, the alarming development of resistance of this microorganism to most classes of antibiotics make *A. baumannii* outbreaks frightening for both patients and healthcare providers [5], with significant levels of mortality (19-54 %) associated with its infections [1]. Furthermore, community-acquired *A. baumannii* infections are also starting to become a concern. This has led to its recent listing as one of the six most dangerous opportunistic pathogens [6]. With considerable clinical and economic costs associated with *A. baumannii* infections, it is concerning that the study of its pathogenic mechanisms remains at an elementary stage. In opposition, several epidemiological studies have been reported for *A. baumannii* infections, with the worldwide infection prevalence shown in Figure 1.1.

In Europe, diseases like pneumonia, bloodstream infections and urinary tract infections have been caused by *A. baumannii* in 11.7-21.8 % of the cases [7]. In 2003, an Italian nationwide study about infections occurring in patients who have been hospitalized in the ICU, showed that *A. baumannii* ranked third among the causative agents and all the isolated strains have showed a high level of resistance (58 %) to all the antibiotics tested [8]–[10]. In America, more precisely in North America, the first appearance of this bacterium was in 1991 in New York City, and since then several outbreaks of multidrug-resistant *A. baumannii* have been reported throughout the United States (US) [11], [12]. A review made by the

Center for Disease Control and Prevention (CDC) reveals that 7 % of pneumonia was caused by *A. baumannii* in 2003, compared to 3 % in 1986, which shows an increase of more than double in less than 20 years [7], [13]. One of the most important contributes to the knowledge of *A. baumannii* was the return of the militaries who have fought on Iraq and Afghanistan. In March 2013, an increase in infections of *A. baumannii* was noted in the US military personnel. An investigation was performed to determine the source of the infections, concluding that it was neither preinjury skin colonization nor introduction of the organism from soil at the time of traumatic injury [14], [15].

In Asia, many outbreaks of pandrug-resistance have been documented and, unfortunately, in this region, the resistance to tigecycline and polymexin B, some of the last resort antibiotics, already exists [3].

Figure 1.1. Worldwide prevalence of Acinetobacter infections [3].

Among infections caused by *A. baumannii*, pneumonia is one of the most common. About 3-5 % of pneumonia nosocomial infections are caused by this bacterium, especially when associated to ventilator-dependent studies, where the reported mortality rate is of about 30-75 % [2], [14], [16]–[18]. *A. baumannii* is also the major causative agent of nosocomial meningitis, an infection of the spinal cord or brain, with high levels of mortality associated (20-27 %) [2], [17], [19], [20]. This bacterium is also

involved in bloodstream infections in the ICU, with the third highest crude mortality rate [2], [17], [19], [21], [22], and in urinary tract infections [2], [17], [19].

1.2 *Acinetobacter baumannii* characteristics

Acinetobacter baumannii is a gram-negative, non-motile, obligate aerobic and rod-shaped bacterium belonging to *Gammaproteobacteria* that is ubiquitous in soil, water, sewage and healthcare settings. Usually, this bacterium has 1.0-1.5 by 1.5-2.5 μm but, in a different phase, can be more coccoid (Figure 1.2). It also has the ability to adapt its metabolism and nutrient needs to adjust to different niches, and that happens because *A. baumannii* has a metabolic flexibility and different specialized systems to acquire nutrition and homeostasis [3], [23]–[26].

This bacterium can be found in two different forms: planktonic cells, when they are a unicellular organism, and biofilm, when bacteria form micro-colonies, which result in a highly structured microbial community. This species does not have special growth requirements, so it can grow at various temperatures and pH conditions. However, for most clinical isolates of *Acinetobacter*, the optimum temperature is 33-37 °C [24], [27].

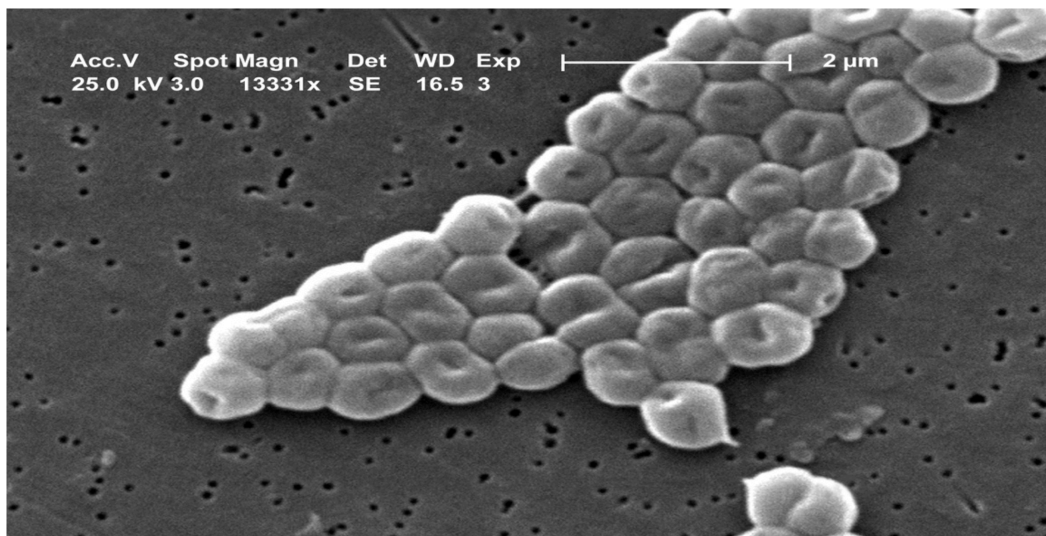

Figure 1.2. Microscopic observation of Acinetobacter baumannii [28].

1.3 Biofilms

Bacterial biofilms can be defined in different manners; however, it is a common agreement that biofilms are composed of multiple bacteria forming a consortium. So a biofilm is a "*coherent cluster of*

bacterial cells embedded in a biopolymer matrix, which compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense" [29].

After many studies, measurements and observations, there is one characteristic that is obvious for everyone: biofilms are remarkably heterogeneous, at structure, at physiological, at chemical and many other aspects [30]. It is also possible to mention some other evident characteristics of biofilms: they have the ability to adapt to environment changes and are very dynamic; bacterial cells have the ability to detach from their biofilm colony (individually or in clumps) and colonize other locals; and biofilms can migrate over the surfaces in many ways [30]–[33].

Biofilms are extremely resistant to host immune defense and antibiotics [19], [34] and, today, they are responsible for 80 % of all microbial infections in the human body [35]. However, biofilm associated-infections rarely cause fatal infections but they can be prejudicial to the quality of life, because they can remain for months, years or a lifetime in different parts of the human body [35]. This type of infections can be divided in two different sources: infections associated to medical devices, and native biofilm infections of host tissues. The first can cause bloodstream or urinary tract infections by biofilms formed in many medical devices like central venous catheters, urinary catheters, cardiac pacemakers and many others. The second, leads to chronic and opportunistic infections, and include the chronic lung infections of cystic fibrosis patients, chronic otitis media, recurrent urinary tract infections and dental caries and periodontitis [36]–[38].

The structure of the biofilms offers them a high resistance to antimicrobial therapies, generally one thousand times greater than in planktonic cells [39]. The formation of biofilm is not a simple process that only involves the adherence of bacterial cells to the surface. To form a biofilm, a highly regulated series of molecular events needs to happen with the behavior of each cell being tightly regulated. Furthermore, many factors can influence biofilm formation, with the most important being bacterial appendages (pili and flagella), bacterial surface components, nutrient availability and quorum sensing [32], [33], [40].

1.3.1 *Acinetobacter baumannii* biofilms

A. baumannii has the ability to adhere and form biofilms in both biotic and abiotic surfaces. In the last 10 years, the concern about biofilm formation on abiotic surfaces has increased, and several studies have been performed in this scope [41]–[43]. Several reports demonstrated that *A. baumannii* clinical

isolates have a high propensity to form biofilms in abiotic surfaces, such as glass and plastic [18], [44]–[46].

The high capacity of *A. baumannii* to form biofilms on abiotic surfaces plays an important role in nosocomial infections because of the bacteria's capacity to form and grow biofilms on medical equipment, such as central venous catheters, urinary catheters and many others [14], [23], [47].

As said above, the process to form a biofilm is a highly orchestrated process that involves a wide range of cellular and environment processes. This is valid for *A. baumannii*, where factors as the presence of antibiotic resistance genes, cell density, growth temperature, concentration of extracellular free iron influence biofilm formation. For example, the presence of an iron-chelating agent contributes to a significant reduction in adhesiveness and biofilm formation on both biotic and abiotic surfaces [45].

1.4 *Acinetobacter baumannii* virulence factors

The virulence factors are the characteristics of an organism that determine its capacity to cause disease or virulence and, in *A. baumannii*, when compared to other gram-negative pathogens, limited virulence factors have been found [2]. Recently, some studies have been pursued to understand how this pathogen remains in the environment and interacts with abiotic surfaces or host cells.

So far, *A. baumannii* pathogenesis has been related to an ability to form biofilms and resist desiccation on abiotic surfaces, and a capacity to adhere to, colonize and invade human epithelial cells [2], [46].

The ability to adhere and form biofilms on abiotic surfaces is a critical feature for *A. baumannii* spread within the hospital ward and between patients [48], with studies documenting its isolation from hospital equipment, bedding, furniture and even potable water [49], [50]. The process of biofilm formation in these surfaces is thought to be multistep, involving several factors that include: the CsuA/BABCDE chaperone-usher pili assembly system required for adherence to the abiotic surface [42]; the polysaccharide poly-β-(1,6)-*N*-acetylglucosamine (PNAG), important for the production of the biofilm extracellular matrix [51]; the outer membrane protein Bap necessary for biofilm maturation [23]; the regulatory elements BfmSR involved in the regulation of biofilm formation and motility [52]; and the outer membrane protein OmpA that plays a role in biofilm development [14].

Regarding the interaction of *A. baumannii* with host cells, the information is even scarcer, and the studies so far have not established a direct correlation between adherence to abiotic and biotic surfaces. This suggests that different molecular mechanisms may be involved. Indeed, unlike biofilm formation on

abiotic surfaces, the Csu chaperone-usher system is not required for adherence to eukaryotic cells [45]. Conversely, the OmpA protein is critical for *A. baumannii* interaction with human cells, being implied in apoptosis induction, adherence and invasion [14] . The Bap protein has also been implied in *A. baumannii* adherence to eukaryotic cells [23]. Other putative virulence factors have been suggested, including a lipopolysaccharide (*lpsB*) [53], the capsular polysaccharide (*ptk* and *epsA*) [54] and phospholipase D [55].

1.5 Treatment of *Acinetobacter baumannii* infections

The National Nosocomial Infection Surveillance (NNIS) showed data from 1986 to 2003, involving many hospitals trough the US, which showed the increase of resistance of *A. baumannii* to amikacin (5% to 20%), ceftazidiem (25% to 68%) and imipenem (0 % to 20 %) [56].

Many studies report the high rate of antibiotic resistance in *Acinetobacter spp.* [7], [9], [57], [58]. However, until the 1970s, the nosocomial *Acinetobacter* infections could be successfully treated by antibiotic, such as ampicillin, nalidixic acid and gentamicin as single antibiotics or in combination [3], [17], [58]. Many clinically isolated *Acinetobacter spp.* have become resistant to older antibiotics, and now they are resistant to most of bacterial drugs [7], [9]. For the new antibiotics, such as amikacin, imipenem and others, a partial susceptibility still remains, but their minimum inhibitory concentrations (MICs) have increased in the last decade [59]. In fact, some studies have demonstrated that imipenem was the most successful active drug but unfortunately, other reports of hospital outbreaks demonstrate the spread of imipenem-resistance. This fact puts a serious threat in the near future to combat *Acinetobacter* infections. In some countries of Europe, like Germany and France, many *Acinetobacter* isolates that were treated by tobramycin and floxacin became resistant to them in less than 5 years from the introduction of these antibiotics.

1.5.1 Antibiotics

The choice of the antibiotics and their dosage for the treatment of *A. baumannii* is crucial to combat and prevent the growth of resistant bacteria. So, it is important to describe the three antibiotics used in this work.

Ampicillin

Ampicillin is a beta-lactam antibiotic that belongs to the class of organic compounds know as penicillins. This antibiotic has antimicrobial activity against Gram-negative (*Salmonella spp., E. coli, Shigella spp.*) and Gram-positive (*Streptococcus spp., Enterococcus spp.* and *Listeria monocytogenes*) bacteria. Ampicillin is also stable against hydrolysis [60].

Its mechanisms of action consist of binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, which inhibits cell wall synthesis. The cell lysis is then mediated by bacterial cell wall autolytic activity [60].

Ampicillin, as a single agent, cannot combat the *A. baumannii* infections, however, a combined treatment may improve the results [60].

Kanamycin

Kanamycin, also known as Kanamycin A, is an aminoglycoside bactericidal antibiotic with antimicrobial activity against Gram-negative bacteria, like *Pseudomonas, Acinetobacter* and *Enterobacter*. Infections caused by Gram-positive bacteria can also be treated by aminoglycosides, but there are other antibiotics more potent and with less damage to the host [3].

Like many others of the class, this antibiotic binds specifically to the 30S-subunit proteins and 16S rRNA. This leads to interference with the initiation complex, misreading of mRNA, and difficulty of the synthesis of the proteins, resulting in bactericidal effect [61].

Usually, the amynoglicosides are not used as single agents to the treatment of *A. baumannii* infections, and the toxicity profiles encumber their use (particularly for longer treatment) [61].

Ciprofloxacin

Ciprofloxacin is a broad-spectrum anti-infective agent that belongs to the class of Quinolones and has antimicrobial activity against Gram-negative and Gram-positive bacteria [62], [63].

The usual mechanism of action is different from other classes of antimicrobial agents. Indeed, the mechanism of action of ciprofloxacin involves the inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV. These two enzymes are required for bacterial DNA replication, transcription, repair, strand supercoiling repair and recombination [62], [63].

There are many studies of quinolones activity against *A. baumannii* infections, and the results were extremely positive. However, through the years, the resistance of *A. baumannii* increased [62], [63].

1.5.2 Bacteriophages

Bacteriophages (phages) are bacterial viruses that infect bacteria and, in the particular case of lytic phages, disrupt bacterial metabolism and cause bacterial lysis. They are the simplest and most abundant organisms on Earth (it is estimated a rate of 10 phages per bacteria). As viruses, they are obligate parasites using the host bacteria to multiply and spread [25].

The discovery of phages is still the subject of extensive debates, including a controversy of claims for priority. Ernest Hankin in 1896, reported antimicrobial activity against *Vibrio cholera*, which he suggested to be responsibility from an unidentified substance. After two years, Gamaleya observed the same phenomenon when he was working with *Bacilus subtilis.* Apart from these results, none of these investigators continued their findings. Almost twenty years later, Frederick Twort continued the investigations of Hankin's, and reported a similar phenomenon and the cause, among other possibilities, was a virus. Financial problems did not allow Twort to continue his investigation and, two years later, phages were "officially" discovered by Felix d'Herelle, a French-Canadian microbiologist [25].

Today, phages have many applications depending on the country they are used. In some countries, as Russia or Poland, phages are already used therapeutically for treatment of bacterial infections that are multidrug resistant (MDR). This method can also be called phage therapy, because it involves the use of a phage to destroy the infective bacteria [64].

In 2010, the first two lytic phages, AB1 and AB2, specific for *A. baummannii* were characterized and reported and, since this date, many others phages with lytic activity against this bacteria were published, although only one was completely sequenced (AB1) [26]. Li *et al.*, investigated and characterized the phage ZZ1, and reported that because of its characteristics (strong heat resistance, efficient antibacterial potential at body temperature) this phage has an increased utility as an antibacterial agent; however, he still recommends further investigation [65].

1.5.3 Combination of Bacteriophages with Antibiotics

Bacteriophages and antibiotics have many differences and many similarities. One of the most important similarities is that both have significant antibacterial activity. However, in theory, the antibiotics have less advantages than therapeutic phages [25].

Several reports and studies discuss the pros and cons of bacteriophages compared to antibiotics. On the pros, the process of selection of new phages is easier, requiring only a few days or weeks compared to the many years necessary to develop new antibiotics. Also, phages do not have any known

side effects while antibiotics have multiple, including allergies, intestinal diseases and secondary effects. In favor of the phages there is also the fact that they are self-multiplying and self-timing; in other words, they multiply when the infection is present and they die after all bacteria are killed. Antibiotics, for their turn, have their concentration decreasing rapidly and thus need to be administered several times to maintain the concentration needed [25], [66].

Phage therapy does not have only advantages, and some issues have been reported. One of the concerns is the potential development of phage resistance; however, there are already some strategies to combat this problem, such as using a consortia of phages to delay this resistance [67]–[69]. Other issue with this therapy, is the influence of phage therapy on the immune system and the high potential for lytic phages to carry genetic material [67]–[69].

Since 2007, phage-antibiotic therapy has been tested and reported in a number of species, like *E. coli* [70] and *Klebsiella pneumonia* [71]. In 2015, Ali *et al.* reported a synergetic effect of a phage combined with antibiotics (half of the MIC of gentamicin, vancomycin and tetracycline) against *Staphylococcus aureus* [72]. Another report concluded that the amikacin-phage combination could have more benefits on *Pseudomonas aeroginosa* biofilms than using phages or antibiotics alone. However, in the same report, they conclude that the combination of meropenem and phage is not recommended for non-growing bacteria in biofilm [73].

In conclusion, the combination of phages with antibiotics has a huge potential for the treatment of infections. However, the variability on the results reported so far highlight the importance to continue the investigation in this area, to find a better therapy to combat *A. baumannii* infections.

1.6 Motivation and aim of the project

Acinetobacter baumannii has emerged as an important nosocomial pathogen in Portugal and worldwide, being mainly implied in hospital-acquired infections. Treatment of these infections is often difficult due to the increasing prevalence of multidrug-resistant isolates, being urgent to develop novel and effective therapies against this bacterium. Furthermore, this bacterial species is able to produce biofilms, which are inherently more tolerant to antibiotics. Phage therapy has been considered as a promising alternative to antibiotics in treating infectious diseases, and it has demonstrated efficacy against biofilms. Recent studies have demonstrated that phages combined with antibiotics can be very

powerful in combating biofilms – however, it is not yet known how antibiotics and strictly lytic phages can interplay when controlling bacterial biofilms.

So, the aim of this project is to understand how antibiotics and lytic phages can interplay in a biofilm system. For this purpose, *A. baumannii* biofilms were used as the case study. Combinations of antibiotics and strictly lytic bacteriophages (belonging to the Center of Biological Engineering (CEB) collection of phages) were assessed towards biofilms. The two more specific goals are:

- Disclose possible synergic or antagonist mechanisms between antibiotics and phages in *A. baumannii* biofilm control;
- Optimize a stepwise phage-antibiotic therapy to treat *A. baumannii* infections.

2. MATERIALS AND METHODS

2.1 Bacteria, bacteriophages and antibiotics

In this work, a strain of *A. baumannii* (Aba1), from BBiG collection, was used for biofilm formation. *A. baumanii* was grown in sterile Tryptic Soy Broth (TSB) and sterile Tryptic Soy Agar (TSA) (1.2 % w/v of agar) was used as solid medium, having both been prepared according to the manufacturer's instructions.

Three antibiotics were used in this study: Ampicillin, Kanamycin and Ciprofloxacin (Sigma Aldrich). The stock solutions were of 100 mg/mL, 50 mg/mL and 25.6 mg/mL, respectively.

Phage Aba1, previously isolated from wastewaters from Braga, and belonging to the BBiG collection, was used in this work. The phage was amplified in solid media. Briefly, 100 µL of an overnight culture of *A. baumannii* Aba 1 was mixed with TSA top agar (0.7% w/v of agar) and added to a TSA plate. After drying, a paper strip was wet on the liquid phage stock and spread on the prepared plates containing the host bacteria. The plates were incubated at 37ºC overnight. Approximately 3 mL of SM buffer (100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl 1M, pH 7.5) were then added to each plate and incubated at 4ºC for about 6 h to recover the amplified phages. The SM buffer containing the phages was then recovered and filtered (0.2 µm). The phage was then purified by PEG 8000/2.5M NaCl, as follows. 0.584 g of NaCl were added for each 10 mL of sample, and the suspension stored on ice for 1h. After centrifugation at 9000g and 4ºC for 10min, the supernatant was recovered, 1g of PEG 8000 added per each 10 mL of initial sample, and stored at 4 °C with gently rocking for 6h. After another centrifugation, the supernatant was discarded and let dry for 10 min. The phage pellet was then resuspended in a small volume of SM Buffer, and chloroform was added in a proportion of 1 vol per 4 vol of sample, vortexing for 30s. After centrifuging at 3500 g for 15 min at 4 °C, the supernatant was recovered, filtered and the concentration of the phage was determined using the double layer agar method. For this, 10-fold dilutions of the phage suspension were done using SM Buffer, and each dilution plated with 100 µL of overnight culture and 3 mL of TSA top agar, onto a TSA plate. After drying, the plates were incubated overnight at 37 °C. The following day the plate forming units (PFUs) were determined as follows:

$$Phage\ concentration\ \left({}^{PFU}/_{mL}\right) = \frac{Number\ of\ Plaques \times Dilution\ factor}{Volume\ of\ plaqued\ phage}$$

2.2 Biofilm formation

For biofilm formation, a pre-inoculum of *A. baumannii* Aba 1 in 5 mL of TSB was grown overnight at 37 °C, with shaking. On the day after, the pre-inoculum's optical density (OD) was adjusted to approximately 0.5 in fresh TSB (about $2x10^5$ cfu/mL) and 20 μL was added to each well of a 96-well plate containing 180 μL of TSB. The plate was incubated at 37 °C with 120 rpm shaking for the time needed for each specific experiment.

2.3 Quantification of biofilm biomass

To quantify the total biomass attached in each well of the 96-well plates, the crystal violet assay was performed. On this procedure, biofilms were washed twice with sterile saline solution (0.9 % wt/vol NaCl) and 200 μL of metanol were added for 15 min, in order to fix the biofilm. Then, methanol was removed and the microplate allowed to dry at room temperature for 20 min. Following, 100 μL of crystal violet were added to each well, and the plate was incubated for 5 min at room temperature. Crystal violet excess was then removed with tap water and the microplate was once again allowed to dry at room temperature. Finally, 200 μL of acetic acid (33% v/v) were added to solubilize the bound die of each well, and the absorbance was read at 570 nm, using acetic acid as blank.

2.4 Quantification of biofilm viable cells by colony forming units (CFUs)

After biofilm formation, the wells were washed twice with 200 μL of saline solution and 200 μL of saline solution were added to each well. Then, the plate was sonicated for 30 min to release the biofilm from the bottom of the wells. Then, 10-fold dilutions were made using sterile saline solution and 10 μL drops of each dilution were plated into a TSA plate, in triplicate. The plates were incubated overnight at 37 ºC, and the CFUs counted and expressed in CFU/mL.

2.5 MIC Determination

In order to determine the MIC of the three antibiotics against *A. baumannii*, the micro-broth dilution method was used. The following protocol was performed by the guidelines of Clinical and Laboratory Standards Institute. First, four colonies of *Acinetobacter baumannii* were inoculated in 5 mL of TSB - in

order to achieve the approximate density of 0.5 in the McFarland standard - and 100 μL of this suspension were added to each well of a 96-well plate. After this, 100 μL of the antibiotics wanted to be tested were added, individually and in different concentrations, creating a gradient (0.031 to 2 μg/mL) that will allow MIC determination. Fresh TSB (200μL) was used as control. Each antibiotic concentration was tested in triplicate. The plates were incubated at 37 °C and 120 rpm, and the antibiotics' MIC were measured after 24 h.

2.6 MBEC Determination

The minimum biofilm eradication concentration (MBEC) was defined as the minimal concentration of antibiotic required to eradicat the biofilm. This experiment was performed following the Innovotech Inc. guidelines. First, three colonies of *A. baumannii* were inoculated in 5 mL of TSB and the density of this suspension was adjusted to 0.5 in the MacFarland Standard. After, 200 μL were added to each well of a 96-well microtiter plate and incubated overnight at 37 ºC and 120 rpm. After 24 h, when the biofilm had been formed, all medium was removed and wells were washed with fresh TSB medium. Then, 100 μL of TSB and 100 μL of antimicrobial solutions (Ampicillin and Kanamycin from 256 μg/mL to 1 μg/mL and for Ciprofloxacin from 64 μg/mL to 0.25 μg/mL) were added to each well. 200 μL of TSB were used as control. After 4 h (or 24 h) of incubation, each well was washed using a saline solution (NaCl 0.9%), in order to remove all the bacteria that were not attached. Following that, 200 μL of fresh saline solution were added to the wells and the microplates were put in a bar water sonicator for 30 min, in order to disintegrate the biofilm. After that, the viable number of cells present in biofilms was determinate by colony-forming unit (CFU) counting, as previously described.

For the combination phage+antibiotic, the same assay was used. However, instead of using 100 μL of antibiotic and 100 μL of TSB, were used 100 μL of antibiotic and 100 μL of phage at MOI=20.

2.7 Phage activity against Biofilm

To determine phage activity against biofilms, *A. baumannii* biofilms were formed and after 24h all medium was removed and the wells were washed twice with fresh TSB medium. Then, 200 μL of phage at different MOI (1 and 10, prepared in TSB) were added to the wells. As a negative control, TSB was used. After 4h or 24h of incubation, the CFUs were determined as described for the MBEC experiments.

2.8 Combinations of phages and antibiotics against biofilms

To evaluate the effect of combinations of phage and antibiotics against biofilms, *A. baumannii* biofilms were formed for 24h. The wells were washed twice with fresh TSB medium and 200 μL of phage with either Ampicillin, Kanamycin or Cyprofloxacin at different concentrations (same range used for the determination of MBEC) were added to the wells. TSB was used as a negative control. After 4h or 24h of incubation, the CFUs were determined as described for the MBEC experiments

2.9 Stepwise treatment of *A. baumannii* infections with phage-antibiotic combinations

In this work, a new strategy to combat *A. baumannii* biofilms was tested, consisting of a stepwise addition of phage and antibiotics, against planktonic cells and biofilms. First, solutions of phage *Aba1* were prepared (MOI = 0.01 and MOI = 10) and Ampicillin, Kanamycin and Ciprofloxacin were used in the same concentrations as before (MIC for planktonic cells and MBEC for biofilms).

Planktonic

A pre-inoculum of *A. baumannii* was adjusted to an OD of approximately 0.5 in fresh TSB medium, in falcon tubes. Then phage (MOI = 0.01), Ampicillin, Kanamycin, Ciprofloxacin or TSB (control) were added to the suspensions, as seen in the "0 h" line of Table 2.1. Then, the falcon tubes were incubated at 37 °C with 120 rpm and the absorbance was measured every 2 h.

After 12 h and 24h of incubation, more phage or antibiotic was added to the falcon tubes, as described in Table 2.1, and the absorbance measured every 2 h.

Table 2.1. Description of the phage and antibiotics addition to the falcon tubes containing A. baumannii

Time (h)	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10	Tube 11
0	Phage	Phage	Phage	Phage	Amp	Amp	Kana	Kana	Cipro	Cipro	TSB
12	Phage	Amp	Kana	Cipro	Phage	Amp	Phage	Kana	Phage	Cipro	TSB
24	Phage	Amp	Kana	Cipro	Phage	Amp	Phage	Kana	Phage	Cipro	TSB

Biofilm

A biofilm of 24h was used in this assay. The wells were washed twice with sterile saline solution and200 µL of TSB containing the phage, Ampicillin, Kanamycin, Ciprofloxacin or TSB (control) were added, as seen in the "0 h" line of Table 2.2, which represents a line of the 96-well plate. Then, the microplate was incubated at 37 °C with 120 rpm and the CFUs were determined after 6h and 12h of incubation. At 12h, more phage or antibiotic (200 µL) was added to the remaining well plates, as described in Table 2.2. The CFUs were once again determined after another 12 h (24 h of total incubation time), and 200 µL of phage or antibiotic were added to each of the remaining wells, as seen in Table 2.2. The plate was once again incubated and the CFUs determined for the 32h time point.

Table 2.2. Description of the phage and antibiotics addition to the microtiter plates containing A. baumannii biofilms

Time (h)	1	2	3	4	5	6	7	8	9	10	11	12
0	Phage	Phage	Phage	Phage	Amp	Amp	Kana	Kana	Cipro	Cipro	TSB	-
12	Phage	Amp	Kana	Cipro	Phage	Amp	Phage	Kana	Phage	Cipro	TSB	-
24	Phage	Amp	Kana	Cipro	Phage	Amp	Phage	Kana	Phage	Cipro	TSB	-

3. Results and Discussion

3.1 Antibiotics against planktonic *A. baumannii* cells

The MIC values of three antibiotics (Ampicillin, Kanamycin and Ciprofloxacin) against planktonic cells of *A. baumannii* were determined using the micro-broth dilution method, with the results for each antibiotic shown in Table 3.1.

Table 3.1 – Values of Minimum Inhibitory Concentration (MIC) of Ampicillin, Kanamycin and Ciprofloxacin against *A. baumannii* Aba1 cells

Antibiotic	**MIC range tested (µg/mL)**	**MIC (µg/mL)**
Ampicillin	0.031-2	0.064
Kanamycin	0.031-2	0.064
Ciprofloxacin	0.008-1	0.016

Analyzing the results obtained it is possible to observe that Ciprofloxacin was the most effective antibiotic, as a lower concentration (0.016 µg/mL) was enough to inhibit the visible growth of *A. baumannii*. Ampicillin and Kanamycin show similar MIC values (0.064 µg/mL).

According to the clinical breakpoints defined by EUCAST for *Acinetobacter spp*. the *A. baumannii* ABA1 strain can be considered as sensitive to the antibiotics tested [74].

3.2 Antibiotics against *A. baumannii* biofilms

The capacity of single different antibiotics to disrupt biofilms of *A. baumannii* was tested at different times (4h and 24h) (Table 3.2).

Table 3.2 – Values of Minimal Bacterial Eradication Concentration (MBEC) of Ampicillin, Kanamycin and Ciprofloxacin after contact for 4 or 24 hours with biofilms of *A. baumannii* Aba1

Antibiotic	MBEC range tested (μg/mL)	MBEC 4h (μg/mL)	MBEC 24 h (μg/mL)
Ampicillin	1-256	> 128	>64
Kanamycin	1-256	32	64
Ciprofloxacin	0.25-64	0.5	0.5

Comparing the results from Tables 3.1 (MICs) and 3.2 (MBECs), it is possible to confirm that biofilms can be up to 1000 times more resistant to antibiotics than planktonic bacteria. After 4 h of antibiotic action, Ciprofloxacin was the most effective in eradicating the biofilm (concentration of 0.50 μg/mL), maintaining the same behavior when used for 24 h. This value is nearly 30 times higher than the concentration needed to inhibit the growth of planktonic cells, which corroborates the higher resistance of biofilms. Nevertheless, the *A. baumannii* ABA1 strain can be considered sensitive to Ciprofloxacin even in the biofilm state. These results are in agreement with other studies that tried to combat biofilms with Ciprofloxacin. Chang *et al.* reported the highest activity of quinolones (ciprofloxacin class) against *A. baumannii* [75].

On the other hand, the opposite outcome is noticeable for Ampicillin and Kanamycin. Both antibiotics, applied for 24 h, are required in a concentration higher than 64 μg/mL to eradicate an *A. baumannii* ABA1 biofilm. However, when applied for 4 h, these antimicrobial agents perform differently: Ampicillin is needed in a concentration higher than 128 μg/mL, in order to be effective; while 32 μg/mL of Kanamycin are enough for this antibiotic to eradicate the formed biofilm. These outcomes can be explained by the different interaction between the antibiotics and the biofilm: Ampicillin is more effective when applied for a longer period, while biofilms of *A. baumannii* gain resistance to Kanamycin, requiring a higher antibiotic concentration when interacting for 24 h with the antimicrobial agent.

3.3 Bacteriophages against A. baumannii biofilms

Previously, it was reported that 80% of human infections could be biofilm-associated, so it is very important to find a way to combat this issue. Bacteriophages emerge as a promising strategy to combat infections involving these organized structures.

In this work, biofilms of A. baumannii were challenged with phages at different multiplicities of infection (MOIs). Figures 3.1 and 3.2 represent the action of phages with MOI=1 and MOI=10 after 4 h and 24 h of action, assessed by CV staining and CFU counting, respectively.

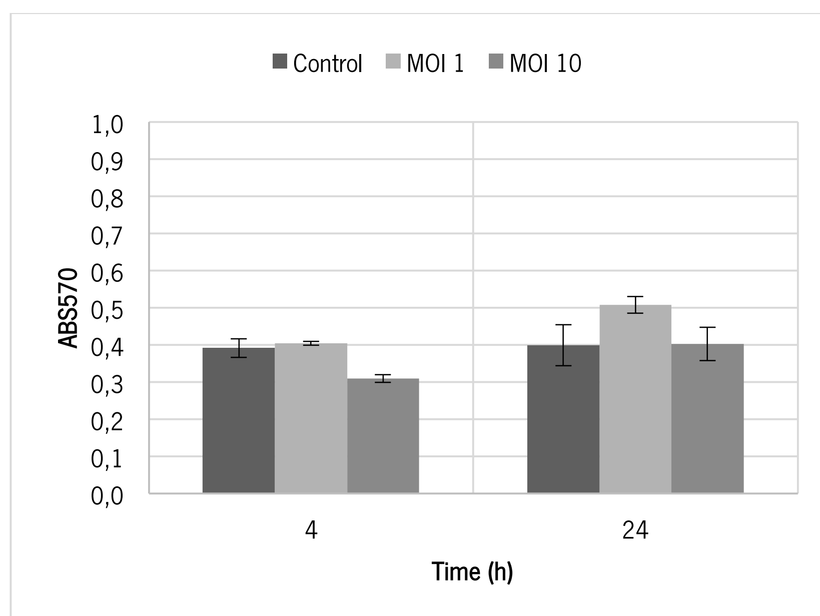

Figure 3.1. Activity of phage Aba1 against *A. baumannii* Aba1 biofilms, measured by crystal violet staining, for 4 h and 24 h of activity, using MOI of 1 or 10.

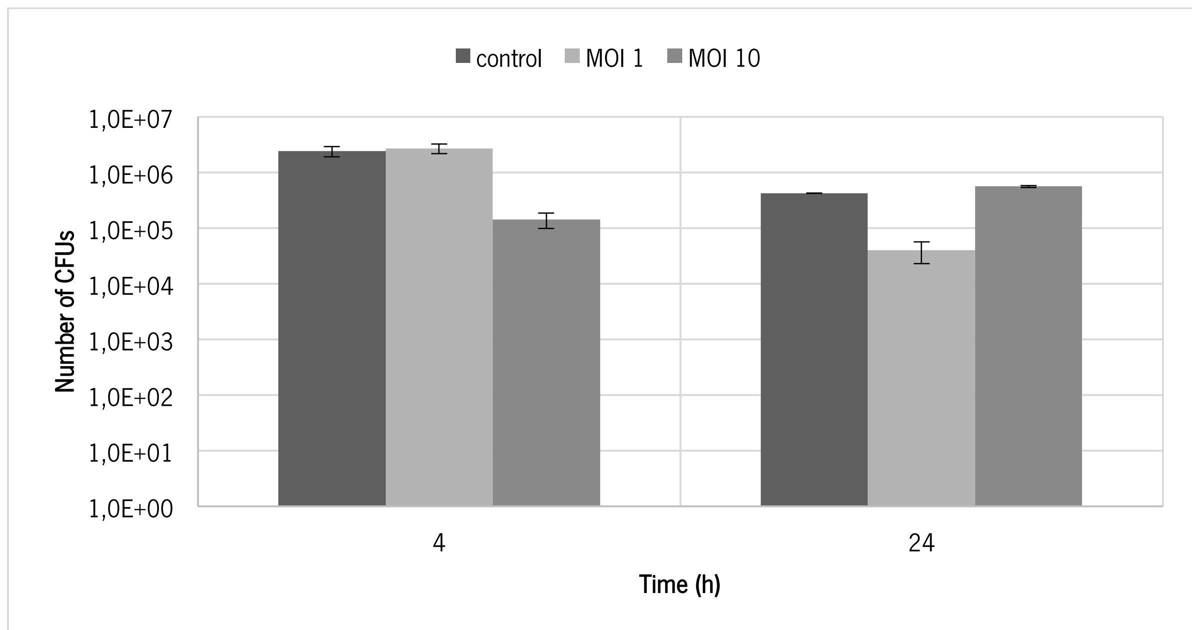

Figure 3.2. Activity of phage Aba1 against A. baumannii Aba1 biofilms, measured by CFUs, for 4h and 24h of activity, using MOI of 1 or 10.

Considering Figure 3.1, and comparing the results obtained for 4 h of phage action, it is possible to conclude that the phage presence at a MOI of 10 has some activity against the biofilm, since a reduction in the absorbance at 570 nm was noted. After 24 h of action, none of the tested conditions was effective, as both retrieved results similar or higher than the control.

It is also interesting to compare the outcomes obtained for the same phage MOI when applied for different periods of time. For both MOIs assessed, the absorbance at 570 nm increased from 4 to 24 h, meaning that the phage is more effective when in action for shorter periods of time. These results indicate that *A. baumannii* biofilms gain resistance to the phage, when the latest is in action for 24 h.

Analyzing Figure 3.2 it is possible to note that after 4 h of phage action, a phage MOI of 10 revealed better results, reducing the number of live bacterial cells by one log. This is in accordance with some studies indicating that the use of higher MOIs cause a higher percentage of biofilm inhibition [76]. Curiously, however, in the present work the lowest MOI (1) was the most effective at 24h, reducing the number of cells by 1 log.

3.4 Combination of Bacteriophages and Antibiotics against *A. baumannii* biofilms

The phage-antibiotic combination is one of the newest potential therapies against planktonic cells and biofilms. This new therapy offers an opportunity for developing treatment strategies for infections caused by *A. baumannii*.

In this work, one of the objectives was to combat the biofilms with this new therapy, assessing the combination of an *A. baumannii* phage (phage Aba1) with different antibiotics to evaluate possible synergist or antagonist effects.

To evaluate these effects, the antibiotics were added to the biofilms with the addition of the phage at an MOI of 10, with further determination of the MBECs for 4h and 24h. The range of antibiotics concentrations used was the same as previously (1-128 μg/mL for Ampicillin and Kanamycin and 0.25-32 μg/mL for Ciprofloxacin). Results are shown in Figures 3.3, 3.4 and 3.5.

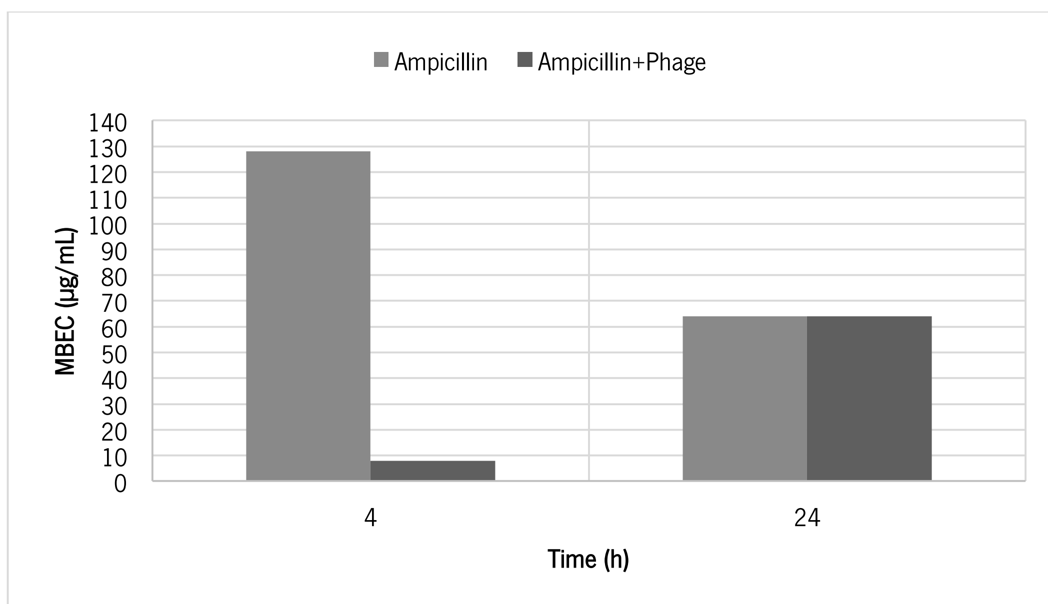

Figure 3.3. Values of MBEC determined for Ampicillin and Ampicillin combined phage Aba1 against A. baumannii Aba1 biofilms.

Looking to the values of the action of Ampicillin with phage (Figure 3.3) for a 4h action, resulted in a reduction in MBEC from >128 μg/mL (as previously determined for Ampicillin alone) to 8 μg/mL. For an action of 24h, the MBEC remained the same. These results indicate that the combination of phage Aba1 with Ampicillin has a positive effect for an action of 4h, with the strain becoming sensitive to the antibiotic. Ampicillin is not currently considered as a treatment option for *A. baumannii* infections due to

the intrinsic resistance of most strains of this species to beta-lactamases; so perhaps the combination with phages may open new perspectives for the application of these previously discarded antibiotics.

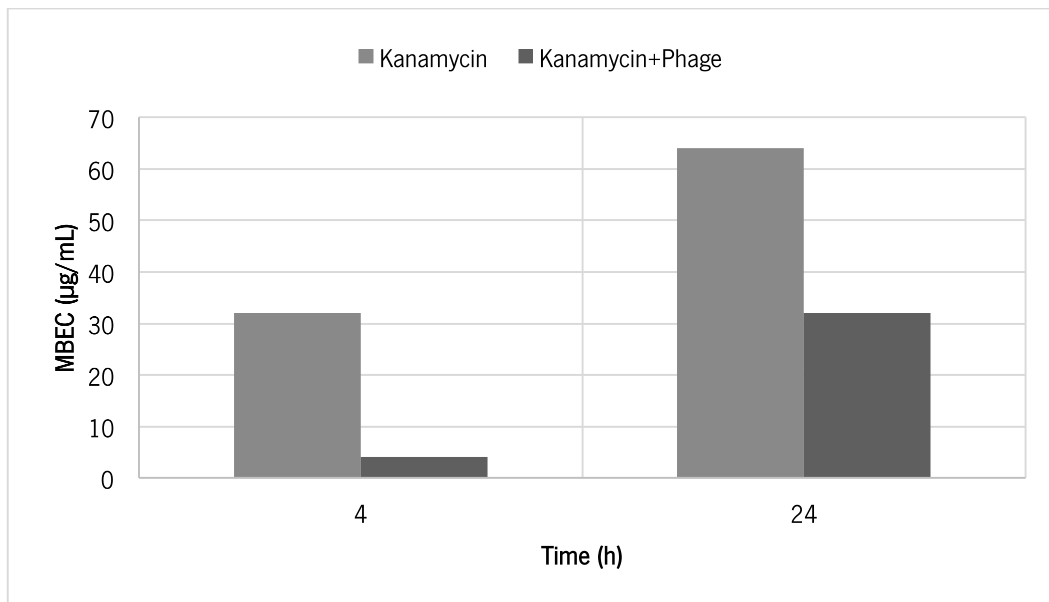

Figure 3.4. Values of MBEC determined for Kanamycin and Kanamycin combined phage Aba1 against A. baumannii Aba1 biofilms.

For Kanamycin (Figure 3.4), the combination with phage Aba1 resulted in a decrease from 32 µg/mL to 4 µg/mL at 4h; and from 64 µg/mL to 32 µg/mL at 24h. Once again, the addition of the phage improved the antimicrobial effect in the combat of the *A. baumannii* biofilms.

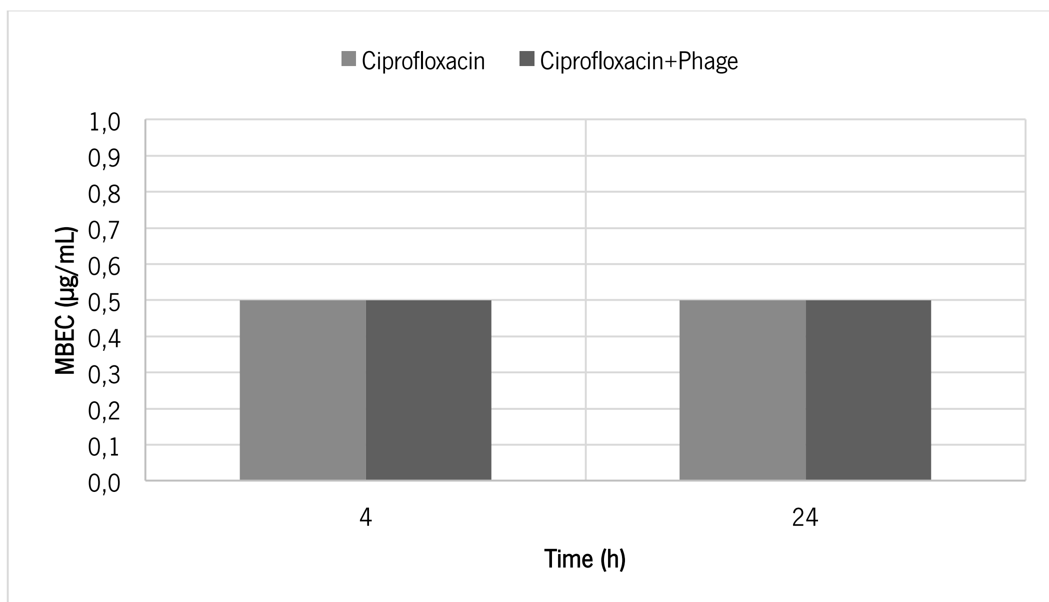

Figure 3.5. Values of MBEC determined for Ciprofloxacin and Ciprofloxacin combined phage Aba1 against A. baumannii Aba1 biofilms.

Ciprofloxacin (Figure 3.5) was the only antibiotic that did not had a decrease in the MBEC values when combined with the phage. Indeed, the MBEC values remained unaltered at 0.50 µg/mL for both 4h and 24h of activity. So the phage used in this study does not seem to have any effect on Ciprofloxacin activity.

The different results obtained for the combination of the phage with the different antibiotics (synergy with Ampicillin and Kanamycin, irrelevant with Ciprofloxacin), could be explained by the high MBEC values of Ampicillin and Kanamycin in comparison with Ciprofloxacin, which is already very efficient on its own. Furthermore, the specific mechanism of action of each antibiotic may also result in different interactions with the phage.

3.5 Stepwise treatment of *Acinetobacter baumannii* infections with phage-antibiotic combinations

In this work, a novel strategy – based on the different time action of phages and antibiotics – was tested against planktonic cells and biofilms of *A. baumannii*. Phage-antibiotic combinations were performed, but, on the contrary of what had been done before, not applied at the same time. For this test, phage *Aba1* was used at a MOI=0.01 for planktonic cells and MOI=10 for biofilms. The three antibiotics (Ampicillin, Kanamycin and Ciprofloxaxin) were used in the same concentrations as before (64 µg/mL for Ampicillin and Kanamycin and 0.5 µg/mL for Ciprofloxacin for biofilms; 0.064 µg/mL for Ampicillin and Kanamycin and 0.016 µg/mL for Ciprofloxacin for planktonic cells).

3.5.1. Planktonic cells

A. baumannii cells were subjected to periodical additions (0h, 12h, 24h) of phage or antibiotics, with measurement of absorbance every 2 hours.

Figure 3.6 represents the results obtained for the Phage-Antibiotic combinations which begun with phage infection (0h). Chart legend can be read as "First addition+12 h addition+24 h addition".

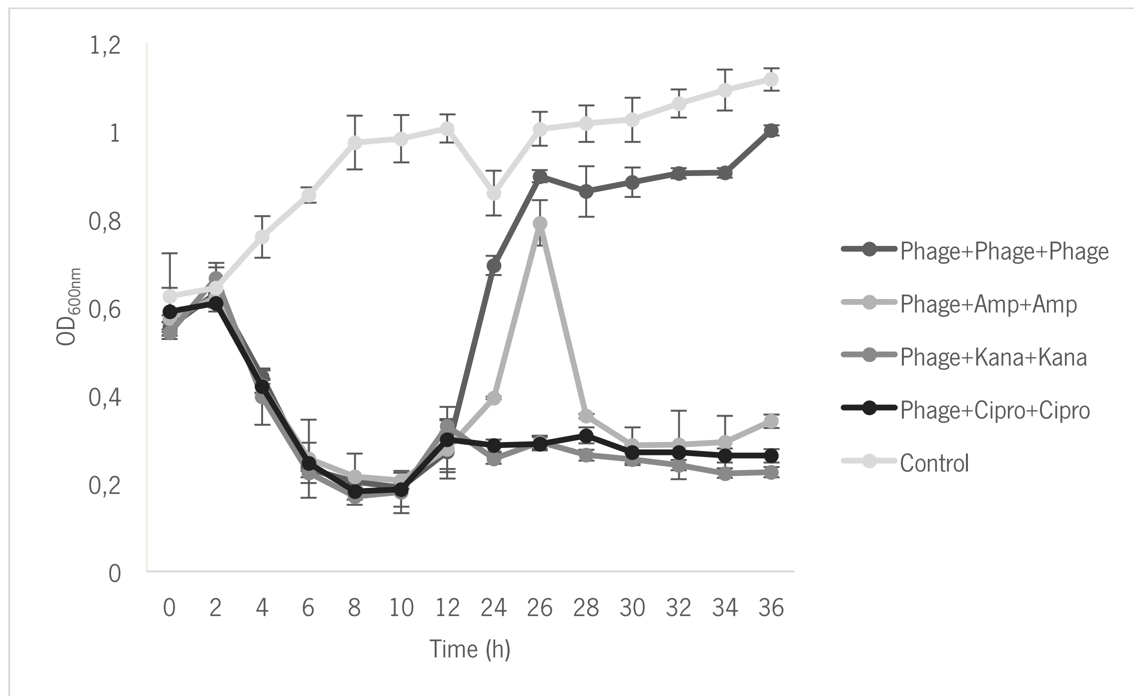

Figure 3.6. Treatment of A. baumannii planktonic cells with phage (0h) followed by the addition of more phage or antibiotic at 12h and 24h, with OD values at 600 nm measured at 2h intervals.

Observing Figure 3.6, it is possible to note that in the end of the first 12 h, all the combinations showed similar values of absorbance, because all of them were in the same conditions (only had the phage at a MOI=10). After the first addition (at 12 h) and until 24 h of total incubation time, the best combinations was the phage with Kanamycin or Ciprofloxacin. In the end of the 36 h, the addition of Kanamycin to the phage (Phage+Kanamycin+Kanamycin) continued to reveal the best results, with an absorbance of 0.22. However, the combination between the phage and Ciprofloxacin showed good results as well, with an absorbance of 0.26. The worst result of this experiment was when only phage was applied, with no antibiotic combined, which had an absorbance of 1, a value similar to the outcome of the control (1.12). This clearly demonstrates the gain of resistance to the phage by the bacteria.

Figure 3.7 represents the results obtained when Ampicillin was the first substance added. Once again, the chart legend can be read as “First addition+12 h addition+24 h addition”.

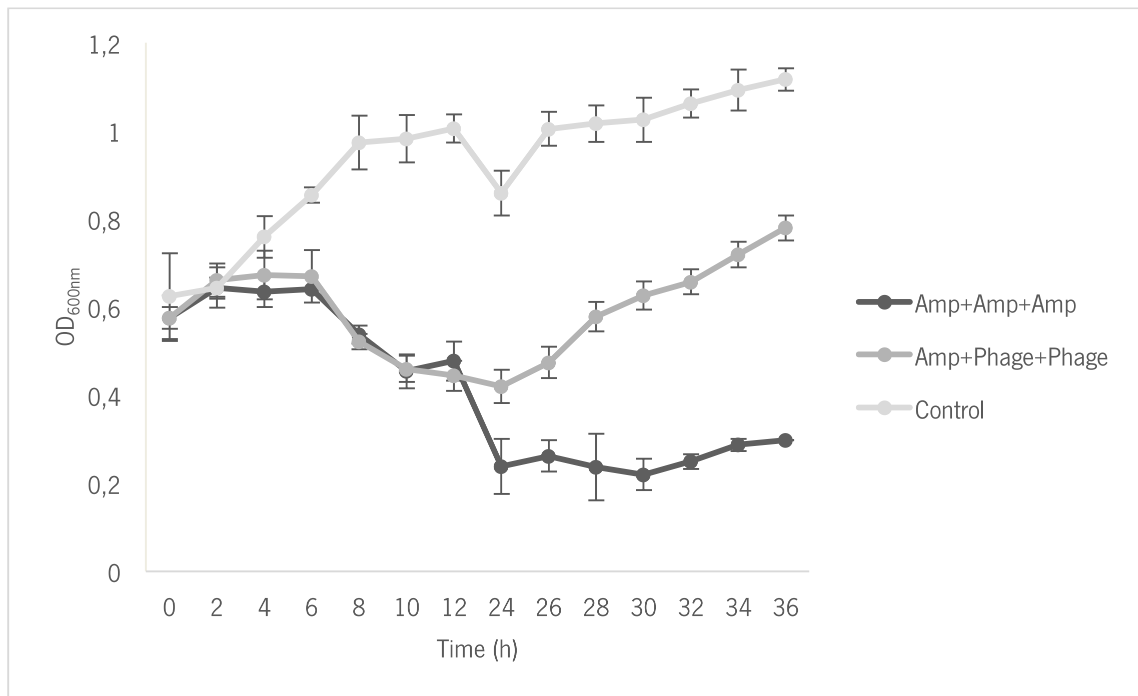

Figure 3.7. Treatment of A. baumannii planktonic cells with Ampicillin (0h) followed by the addition of more antibiotic or phage at 12h and 24h, with OD values at 600 nm measured at 2h intervals.

To study all the possibilities of treatments, different combinations between phages and antibiotics were tested. In Figure 3.7, the first addition was the Ampicillin, followed by phage or another dose of Ampicillin after 12h and 24h of incubation. After 36h of incubation, the combination with the lowest absorbance was the Ampicillin+Ampicillin+Ampicllin, with 0,29. With a higher absorbance was the combination with phage, with 0,78. So clearly, the combination of Ampicillin followed by Ampicillin is advantageous.

Figure 3.8 represents the results obtained when Kanamycin was the first substance added.

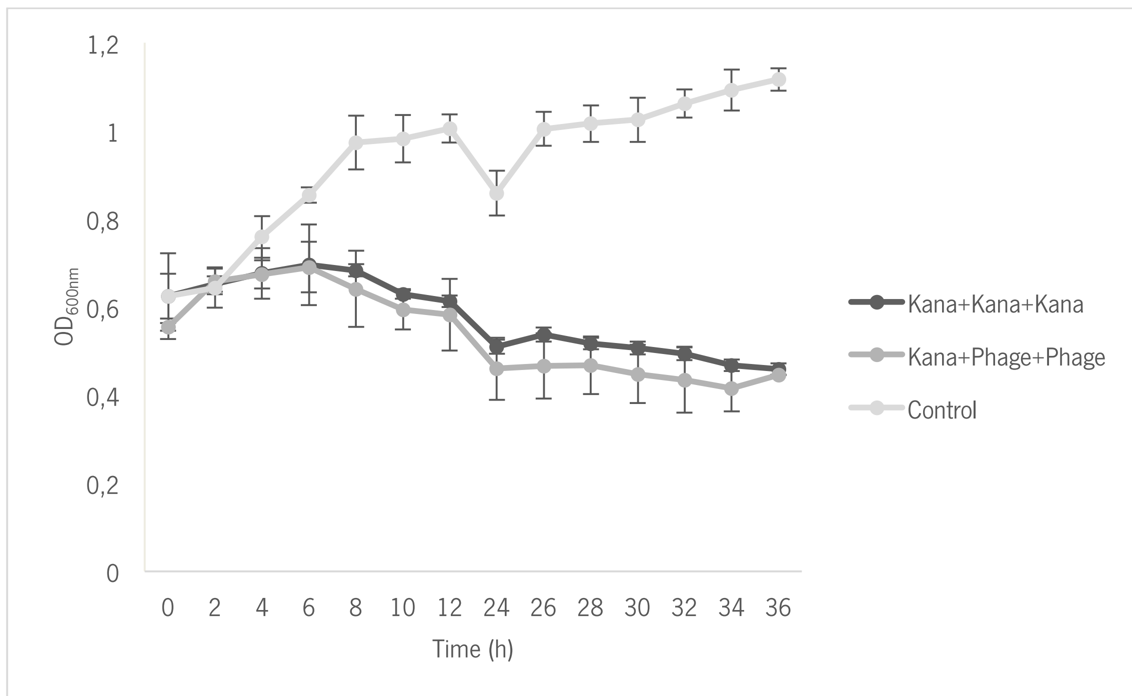

Figure 3.8. Treatment of A. baumannii planktonic cells with Kanamycin (0 h) followed by the addition of more antibiotic or phage at 12h and 24h, with OD values at 600 nm measured at 2h intervals.

Looking at Figure 3.8, it is possible to observe that the combinations had an equal effect (0.44 to phage and 0.46 to Kanamycin), with a good reduction comparing to the control cells.

Figure 3.9 represents the results obtained when Ciprofloxacin was the first substance added.

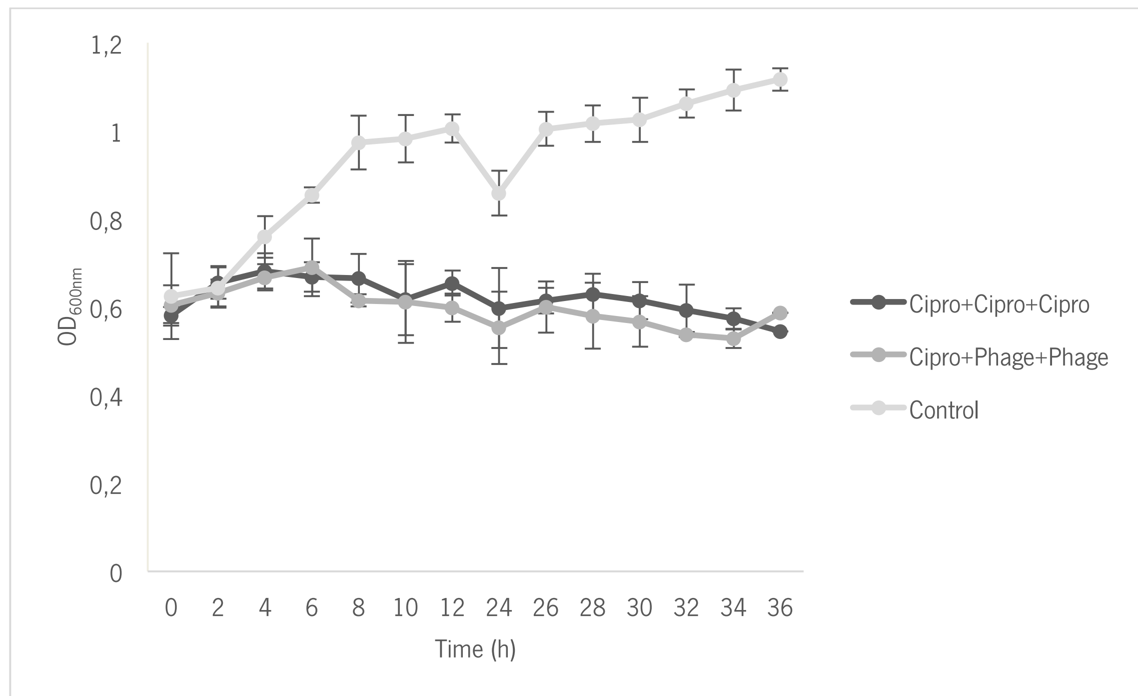

Figure 3.9. Treatment of A. baumannii planktonic cells with Ciprofloxacin (0h) followed by the addition of more antibiotic or phage at 12h and 24h, with OD values at 600 nm measured at 2h intervals.

Once again, the results are very similar between combinations (0.54 for Ciprofloxacin and 0.58 for phage), both resulting in a significant reduction of cell growth comparing to the control.

Analyzing all the figures, it is possible to conclude that the best combination in this novel treatment is the Phage+Kanamycin+Kanamycin, with an absorbance of 0.22. Furthermore, the best results were of the figure 3.8, in other words, the combinations which had the phage *Aba1* as first addition. It is possible that by adding phage initially some of the bacteria resistant to the antibiotics are eliminated and, therefore, the antibiotics can then be more effective.

3.5.1. Biofilm

After biofilm formation, phage Aba1 or one of the antibiotics was added to the biofilm. After 12 h and 24h of incubation more phage or antibiotics was added to the biofilm, with periodic (6h, 10h, 24h and 32h) measurement of viable cells (CFUs).

Figure 3.10 represents the results obtained for the Phage-Antibiotic combinations which begun with phage infection (0h). Chart legend can be read as “First addition+12 h addition+24 h addition”.

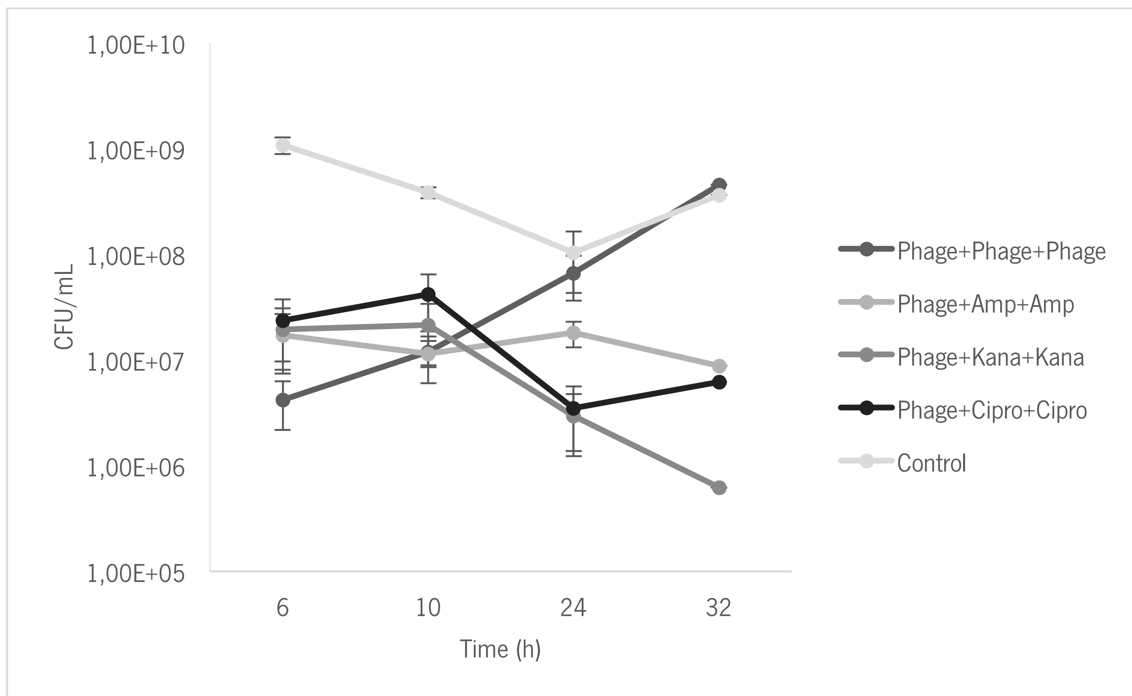

Figure 3.10. Treatment of A. baumannii biofilms with phage (0h) followed by the addition of more phage or antibiotic at 12h and 24h, with CFU counting at 6h, 10h, 24h and 32h.

Observing Figure 3.10 it is possible to note that the use of only phage results in no decrease of *A. baumannii* biofilm cells since the CFUs are identical to the control after 32h. For its turn, the combination of phage followed by kanamycin seems to be the most beneficial, with a reduction of about 3 logs in biofilm cells. The remaining two combinations had similar results with a reduction of about 1.5 logs in biofilm cells.

Figure 3.11 represents the results obtained for the Phage-Antibiotic combinations which begun with Ampicillin addition (0h).

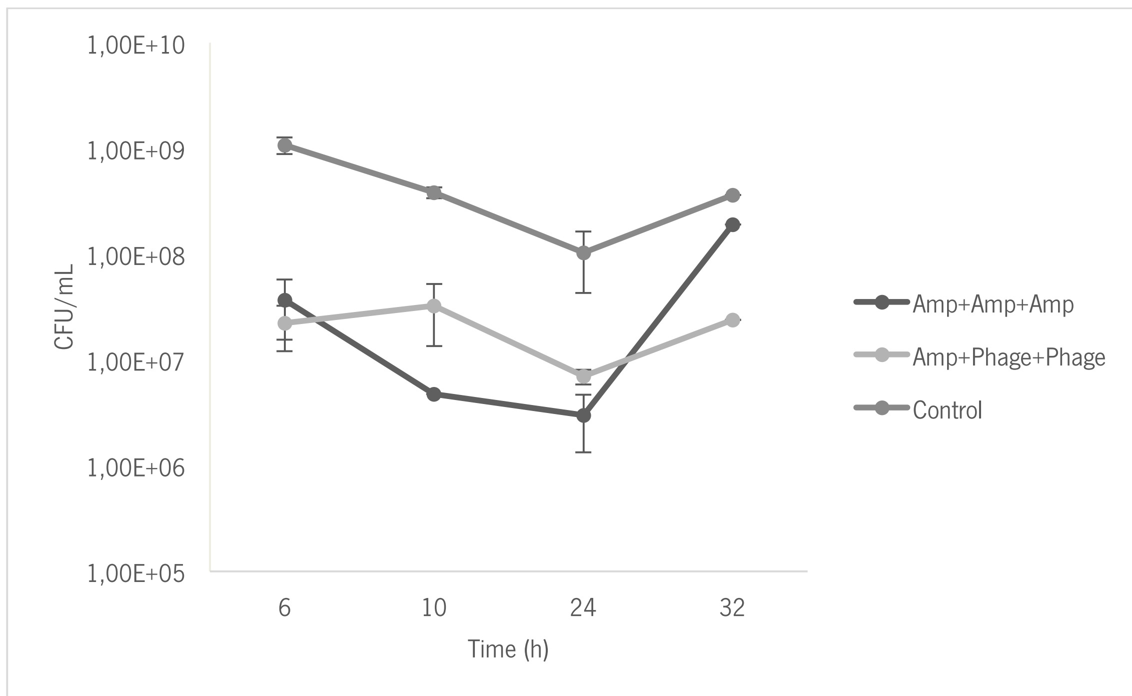

Figure 3.11. Treatment of A. baumannii biofilms with Ampicillin (0h) followed by the addition of more antibiotic or phage at 12h and 24h, with CFU counting at 6h, 10h, 24h and 32h.

Observing Figure 3.11 it is possible to note that these combinations are not so efficient as the previous ones that begun with phage. It appears that in both Ampicillin combinations tested there is a tendency of increase of the biofilm cells, which indicates the loss of efficacy of the treatment (with probable gain of resistance by the bacterial cells). Nevertheless, Ampicillin with phage has a better effect that only Ampicillin, showing a 1 log reduction.

Figure 3.12 represents the results obtained for the Phage-Antibiotic combinations which begun with Kanamycin addition (0h).

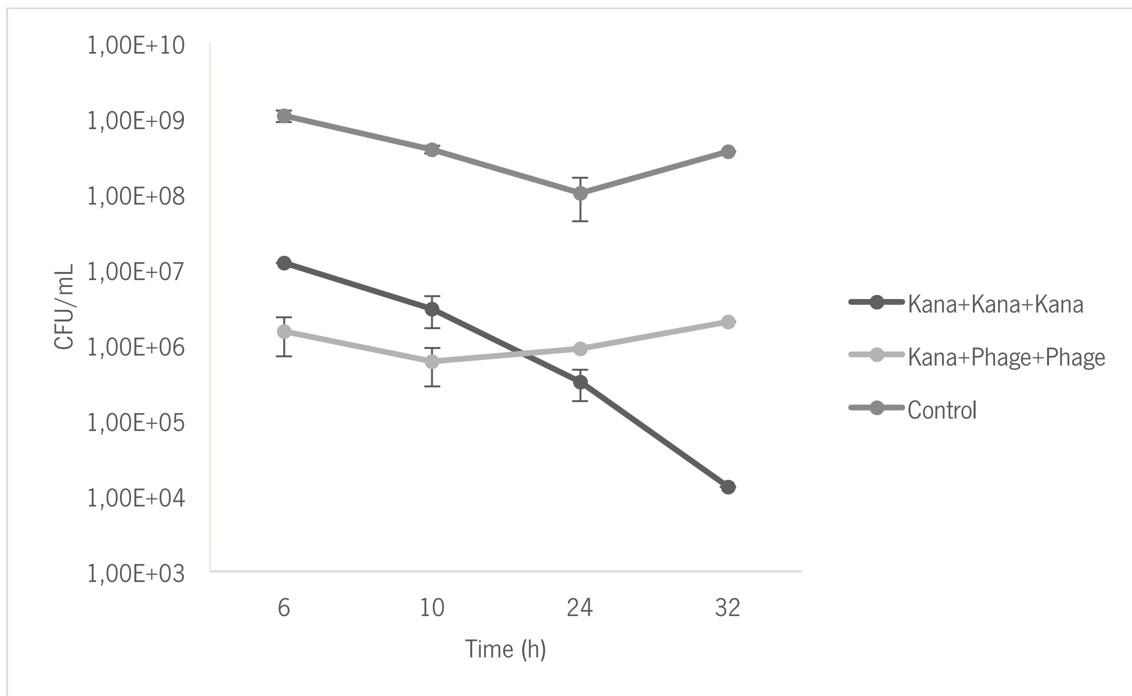

Figure 3.12. Treatment of A. baumannii biofilms with Kanamycin (0h) followed by the addition of more antibiotic or phage at 12h and 24h, with CFU counting at 6h, 10h, 24h and 32h.

Figure 3.12 demonstrates that a stepwise addition of Kanamycin results in almost 5 log reduction of biofilm cells, with a constant tendency to reduce biofilm. The combination of Kanamycin followed by phage also reduces biofilm cells by about 2 logs.

Figure 3.12 represents the results obtained for the Phage-Antibiotic combinations which begun with Ciprofloxacin addition (0h).

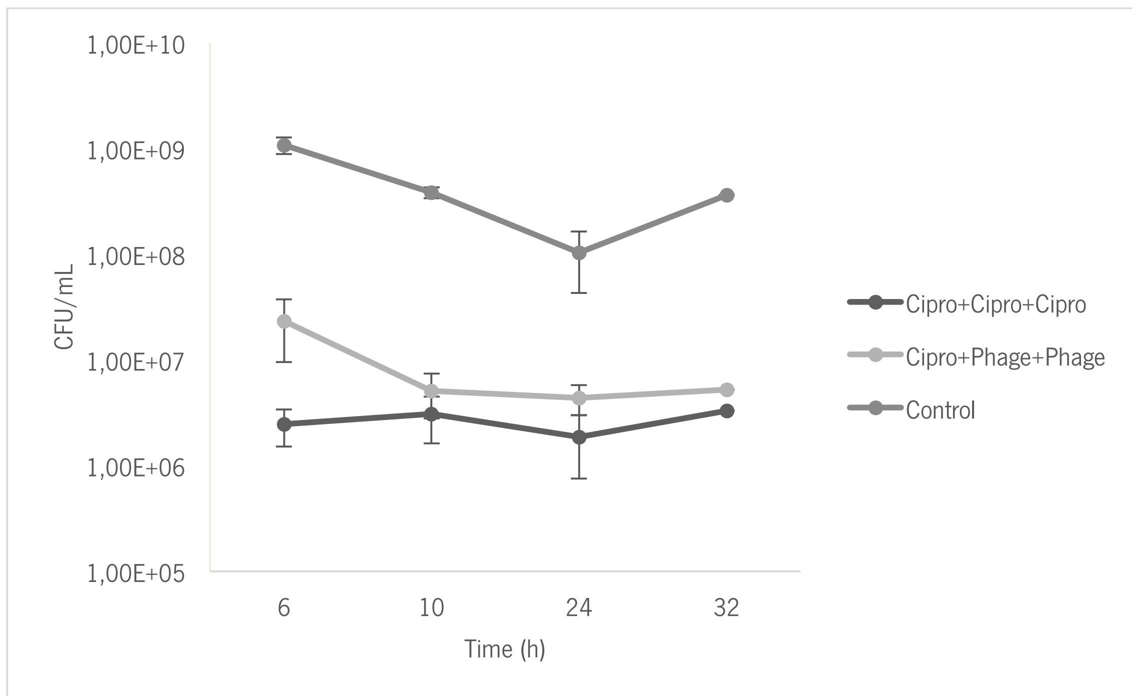

Figure 3.13. Treatment of A. baumannii biofilms with Ciprofloxacin (0h) followed by the addition of more antibiotic or phage at 12h and 24h, with CFU counting at 6h, 10h, 24h and 32h.

The stepwise addition of Ciprofloxacin or Ciprofloxacin with phage presents similar results, with the reduction of almost 2 logs of the biofilm cells. Interestingly, a more accentuated decrease was expected since Ciprofloxacin had the lowest MBEC. However, it seems that the stepwise addition of this antibiotic does not result in more efficacy against the biofilm cells, only in the maintenance of the same activity.

Comparing all the Figures it is possible to note that the best treatment was the stepwise addition of Kanamycin, with a 5 log reduction, immediately followed by the combination of Phage with Kanamycin, with a 3 log reduction. The better results of Kanamycin comparing to the other antibiotics may result from a slower gain of resistance from the bacteria to this antibiotic than to Ampicillin or Ciprofloxacin. So, using this strategy of stepwise addition of the antibiotic, the bacteria are not allowed enough time to acquire resistance and therefore the antibiotic shows increased efficacy.

The results from the biofilm experiments are in agreement with the planktonic, with Kanamycin combinations having the best effect to combat *A. baumannii*.

4. CONCLUSIONS AND FUTURE WORK

The main objective of this work was to analyze to process of synergy between antibiotics and phage *Aba1* against planktonic cultures and biofilms of *A. baumannii*. In a first step, the MIC and MBEC breakpoints of the antibiotics against planktonic cells and biofilm of this bacteria were determined. The MIC values for the three antibiotics tested (Ampicillin, Kanamycin and Ciprofloxacin) indicated that the strain evaluated is sensitive to their activity. The MBEC values for Ampicillin and Kanamycin were upon 1000 times higher than the MIC, which clearly indicates resistance from the biofilm cells to these antibiotics. Ciprofloxacin was the exception, with a MBEC that although 30x higher than the MIC can still be considered as efficient against the biofilms.

In a second step of the work, phage *Aba1* was tested against *A. baumannii* biofilms with a MOI of 1 or 10, and an incubation time of 4h or 24h. For both periods, the phage at MOI=10 had a better activity, which corroborates some published studies. Furthermore, the activity of the phage was better for a short period of time indicating a gain of resistance by the biofilm, independently of the MOI used.

In a third step, the combination of phage-antibiotic was assessed against *A. baumannii* biofilms. The results demonstrated a synergy between Ampicillin and phage *Aba1*, especially for a 4h action. This result opens a new opportunity for this antibiotic, because the isolated action of Ampicillin had the worst results against *A. baumannii* biofilms. The combination of phage *Aba1* and Kanamycin also had a synergist effect, while the combination of phage with ciprofloxacin had no effect. These results clearly demonstrate that the result of phage-antibiotic combination depends on the antibiotic used (and also of the phage), and studies for each application are necessary because no general conclusion can be assumed. In general, the combined treatments of antibiotics and phages has potential in the combat of biofilms, and this treatment could be used to combat nosocomial infections caused by *A. baumannii*.

In a fourth step of this work, a novel strategy was tested. It consisted of the stepwise addition of phage and/or antibiotics against planktonic cells and biofilms of *A. baumannii*. This novel treatment showed that the stepwise addition of Kanamycin or Phage+Kanamycin resulted in significant (3-5 log) reductions on biofilm cells. The combinations involving Kanamycin were also the best strategy against planktonic cells. This is a very interesting result, since the MBEC of Kanamycin indicated biofilm resistance, and the application of this novel treatment reveals a new possibility for the efficient use of Kanamycin against *A. baumannii* infections.

This work opens new questions to be addressed in future works. The following experiments are suggested:

- Evaluate if the effect of phage-antibiotic combination is related to the antibiotic class (and therefore, mechanism of action);
- Evaluate if the type of phage can also influence the effect of phage-antibiotic combinations;
- Improve the novel strategy here evaluated to combat *A. baumannii* biofilms, using the stepwise addition of phages and/or antibiotics. For example, determine the time that bacteria take to develop resistance to each antibiotic or phage, and optimize the stepwise procedure to avoid the appearance of the resistant phenotype and therefore, have an improved effect against biofilm cells.

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