Universidade de Aveiro Departamento de Biologia
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Nádia Castanho Valério

Potential effects between bacteriophages and antibiotics to inactivate *Escherichia coli*

Potenciais efeitos entre bacteriófagos e antibióticos para inactivar *Escherichia coli*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

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“A life path may have strange twists and turnings, and we do not always end up where we intend to go....”

Marion Zimmer Bradley, **The Forest House**

O júri

Presidente	**Professora Doutora Maria Ângela Sousa Dias Alves Cunha** Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro
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Palavras-chave Terapia fágica, resistência a antibióticos, bactérias patogénicas, *Escherichia coli*, infeções urinárias.

Resumo

Escherichia coli é uma bactéria oportunista que pode ser encontrada como parte da flora normal do trato gastrointestinal humano e de alguns mamíferos. Este microrganismo é capaz de provocar diversas infeções, sendo responsável pela maioria das infeções do trato urinário (ITU). *E. coli* é resistente a uma grande variedade de antibióticos, tornando difícil o tratamento de infeções por ela causadas. Deste modo, a terapia fágica pode ser uma ferramenta útil no tratamento de infeções causadas por estirpes de *E. coli* resistentes aos antibióticos. Contudo, também a terapia fágica também leva ao desenvolvimento de bactérias mutantes resistentes aos fagos. Por esta razão, neste trabalho, foi avaliada a combinação de duas terapias, quimioterapia e terapia fágica, de modo a avaliar possíveis efeitos sinérgicos e atenuar o desenvolvimento de resistências aos fagos e antibióticos. Foi usado o fago ECA2, isolado num estudo prévio, e vários antibioticos (ampicilina, canamicina, piperacilina, ciprofloxacina tetraciclina e cloranfenicol) com diferentes mecanismos de ação. A estirpe de *E. coli* usada é sensível aos antibióticos ciprofloxacina, tetraciclina e cloranfenicol e resistente aos antibióticos ampicilina, canamicina e piperacilina. O fago ECA2 inativou eficientemente a bactéria *E. coli*, causando uma redução de ≈4,5 log na concentração da bactéria após 2 horas de tratamento em phosphate buffered saline (PBS). A inativação bacteriana com a mistura de fago e antibióticos ampicilina, canamicina e piperacilina foram similares aos resultados obtidos apenas com o fago. Como a estirpe bacteriana apresentava resistência a estes antibióticos, a inativação bacteriana resultante foi devida apenas à ação do fago. As misturas do fago ECA2 com cloranfenicol e com tetraciclina mostraram ser menos eficazes na inativação da bactéria do que o fago sozinho. A conjugação do fago com a ciprofloxacina resultou numa inativação bacteriana de cerca de 8,3 log, em detrimento dos ≈ 4,5 log de inativação bacteriana obtidos com apenas o fago. Além disso, a conjugação do fago ECA2 com a ciprofloxacina resultam numa diminuição das resistências bacterianas obtidas em relação ao fago e ao antibiótico individualmente. A terapia fágica também foi avaliada em urina com vista a avaliar o uso desta terapia no controlo de infeções urinárias. A inativação de *E. coli* na urina foi semelhante à obtida nos ensaios em PBS, tanto para o fago como para a conjugação do fago ECA2 com a ciprofloxacina. Foi ainda testado na urina um cocktail com dois fagos, o fago ECA2 e com outro fago específico para esta bactéria, o fagophT4A (previamente isolado pelo grupo de trabalho). Observou-se numa redução bacteriana de 3,5 log. Os resultados indicam que a combinação fagos e antibióticos pode resultar num efeito sinérgico na inativação de bactérias, mas apenas quando a bactéria é sensível ao antibiótico. Além disso, a combinação de antibióticos com fagos contribui para a gestão dos níveis de resistência, controlando a resistência aos antibióticos e os mutantes resistentes ao fago. Os fagos limitam o desenvolvimento de variantes resistentes a antibióticos em tratamentos combinados independentemente do tipo de antibiótico, mas os antibióticos limitam a resistência de mutantes aos fagos apenas quando as bactérias são sensíveis ao antibiótico. Contudo, em geral, na presença de antibióticos, a resistência dos mutantes aos fagos foi a mesma ou menor do que quando os fagos foram testados isoladamente. A elevada eficiência de inativação bacteriana por fagos combinada com uma maior inativação bacteriana na presença de antibiótico, e a elevada sobrevivência dos fagos em urina, abre o caminho para estudos mais aprofundados para controlar a UTI e o desenvolvimento de resistências em *E. coli*, a bactéria mais frequentemente isolada em UTI ao nível da comunidade e em ambientes hospitalares.

Keywords Phage therapy, antibiotic resistance, pathogenic bacteria *Escherichia coli*, urinary infections

Abstract

Escherichia coli is part of the normal flora of the gastrointestinal tract of humans and various mammals. This opportunistic microorganism is capable of cause several infections, such as urinary tract infections (UTI). *E. coli* is resistant to a large number of antibiotics, becoming harder the control of infections caused by this bacterium. Phage therapy may be a useful tool to control infections caused by antibiotic resistant strains. However, the major concern of the phage therapy is also the emergence of phage resistant bacteria. In this study, was evaluated the combination of two different therapies, chemotherapy and phage therapy, to evaluate the possibility of synergic effects between them. It was used the phage ECA2 (a phage previously isolated by the research group) and various antibiotics (ampicillin, kanamycin, piperacillin, tetracycline, chloramphenicol and ciprofloxacin) with different mechanisms of action. The *E. coli* strain used in this study is sensitive to the antibiotics ciprofloxacin, tetracycline and chloramphenicol and resistant to the antibiotics ampicillin, kanamycin and piperacillin The phage ECA2 caused a reduction in *E. coli* concentration of ≈ 4.5 log after 2 hours of treatment in phosphate buffered saline (PBS). The results obtained with the mixtures of the phage with ampicillin, kanamycin and piperacillin did not cause significantly differences when compared with the results obtained just with the phage. As the bacterium *E. coli* showed resistance to those antibiotics, the bacterial inactivation was just due the action of the phage. Otherwise, the results obtained using the mixtures of ECA2 with tetracycline and chloramphenicol were worse than the results obtained just with the phage. The conjugation of the phage with ciprofloxacin resulted in a bacterial inactivation of about 8.3 log, compared to the ≈4.5 log of bacterial inactivation obtained with the phage alone. In addition, the conjugation of the phage ECA2 with ciprofloxacin resulted in a decrease of the bacterial resistances obtained the phage and the antibiotic individually. The efficacy of phage therapy in urine was also evaluated, with the phage and the mix of phage and ciprofloxacin. The inactivation of *E. coli* in urine samples was similar to that obtained in PBS. It was observed a decrease of 4.3 log after 4 hours of treatment. Furthermore, a cocktail with two phages, the phage ECA2 and another *E. coli* specific phage, previously isolated by the research group, the phage phT4A, was also tested. The *E. coli* inactivation was 3.5 log after 4 hours. The results indicate that phage and antibiotic combinations could result in synergistic effect in the inactivation of bacteria, but only when the bacterium is sensitive to the antibiotic. Also, the combination of antibiotics with phages contributes to managing resistance levels, controlling the antibiotic resistance and phage-resistant mutants. The phages limit the emergence of antibiotic resistant variants in combined treatments independently of antibiotic type, but the antibiotics limit the resistance of phage-mutants only when bacteria are sensitive to the antibiotic. However, overall, in the presence of antibiotics the resistance of phage-mutants was the same or less than when phages were tested alone. The high bacterial inactivation efficiency with phages combined with a higher bacterial inactivation in the presence of antibiotic and the long periods of phage survival in urine samples, paves the way for depth studies to control urinary tract infection and to overcome the development of resistances by *E. coli*, the bacterium most frequently isolated in UTI at the community level and at hospital settings

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List of Acronyms and Abbreviations

ANOVA	**Analysis of variance**
CFU	Colony forming units
DHF	Dihydrofolate
DNA	Deoxyribonucleic acid
ds	Double-stranded
EUCAST	European Committee on Antimicrobial Susceptibility Testing
G	Gravitacional force
ICTV	The International Committee on Taxonomy of Viruses
log	Logarithm
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
ºC	Degrees Celsius
PABA	Para-aminobenzoic acid
PBP	Penicillin-Binding-Proteins
PBS	Phosphate-Buffered Saline
PFU	Plaque forming units
PFU/mL	Plaque forming units per milliliter
rRNA	Ribosome ribonucleic acid
ss	Sigle-stranded
THF	Tetrahydrofolic acid
tRNA	Transfer ribonucleic acid
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UTI	Urinary tract infection
WHO	Word Health Organization

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Thesis outline and objectives

Objectives and Thesis Outline

This present work foccuses in the utilization of phage therapy combined with antibiotics to inactivate *E. coli*. This bacterium is responsible for many urinary tract infections and it is resistant to most antibiotics used worldwide.

This document has two chapters. Chapter 1 includes a literature review, serving as a basis for the following experimental work. The Chapter 2 includes an experimental work about the efficiency of phage therapy in the control of *E. coli* growth, in order to assess the suitability of phage therapy in the treatment of urinary tract infections. It was also tested the possibility of a synergic effect between bacteriophages and antibiotics with clinical use, focusing on the emergence of resistances to both antibiotic and phages.

Chapter 1- **General Introduction**

1.1. *Escherichia coli*

Escherichia coli belongs to the *Enterobacteriaceae* family, which is the largest and most heterogeneous family of gram-negative bacilli. The primary difference between Gram-positive and Gram-negative focuses on the cell wall [1].

E. coli is a nonspore-forming bacteria with motility due its peritrichous flagella and it is a bacteria with fimbriae. Most *E. coli* strains are capable of growing over a large range of temperature (approximately 15 – 48 °C). The growth rate is maximal in the narrow range of 37 – 42 °C [2].

This bacterium is facultative anaerobe, capable to reduce nitrates to nitrites. Also, it is capable of fermenting glicose with gas production and capable of produce catalase. *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulfide negative. The ability of *E. coli* to ferment lactose is a classic differencial test to separate this species from *Shigella* and *Salmonella* [2].

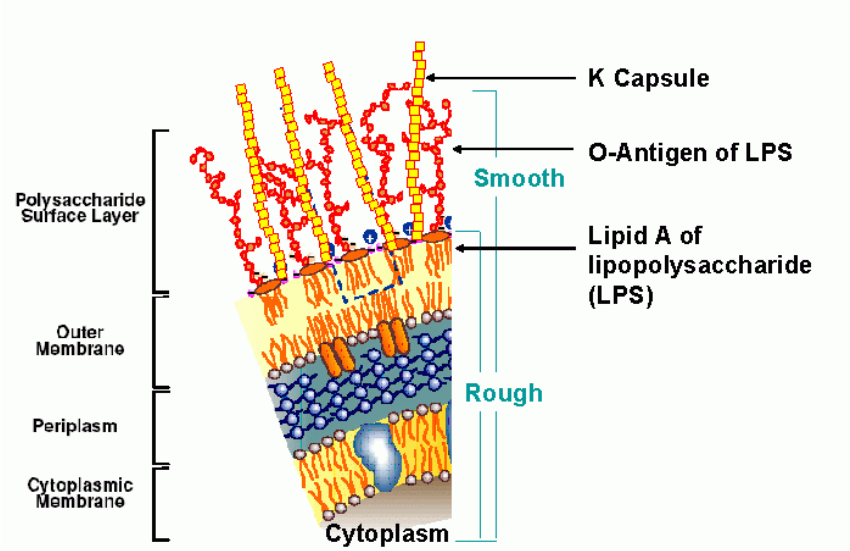


Figure 1: Schematic representation of characteristics and outer membrane antigens [3]

This bacterium can be found at the commensal flora of the intestinal tract of humans and many other animals. *E. coli* is one of the most frequent causes of common bacterial infections, including intra-abdominal like cholecystitis and cholangitis. It also cause enteric infections, like traveler diarrhea [4].

E. coli is the bacteria most implied urinary tract infections (UTI), including uncomplicated urethritis/cystitis, symptomatic cystitis pyelonephritis, acute prostatitis,

prostatic abscess, and urosepsis. It is also responsible for other clinical infections such as pneumonia and meningitis [4].

1.2. Urinary Tract Infections

The bladder is typically sterile, but the epithelial cells coating the urethra, downstream, are colonized by rods and aerobic facultative gram-negative cocci. The presence of microorganisms in the terminal urethra, and its multiplication and invasion of tissues can cause the beginning of a UTI (urinary tract infections). Although the urinary tract is traditionally considered to be sterile, advances in metagenomics and other technologies allowed the discovery of a 'urinary microbiome', which may alter the way in which we think about UTI[4].

UTI can be classified in two types, infections acquired in the community, between non hospitalized people and nosocomial infections that affect hospitalized patients in health institutions.

The most prevalent microorganisms are aerobic gram-negative. *Escherichia coli* is the prevailing bacterium in both UTI types, being responsible to 75 to 90% of acute UTI in the community and around 50 to 60% in the hospital infections, with significant morbility and mortality wordwide[5]. Strains of *Staphylococcus*, *Proteus, Klebsiella pneumoniae, Pseudomonas, Enterococcus and Enterobacter* can also be found as responsible of the UTI [6,7].

There are many factors implicated in the occurrence of UTI such as urinary stasis, pregnancy, diabetes, urinary obstruction, poor hygiene, the insertion of foreign objects, menopause, neurological disorders and sexual diseases[8]. Females have higher incidence in UTI due to the anatomical structure of the female urethra. The female urethra is shorter and close to anus than the male urethra, so it is easier for women to commonly have an UTI [9,10].

The bacterial growth may be promoted by the presence of certain nutrients, such as glucose, amino acids and uric acid in urine and by an increase in its pH. The presence of 10^5 CFU/mL of the same organism in a sample of urine is defined by significant bacteriuria. The main symptoms of a urinary tract infection are numbered as dysuria, increase of urinary frequency, burning urinate sensation, fetid urine odor, color changing, difficulty to urinate,

presence of blood in urine, pain in the abdomen, fever, chills, back pain, nausea and vomit[11].

The empiric treatment for UTI is the use of broad-spectrum antibiotic and the recommended oral antibiotic options include fluoroquinolones, aminopenicillines and aminoglycosides[12]. Multidrug resistant *E. coli* are widely distributed in hospitals and actually an increase of this bacterium in the community is observed. *E. coli* is found to have high resistance to several antibiotics, such as aminoglycosides (e.g. Kanamycin, Streptomycin, Gentamicin), aminopenicillin (e.g. Ampicillin), sulfonamides, tetracycline and fluoroquinolones[13].

1.3. Bacteriophages

Bacteriophages, or phages, are viruses that infect only prokaryotes (bacteria and archaea)[14], resulting usually in propagative lyses (lytic cycle) or lysogenization (lysogenic cycle) of the infected cell. Bacteriophages are the most abundant microorganisms at biosphere [16-19].

Although phages can have two different life cycles, the lytic phages are the best candidates for phage therapy because they replicate fast within their hosts and lyse them[15]. These biological entities can be defined as a capsid-encoding organism that is composed by proteins and nucleic acids, self-assembled in a nucleocapsid that needs a prokaryotic organism to completing its life cycle[16,17].

The main role of phages in biological ecosystem aims the coevolution of phages with their host bacteria and provides the earth ecological equilibrium in several environmental or ecological niches[22,23,25]. The most of the phages are tailed phages, which accounts for 96% of all phages present on earth, belonging to the order Caudovirales (families *Myoviridae, Siphoviridae* and *Podoviridae*)[18].

1.3.1. Bacteriophage morphology

Bacteriophages feature a variety of morphological types, like various shapes ant sizes, but generally most have a capsid, a colar and a tail **Figure 2**[18]. They are essentially constituted by two main components: nucleic acids and proteins.

Some constituents of bacteriophage have a specific terminology, as is the case of nucleic acid that is called core, envelope which is called capsid and the morphological subunits holds the name of capsomeres. Also viral infectious particle has a specific nomenclature, being called virion[19].

The core of phages is encapsulated with a protein or a lipoprotein capsid, that usually is the shape of an icosahedron, is connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers.

The capsid has three important functions during the phage life cycle: (1) protect the phage genome during its extracellular phase, (2) enable the adsorption of the phage, fixing the virus to the host bacterium (in *Caudovirales*), and (3) the subsequent delivery of the phage genome into the host cytoplasm[20].

Usually phages have a size between between 24 and 200 nm long and can vary between 17kb and 5000 kb. Its genome can also be very diversified, so can be found phages with double-stranded DNA, single-stranded DNA, or single stranded RNA[14].

The tail may or may not be a contractile structure bounded to six fibres, that contain receptors on their end that are capable of recognize binding sites on the surface of the host cell. Nevertheless, not all phages have tail fibers, so in these cases the organism has other attachment mechanisms [18,20].

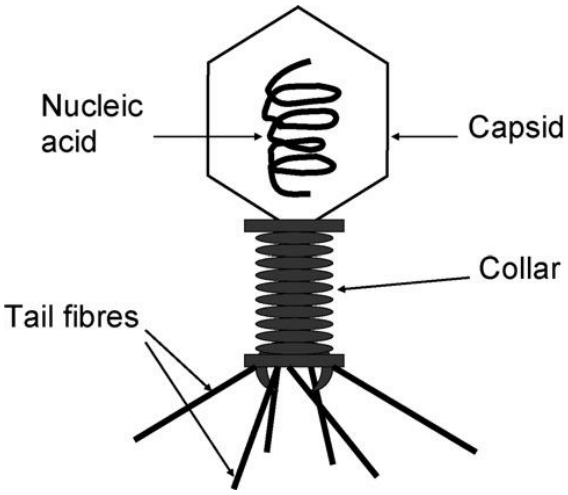


Figure 2: Representation of a typical bacteriophage [18]

1.3.2. Taxonomy of bacteriophages

The *International Committee on Taxonomy of Viruses (ICTV)* is the responsable for the classification of viruses. That classification is based on morphological, like size and shape, and molecular characteristics, type of nucleic acid **Table 1** [14].

Nowadays, it can be found around 13 families and 30 genera, belonging to the *Caudovirales* order. Three major families are divided based on tail structure:

1. *Myoviridae*- viruses have a contractile tail constituted by hem, a central tube and a big capsid head (150 nm);
2. *Siphoviridae*- viruses have long not contractible tails, and a relativelly small capsid head (50-60nm)*;*
3. *Podoviridae*- viruses have no contractile tail and have short tails and a small capsid head (50-60 nm).

The non-tailed phages are classified into ten families and they are polyhedral, filamentous or pleomorphic [18,21–23].

Table 1: Major characteristics of phage families (adaptated from Ackermann 2007)

Shape	**Nucleic acid**	**Family name**	**Characteristics**
Tailed	DNA, 2, L	*Myoviridae*	Contractil tail
	DNA, 2, L	*Siphoviridae*	Long non-contractil tail
	DNA, 2, L	*Podoviridae*	Short non-contractil tail
Polyhedral	DNA, 1, C	*Microviridae*	Icosahedral capsid
	DNA 2, C, S	*Corticoviridae*	Complex capsid with lipid layer
	DNA 2, L	*Tectiviridae*	Icosahedral capsid with inner lipid vesicles
	RNA, 1, L	Leviviridae	Quasi-icosahedral capsid
	RNA 2,L, seg	Cystoviridae	Icosahedral capsid, lipids
Filamentous	DNA 1, C	Inoviridae	Rod-shaped with helical symmetry
	DNA 2, L	Lipothrixviridae	Enveloped filaments, lipids
	DNA 2, L	Rudiviridae	Helical rods
Pleomorfic	DNA 1,C,S	Plasmaviridae	Envelope, lipids, no capsid.
	DNA 2,C,S	Fuselloviridae	Envelope, lipids, no capsid.

C-Circular; S-superhelical; seg-segmented; 1-single-stranded; 2- double-stranded

1.3.3. Life cycle of bacteriophages

Bacteriophages are metabolically inert in their extra cellular form. They are only able to self-reproduce as long as the host bacteria is present and their replication depends exclusively on the host intracellular machinery to translate their own genetic code.

Bacteriophages can have one of two major and distinctive types of life cycle, the lytic or virulent cycle, the temperate or lysogenic cycle and more sporadically through pseudolysogeny. According to this, phages are classified, based on their life cycle, as lytic (virulent) or lysogenic (temperate) [17,24].

Lytic phages infect prokaryotic cells, causing inhibition of host metabolism and subverting it to the production of phage offspring. The lytic cycle results in the lysis of the bacterium accompanied by the release of new multiple phage particles. The new phages produced by the host bacteria spread to infect other cells. First, the phage binds to specific receptors of bacteria, this phase is called adsorption [17,25]. Phages can use different parts of lipopolysaccharide (LPS), flagella, fimbriae and many other surface proteins as receptors. Bacteriophages may also use enzymes to break down the bacterial surface[14,26]. Then the phage genome is injected into the host bacterium and occurs early gene expression. Most of the proteins produced in this phase are involved in the shutting down of the host bacterial systems and phage genome replication[25]. After replication of the phage genome, occurs the expression of the phage late proteins that are involved in the formation of new phage particles and lysis of host bacteria[27]. The phage head and tail are assembled and the phage genome is packaged. The bacteria are destroyed through lysis, resulting in a realease of the new phage particles.

On the other hand, in lisogenic cycle, the phage genome is integrated into the host cell DNA. Prophage DNA will be replicated when the host cell genome replicates and so daughter cells will inherit the viral DNA (**Figure 3**). The prophage can stay in a dormant state for long periods of time and may become activated and turn on the lytic cycle. The lytic cycle is induced spontaneously by chemical or physical agents such as radiation, pollutants, changes in temperature and nutrient concentrations[25,28]. At the end the newly formed phage particles will lyse the host cell. Lysogeny might be a viral survival strategy to ensure periods of low host density during nutrient starvation [25,29].

The life cycle and the genome of many temperate phage is already studied and there is evidence that several phages have genetic sequences that induce the production of virulence factors in bacteria, so the use of temperate phages in human therapy is inappropriate. Since virulent phages do not provide prophage state and therefore do not have or transfers genes coding for the production of virulence factors in their hosts[30].

Over the two main life cycles of bacteriophages, it is possible to find another phenomenon known as pseudolysogeny. Yet, unlike true lysogeny, the phage genome does not integrate to the host. Pseudolysogeny is a condition in which the bacterial cells coexists with the viruses **Figure 3**. In the host cells, there is insuficiente energy available for the phage initiate genetic expression leading to a temperate or lytic response[31]. When the nutrientes are supplied to the bacteria, the pseudolysogens evolve into one of the two phage life cycle, lytic or lysogenic. The frequency of pseudolysogen phages is correlated with the concentration of nutrientes available to their host[25,31].

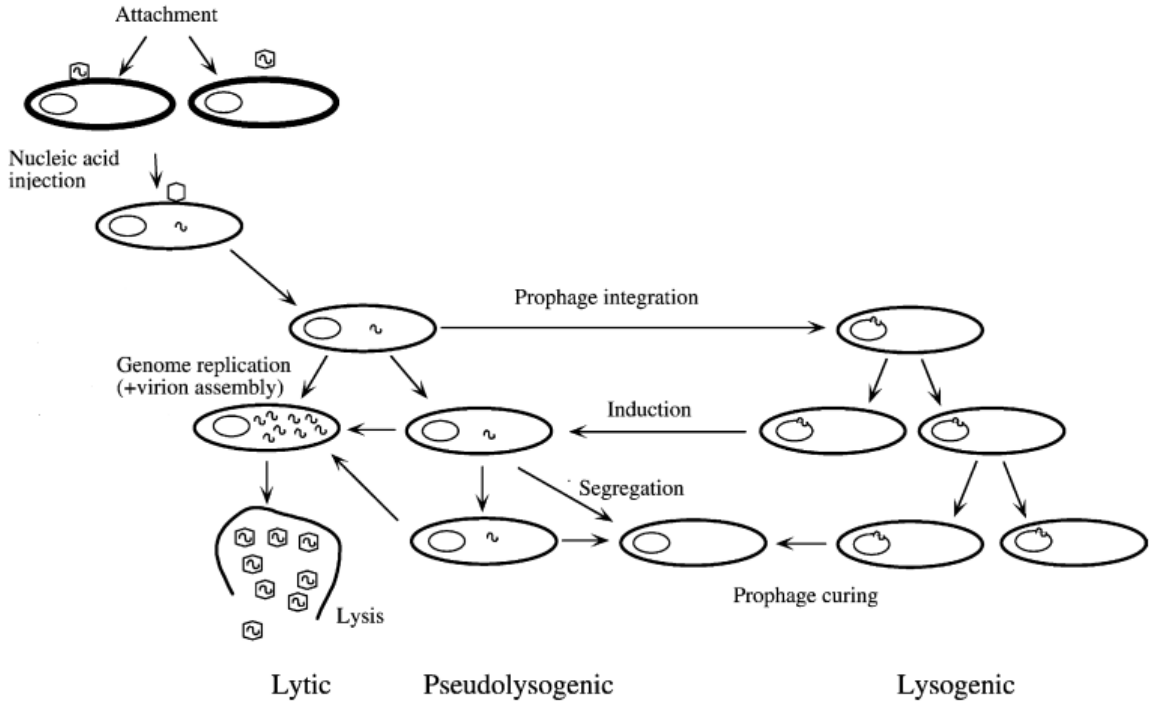


Figure 3: General phage life cycle (Adapted from Weinbauer, 2004)

1.4. Phage Therapy

Phages therapy consists in the utilization of bacteriophages to inactivate pathogenic bacteria. Soon after its discovery by Twort in 1915 and d'Herelle in 1917, in which the latter named this type of virus, phages soon began to be exploited and used to control infections by pathogenic bacteria[25,32]. This method of treatment was used to treat and prevent bacterial infections in the former Soviet Union and Eastern Europe, however, was abandoned by the west due to the appearance of antibiotics[33].

Antibiotic use increased rapidly. The mastery of production of antibiotics, the relatively broad spectrum of action of antibiotics, and the stability of the preparations were advantages over phages, but the emergence of pathogenic bacteria resistant to antibiotics has recently teased the western scientific community to reevaluate phage therapy as an option for bacterial infections treatment[30,33].

1.4.1. Phage seleccion

The selection of appropriate phages is a critical factor to the success of the phage therapy as a successful phage-mediated control of pathogenic bacteria. Beside the efficiency on bacterial inactivation, it is necessary to have atention to other important characteristics when selecting viruses for phage therapy [14,28].

Therapeutic phages should have a very abroad host range, that means that a single phage should be virulent to various strains of bacteria[34,35]. The selection of phages with a large host range limits the number of phages in the library and also reduces the cost for clinical trials[35].

The phages used in phage therapy should be characterized in detail. It is importante to sequence the genome of the phage, to identify its structure, test its behavior *in vitro*, and to prove their efficiency *in vivo* [14]. For phage therapy, lytic phages should be elected and the development of lysogeny must be avoided. When lysogeny is established the host becomes immune to an infection caused by the same or similar phages [36]. Additionally, lysogenic phages may transfer genes potentially dangerous between hosts, such as genes that encode toxins or virulence factors, which may be toxic to humans[14,32,37].

The adsortion is an important phase in the phage infective process to the host cell. Studies on T-even (like T2 and T4) and T-odd (like T1 and T3) phages have reveled that a

number of environmental factors, such as, ion concentrations, organic cofactors, pH and temperature, may have a substantial impact in the adsorpion of the phage to its host cell[38]. The increased of the adsorption efficiency had a similar effect to increasing the initial multiplicity of infection. It can be observed a decresing of the number of phages during amplification [38].

The phage burst size (number of phages produced by each host cell) and the latent period (time spent from virus entry into the cell until the first offspring are released) are also important factors to consider when phages are selected. Selecting phages with high burst size (i.e. producing a large number of descendents) is very important. Phages cannot be administered in high doses because they dessiminate badly. Wherefore, a high burst size increases the probability that phages reach target bacteria, which is crucial for achieving an efficient viral infection. If phages can eliminate bacteria faster than they can replicate, a high burst size also results in a lower risk of selection for phage resistant bacteria[35].

1.4.2. Multiplicity of infection

The most adequate multiplicity of infection (MOI), number of viruses used per host cell, to be used in phage therapy is yet a controversial aspect. It has been stated that, contrarily to the case of chemicals and other substances, precise initial doses may not be essential in treatment, because of the selfperpetuating nature of phages, revealed by an increasing of phage titers along with bacteria. The MOI has been mentioned as an important factor influencing the efficiency of phage therapy differs among the various animals used for *in vivo* experiments, particulary due to the physical-chemical complex environment and host defenses, but differing from the results obtained *in vitro*[39,40].

1.4.3. Advantages and disadvantages of phage therapy

Nowadays, most pathogenic bacteria are resistant to several antibiotics, becoming imperative the development of alternative antibacterial alternatives. Nowadays, western countries revived the interest in phage therapy [32].

Phage therapy is a potential alternative to antibiotics and to other antibacterial compounds to inactivate pathogenic bacteria [28]. The major advantages of bacteriophages over antibiotics include:

1) The specificity to the host, since the remaining microbiota is not affected, which is an advantage relatively to other antibiotics that can provoke damage in the microbiota [41,42]. That characteristic is due the fact that phages only infect a few bacterial strains [36].
2) Low-cost and rapid production of phage suspensions;
3) Self-replication, meaning that low or single dosages will multiply as long as there is still a host present;
4) Co-evolution of phage and bacteria result in a fitness cost to the host.
5) Although some phage-resistant mutants appeared after treatment, it is comparably easier to find new phages because phages coevolve with their host bacteria, outnumbering bacteria in the environment, what makes possible the rapid isolation of new lytic phages from the environment for phage-resistant bacterial mutants. So, even if the bacteria acquire phage resistance, new mutant phage that acts lytically against these bacteria can be used against the targeted bacteria[43,44].

It is safe the use of phage due the fact that no serious side effects have been described, because phages or their products (amino acids and nucleic acids) do not affect eukaryotic cells [51, 52].

Although phage therapy have many advantages, also have some limitations.

The release of bacterial endotoxins bound to the membrane during cell lysis can cause side effects, though, purification of the phage suspensions is a simple process [45].

Also, the lysogenic conversion can be another problem associated to this therapy. The lysogeny can induce a phenotype modification of the host cell such as toxin production and antibiotic resistance but also resistance to infection by similar phages [14,37]. The phage infection starts with the attachment of the phage into specific receptors of bacteria. If occurs a mutation on the bacteria receptors, the phage fails to recognize bacteria. Despite this obstacle to the application of phage therapy, the induction of resistance of bacteria is not considered a major concern, since the mutation rate of phage keep up with the mutation rate of bacteria, sufficiently to maintain the effectiveness of phage therapy [46]. Furthermore, it is possible that the receptor used by the phage is a virulence determinant thus the loss of receptor would reduce the virulence of bacteria, then it would be easier for the host immune system to overcome the infection [14]. The use of cocktails of phages can also reduce the phage resistance [47,48].

1.5. Antibacterial therapy

In 1928, Alexander Fleming made accidentally an importante discovery for humanity, by observing the inibition of a bacterial culture of *Staphylococcus aureus*, by the action of a fungus, *Penicillium notarum*. Decades later, penicilin was the first antibiotic [49].

Most antibiotics can be classified due to its bactericidal or bacteriostatic activity[50]. However, usually the antibiotics are classified by its mechanism of action, which means, the target in bacteria where the antibacterial exerts its effect [51,52].

1.5.1. Mechanism of action of antibiotics

Nowadays, antibiotics are classified into five major groups based on its intracellular target and its mechanism of action (**Figure 4**)[53].

I. **Cell wall synthesis inhibition** (e.g. penicillin and derivatives, cephalosporins, carbapenems and glycopeptides). These compounds are more effective against infection by Gram positive bacteria, because of the cell wall that coats this type of bacteria. That kind of antibiotics have the peptidoglycan present on the cell wall as target, which means that prevent the synthesis of peptidoglycan, leading to weakening of the cell wall and lysing the bacterial cell [59-60].

II. **Cell membrane disruption** (polycationic peptide antibiotics like polymyxins). Some antibiotics have the capacity to interact with the membrane phospholipids of the bacteria cell, distorting the cell surface. Consequently, this disruption leads to leakage of cellular contents, thus killing the bacterium. Amtimicrobial agents that interfere with cytoplasmatic cell membrane are bactericidal[54].

III. **Nucleic acid synthesis inhibition** (quinolones, rifampicin and sulphonamides). Certain antibiotics interfere with DNA replication and transcription processes in microorganisms. This mechanism focuses in DNA gyrase and bacterial topoisomerase IV[51,52]

IV. **Protein synthesis inhibition** (tetracycline, aminoglycosides, chloramphenicol and macrolides). In the ribosomes of bacteria is made the translation of the mRNA, allowing protein synthesis. The bacterial ribosomes have two subunits 30S and 50S, differing from eukaryotic cells which have the subunits 40 S and 60s.

Antibiotics with this mechanism, bind to ribosomal subunits, interfering with a phase of the protein synthesis[51,52].

V. **Metabolic process inhibition** (e.g. sulfonamides). Folic acid is a vitamin which functions as a coenzyme in the synthesis of purines and pyrimidines. The bacteria need to synthesize this nutrient, because they cannot get it from the surroundings. Therefore, the antibiotics of this group act by mimicking the substrate of two enzymes (para-aminobenzoic acid, PABA and dihydrofolate, DMF) to produce the active form of folic acid, the tetrahydrofolic acid (THF)[51,52].

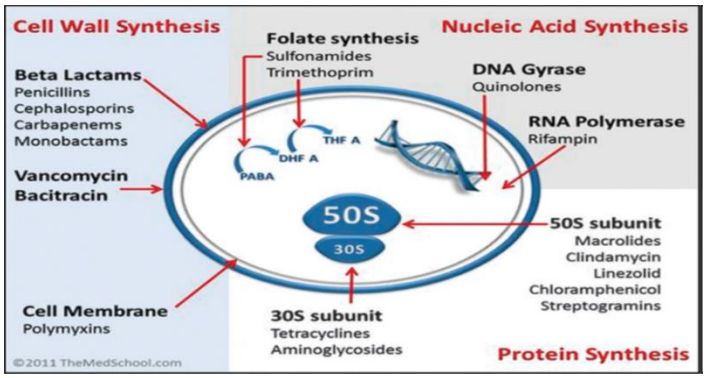


Figure 4: Classification of antibiotics by mechanism of action (Li, Collins, and Keene 2015)

A. β-Lactams

The β-lactams are antibiotics that have in their chemical composition a β-lactam ring [55]. Currently there are several types of β-lactam, which differ in structural position of the radical R. Penicillin G and V are natural, produced by fungus of the *Penicillium* genus, while some other β-lactam are semi-synthetic, being classified as amino-penicillins (ampicillin and amoxicillin), carboxi-penicillins (carbenicillin, ticarcillin, meticiclin, cloxacillin, flucloxacillin and oxacillin) and ureido-penicillins (azlocillin, mezlocillin, and piperacillin). Antibiotics classified as cephalosporins, carbapenems and monobactams are also β-lactams antibiotics.[56,57].

β-lactams antibiotics are antimicrobial agents that irreversibly inhibit the transpeptidase enzymes, whose function is to catalyze the transpeptidation reaction between the peptidoglycan chains of the bacterial cell wall. This enzyme activity leads to the formation of crosslinks between the peptide chains of the peptidoglycan structure, which is

capable of producing a significant rigid structure to protect the bacterial cell against changes in osmotic medium. The enzymes like transpeptidases and carboxiprptidases, named Penicillin- Binding- Proteins (PBPs) are the targets of the β-lactam antibiotics. All the β-lactam antibiotics have bactericidal activity [56,58].

Ampicillin is an amino penicillin, an antibiotic with large spectrum, but it is particularly effective against Gram negative bacteria non producers of β-lactamases. The intensive and continuous use of these antibiotics leads to a reduction of the bacterial strains susceptible to ampicillin[58,59].

Piperacillin is an ureidopenicillin, an antibiotic with a very large action spectrum. It is very effective against both Gram negative and Gram positive bacteria.When compared to the natural penicillins and aminopenicillins, piperacillin is more slowly inactivated by β-lactamases.

B. Chloramphenicol

Chloramphenicol is an inhibitor of protein synthesis, capable of binding to the peptidyltransferase centre of the 50S ribosomal unit, preventing the formation of peptid bonds. As a result of the binding process to enzymes, the antibiotic will prevent the elongation of the peptides. Chloramphenicol is a large-spectrum antibiotic against both Gram positive and Gram negative bacteria, with bacteriostatic characteristic. A few years ago, this antibiotic was used in human and veterinary medicine [13].

C. Tetracycline

Tetracycline is a bacteriostatic and broad-spectrum antibiotic quite effective against various aerobic and anaerobic bacteria, Gram positive and Gram negative[61,62].

Tetracyclines inhibits protein synthesis by binding to the 30S ribosome subunit, preventing the binding of aminoacyl-tRNA, causing misreading of the mRNA and blocking the addition of new amino acids. The release of proteins is also inhibited[57,61].

D. Aminoglycosides

Aminoglycoside antibiotics correspond to a group formed by two or more amino sugars connected by a glycosidic bond to a hexose amino. Most of aminoglycosides have a natural origin, but kanamycin is a semi-syntetic antibiotic[53].

The aminoglycosides exert their activity by binding irreversibly to the aminoacyl site of 16S ribosomal RNA (rRNA) within the 30S ribosomal subunit, leading to protein synthesis inhibition. Meanwhile, unlike other antibiotics with inhibition of the protein synthesis, aminoglycosides holds bactericidal activity[63].

The kanamycin was formerly used in the treatment of severe infections caused by Gram-negative, but currently this antibiotic has low clinical use due to its nephrotoxicity and ototoxicity and the bacterial resistance to this antibiotic [63,64].

E. Fluoroquinolones

Fluoroquinolones are antimicrobial agents that are capable of interfere with the DNA synthesis by blocking the enzyme topoisomerase II and topoisomerase IV. The enzyme helps to roll up and unroll DNA durinf the DNA replication. DNA gyrase binds to DNA and introduces double stranded breaks that permits the DNA to unroll. Fluoroquinolones binds to the complex DNA gyrase- DNA and enable the broken DNA strands to be released into the cell, causing the cell death[65].

Ciprofloxacin is a Class II fluoroquinolone, with bactericidal activity. It has activity against a wide range of Gram-negative and Gram-positive microorganisms. Ciprofloxacin is slightly less active when at acidic pH. That antibiotic is used to treat several bacterial infections, namely UTI, gastroenteritis, nosocomial infections and sexually transmitted diseases[66].

1.5.2. Antibacterial resistance to antibiotics

Antimicrobial agents are compounds that are capable to kill or inhibit bacterial growth[67]. Moreover, these compounds also act as selective forces for the bacterial evolution. Thus, the microorganisms adapted through different mechanisms, reducing the effectiveness of antimicrobial agents[68].

However, beside the health impact, the antimicrobial resistances have a huge economic impact, because infections with multidrugs resistant microorganisms because multiresistant microorganisms require longer treatment, with higher costs and an increased risk of mortality[69].

The resistance of a bacterium to an antimicrobial drug can result from intrinsic or acquired mechanisms. The intrinsic mechanisms occur naturally in genes located on the bacterial chromosome and they are inherent to bacteria. These intrinsic mechanisms are related to the absence of the target or with the presence of low affinity targets, low cell permeability and also to multidrug efflux systems of multiresistant bacteria[70]. In the natural resistance, the bacteria are always resistant to the antimicrobial drug.

Otherwise, in the acquired resistance, the bacteria are initially sensitive to a particular antibiotic, but due to various mechanisms it becomes resistant[71]. Acquired antimicrobial resistance can occur due to a mutation in the gene of the chromosome, consisting of the antibiotic target or by horizontal transfer of genes, specifically mobile genetic elements that are most likely to be transmitted[72].

Genes that confer bacterial resistance to antimicrobial drugs can be disseminated. With some exceptions, the intrinsic resistance and the resistance resulting from a mutation are resistance mechanisms that are unlikely to be transmitted[73]. The most frequent mechanism of acquisition of resistance genes corresponds to horizontal transfer, which may occur within the same strain or between different strains or even different bacterial genera[74]. There are three different mechanisms of horizontal gene transfer, transformation, conjugation and transduction **Figure 5**:

I. **Transformation-** bacteria capture and incorporate segments of DNA from bacterial lysates, the resistance gene may be incorporated into the chromosome or plasmid in the recipient cell. The ability of bacteria to include extracellular genetic material and go through transformation is called competence [51].
II. **Conjugation**- process occurred as a result of direct contact of two bacterial cells, of the same kind or even of different species. In this mechanism exists the transfer of fragments of genetic material, usually through plasmids. That is dependent of the conjugative pili, that may or may not be present in the bacterial cell[75].
III. **Transformation**- mechanism of DNA acquisition by which non-viral DNA can be transfer mediated by bacteriophages. Although bacteriophages only can infect the

surrounding bacterial hosts, this mechanism has the advantage that transducing phages can the persistant during environmental conditions [74].

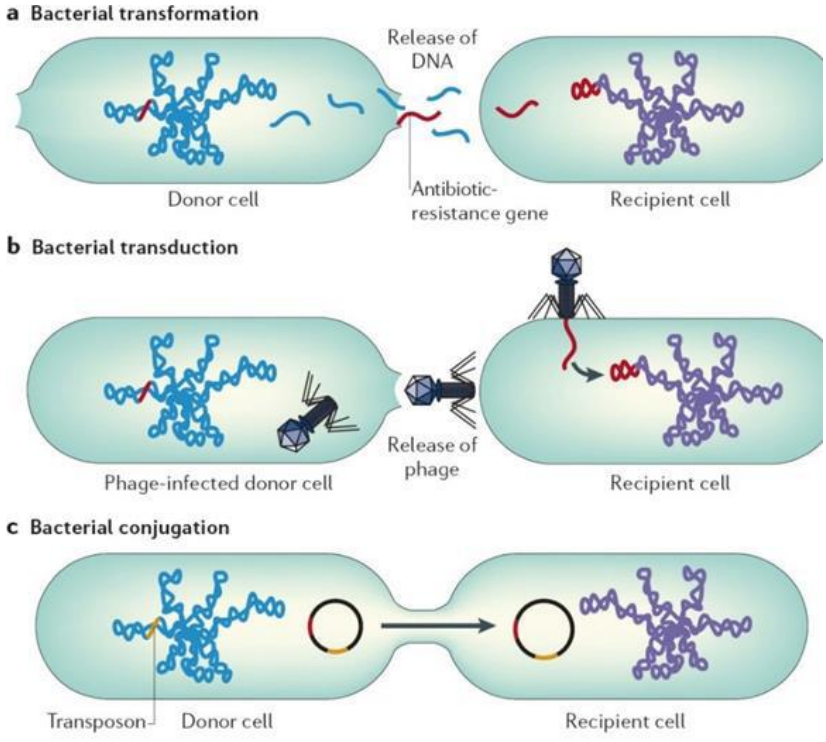
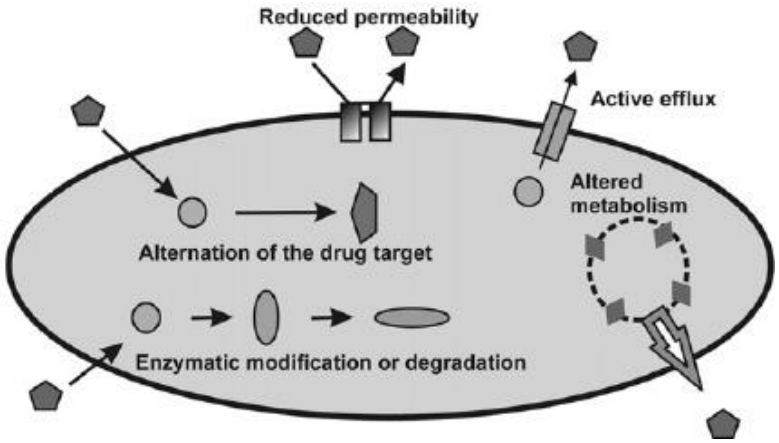


Figure 5: Horizontal gene transfer between bacteria (adapted from Furuya 2006)

After the bacteria gains resistant genes, they can use several biochemical types of antibiotic resistance, namely antibiotic inactivation (interference with cell wall synthesis),



target modification (inhibition of protein synthesis), altered permeability (changes in outher membrane or formation of new membrane transporters) and efflux pumps **Figure 6.**

Figure 6: Diagram representing the major machanisms of antibiotic resistance (Vranakis 2013)

1.5.3. *Escherichia coli* genes of resistance to antibiotics

Escherichia coli is a bacterium capable of acquire multiple resistant genes to antibiotics, becoming a problem of public health. Over the years, most strains of *E. coli* have gain resistance to most antibiotics available. Some surveillance data show that resistance in *E. coli* is higther for antimicrobial agents that have been in use for longest time [13,67]. The principal genes responsible for antibiotic resistance found in *E. coli* strains are represented in **Table 2**[76–79].

Table 2: Major genes responsible for antibiotic resistances in strains of *E. coli*

Antibiotic resistance	Gene
Tetracycline	• *tetA;* • *tetB;*
Sulfamethoxazole-trimethoprim	• *Sul-1;* • *Sul-2;*
Penicillins + cephalosporins	• bla_{TEM-1}*;* • bla_{CTX-M}*;*
Oxapenicillins	• *OXA-1*
Chloramphenicol	• *cat I- IV*

Chapter 2- **Potential effects of bacteriophages and antibiotics to inactivate *Escherichia coli***

2.1. Introduction

Escherichia coli is one of the most frequent causes of many common bacterial infections, including abdominal infections, urinary tract infections (UTI), enteric infections, pneumonia, bacteremia and meningitis[73]. This bacterium is the leading cause of both community-acquired and nosocomial UTI. Up to 50% of females eventually experience at least one episode of UTI. *E coli* causes 12 - 50% of nosocomial infections and 4% of cases of diarrheal disease [5,80].

After the discovery of antibiotics, in the decade of 30, their use increased exponentially, but microorganisms adapted through mechanisms of acquisition and transfer of resistance genes, reducing the effectiveness of these antibacterial agents. *E. coli* is capable of acquire multiple resistant genes to antibiotics, becoming a problem of public health [67].

Phage therapy, which uses lytic phages to inactivate bacteria, can be an alternative to antibiotics or used in combination with antibiotics to control infections. Several studies already shown that phages efficiently inactivate *E. coli*, even antibiotic resistant strains[81–83].

A major concern regarding the use of phages to control infections is the emergency of phage-resistant mutant [82,84,85]. Resistance may result from the alteration or loss of the bacterial cell surface receptors; inhibition of phage DNA penetration; production of restriction endonucleases which degrade the phage DNA, clustered regularly interspaced short palindromic repeats (CRISPR) system, a widespread microbial response to by-pass the selective pressure exerted by phage infection, among others [86]. This limitation can be overcomed by the combined use of phages and antibiotics. Some studies shown a synergetic effect of the combined use of antibiotics and phages[87–89]. Zhang et al (2012), showed that the combination of phage SBW25φ2 and Kanamycin reduced the resistance evolution of a strain of *Pseudomonas fluorescens* SBW25 relatively to the antibiotic [88]. It has been stated that the decrease in bacterial resistance to phages and/or antibiotics in dual therapy is due to the fact that a strain non susceptible to one antimicrobial agent can be eliminated by the second one[84]. Another study performed by Comeau et al (2007), showed a synergism between the phage φMFP and several antibiotics, like aztreonam and cefixime against an uropathogenic *E. coli* (MFP). The combination of the phage with those antibiotics resulted in an increase of the phage lysis plaques production by the host bacterium. That result was not shown to antibiotics which the bacterium was inicially resistant, namely most penicillins

(e.g. piperacillin, ticarcillin and amoxicillin)[90]. The synergic effect between antibiotics and bacteriophages was also assessed by Verma et al (2009). On that study, the authors demonstrated a decrease in the bacterial density on the biofilm of *Klebsiella pneumoniae* B5055 with the lytic bacteriophage KPO1K and the conjugation of the phage with the antibiotic ciprofloxacin. Although the results of the treatments with the phage and the conjugation were equally effective, the combination had a decrease in the formation of resistant variants to that antibiotic [87].

It has also been shown that bacterial mutations induced by phages may lead to loss of pathogenic properties[91–93] and also decrease bacterial growth[94–96]. The loss of pathogenic properties and the decrease in the bacterial growth after phage exposure could be fitness cost which can contribute to their elimination from the environment faster than their wild-type parents. These results suggest that the remaining bacterial mutants maintained their viability in the presence of phages but their phenotypes and/or genotypes were affected [94–96].

The aim of this study was to evaluate the potential synergistic effect of phages and antibiotics in the inactivation of *E. coli* in order to control infections, namely UTI, and to reduce the development of resistance to phages and antibiotics.

2.2. Materials and methods

2.2.1. Bacterial strain and growth conditions

The bacterial strain *Escherichia coli* ATCC 13706 was used as phage host. The *E. coli* strain was purchased from ATCC collection. The bacterial culture was stored in Tryptic Soy Agar (TSA; Liofilchem) at 4 ºC. Before each assay, one isolated colony was transferred to 30 mL of Tryptic Soy Broth (TSB; Liofilchem) and grown overnight (16 - 18 hours) at 37 ºC. Then, 300 µL of fresh culture was transferred to 30 mL of TSB and incubated overnight to reach the optical density (O.D. 600 nm) of 0.8, which correspond to about 10^9 cells per ml. All the procedures were done aseptically.

2.2.2. Phage selection and quantification

A sewage water sample (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro) was used to select the somatic bacteriophage of *E. coli* in a previous work by Pereira et al (2016). The authors have isolated two phages phT4A and ECA2 (Pereira et al. 2016). An isolated and morphologically representative plaque of each phage was picked out with a *Pasteur* pipette, by aspiration, and was added to 50 mL of *E. coli* culture in the exponential growth phase. The mixture was incubated at 37 ºC for 6 - 8 hours. After that, the mixture was centrifuged at 13 000 G (Heraeus Megafuge 16R Centrifuge; Thermo Scientific) for 10 min to remove non-infected bacteria and bacterial cell residues. The supernatant with the phage particles was stored at 4 ºC after the addition of 1% chloroform.

The quantification of phages was determined, in duplicate, by the agar double layer technique, using TSA medium [98]. The plates were incubated upside-down at 37 ºC and after 18 h of incubation the number of lysis plaques was counted at the most convenient dillution (with around 30 - 300 lyses plaques per plate). The results were expressed as plaque forming units per mililitre (PFU/ mL).

2.2.3.Bacterial kill curves in PBS

Bacterial inactivation was determined using the ECA2 phage suspension and the *E. coli* ATCC 13706 as host, at a MOI of 100. In order to obtain a MOI of 100, it was added 2.5 µL of bacterial culture at a concentration of 10^8 CFU/mL and the phage suspension at a concentration of 10^8 CFP/mL to 30 mL of TSB in sterilized erlenmeyer. For each assay two control samples, the bacterial control (BC) and the phage control (PC) were included. The bacterial control was inoculated just with bacteria and without phage, and the phage control was inoculated with phage and without bacteria. The controls and test samples were incubated exactly in the same conditions, at 37 ºC. One mililiter of the test sample and of the bacterial and phage controls was collected at time zero and after 2, 4, 6 and 8 hours of incubation. The bacterial concentration was determined in duplicate, by pour plating, on TSA after an incubation period of 24 h at 37 ºC. The phage title was determinated, in duplicate, through the double agar layer method after an incubation period of 4 - 8 h at 37 ºC. Three independent assays were done in different dates.

2.2.4. Bacterial kill curves with phage and antibiotics in PBS

In order to perform phage therapy with antibiotic, it was tested antibiotics with different mechanisms of action. Previously, it was done an Antimicrobial Susceptibility Test to guide the selection of the antibiotics to be used. It was selected the ampicillin (Applichem Panreac ITW companies) and piperacillin (Fluka Analytical) as β-lactams, kamanycin (Applichem Panreac ITW companies) as aminoglycoside, tetracycline (Sigma Life-Science), chloramphenicol (Applichem Panreac ITW companies) and the fluoroquinolone Ciprofloxacin (Sigma Life-Science). The phage therapy assay was performed as previously described, with the difference that was added an antibiotic control for each antibiotic used and a new test sample, added of phages and antibiotics. The antibiotic control (AC) was inoculated with bacteria and antibiotic at the MIC (according EUCAST 2015) for *E. coli*.. After 0, 2, 4, 6 and 8 h of incubation, aliquots of the test samples and of the controls were collected to quantify bacteria and phages.

It was also performed a short assay, using the ciprofloxacin at MIC (0.5 mg/mL). The samples were collected at 0, 30, 60, 90 and 120 min.

2.2.5. Antimicrobial Susceptibility Tests

The antimicrobial susceptibility tests were done according the EUCAST standards [100].

A bacterial culture with 5 hours of growth at 37 ° C (1.0 x 10^8 CFU / ml) was diluted 1:100 in 0.85% saline solution to obtain a density of 0.5 MacFarland. After that, it was dipped a sterile cotton swab into the suspension and spreaded the inoculum over the entire surface of the plate of Muller-Hinton by swabbing in three directions. Then, the disks with antibiotics (ampicillin, kanamycin, piperacillin, tetracycline, chloramphenicol and ciprofloxacin) (OXOID) were placed at the plate and incubated inverted at 37 ºC for 16 – 20 h. The diameters of inhibition zones were measured and the results were interpreted according information EUCAST (2015).

2.2.6. Determination of the rate of emergence of phage-resistant bacteria

In order to determinate the frequency of phage-resistant bacteria the procedure described at **Figure 7** was used. Ten isolated colonies from a plate with sensitive bacteria were selected and were inoculated into 5 mL of TSB medium, then, it was incubated at 37 ºC for 24 h. One hundred µL of the 10^0 to 10^{-2} dilutions and 100 µL of the phage suspention was plated on TSA by the the double agar layer method. The plates were incubated until colonies of bacteria are seen (3 - 5 days). Simultaneously, it was spread 100 µL of the 10^{-5} to 10^{-7} dilutions on TSA plates without phage. The plates were incubated for 24 h[93]. The previously prepared ten TSB cultures of bacteria were also used to determine the development of phage-resistant bacteria in the presence of antibiotics. An aliquot of 100 µL of culture was added to a new tube of TSB added of antibiotic at MIC. Then it was followed the same procedure (described above) (**Figure 7**) used to determine the development of phage-resistant bacteria in the presence of just phages [95].

The calculation of the frequency of mutants was done by dividing the number of resistant bacteria by the number of sensitive bacteria. This formula was used to calculate the

frequency of antibiotic resistant mutants, phage resistant mutants and the frequency of mutants for the mixture of phage and antibiotic.

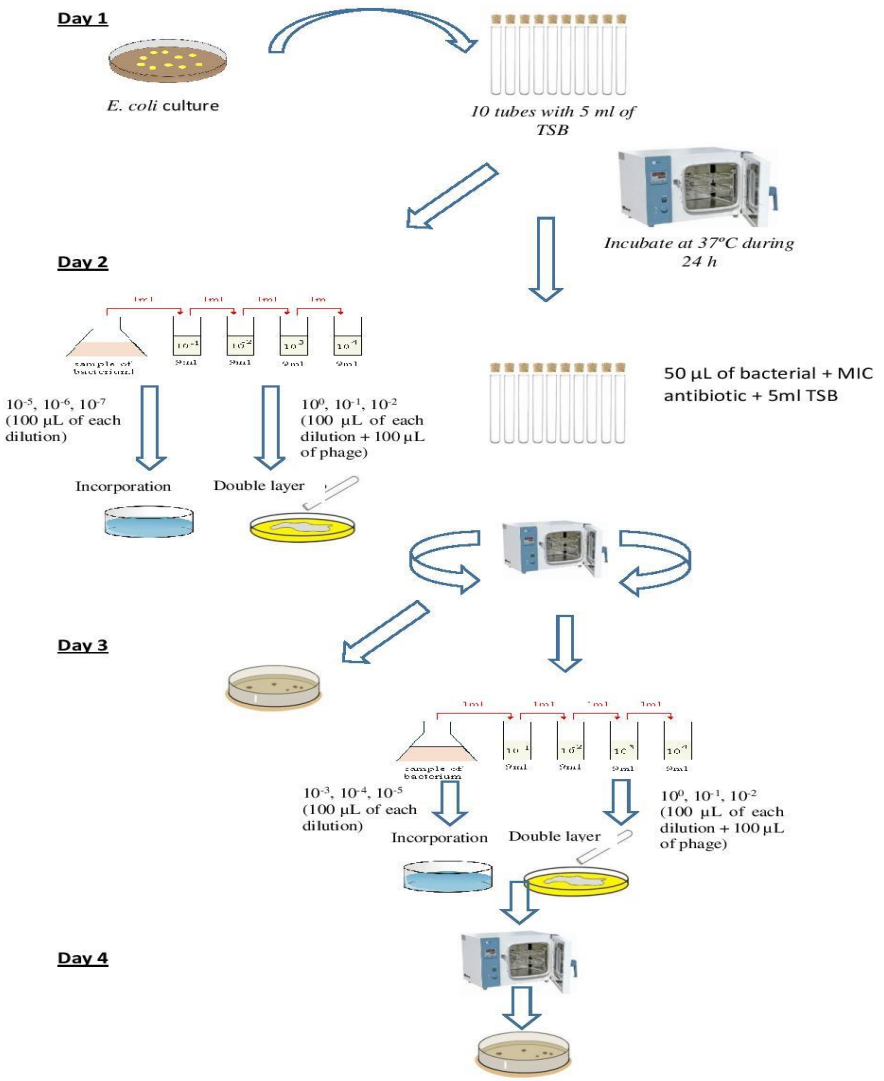


Figure 7: Design of the experimental work to test the emergence of bacterial resistances {adapted from (Haddix 2000) and (Filipov 2011)

2.2.7. Determination of catalase and indol activity

It was selected one colony of a phage-resistant bacteria, grown inside a lysis plaque of a spot test, which was inoculated in TSA medium. Simultaneously, was selected one bacterial colony without phage contact that was used as control. Then, physiological parameters were determined as described below.

Catalase test

The production of enzyme catalase was tested by placing three drops of the hydrogen peroxide (H_2O_2) reagent in microscope slides. After a bacterial colony was picked up with a stick and putted on the H_2O_2 drops. The presence or absence of the oxygen released was checked. The release of oxygen was considered as a positive test for catalase production.

Indole test

The production of indole due to the degradation of the acid tryptophan by tryptophanase enzyme was carriet out by inoculating the bacteria in TSA medium which contains tryptophan after incubation, 0.5 mL of Kovac´s reagent (aqueous solution of p-dimethyl aminobenzaldehyde) were added to the TSA plate. The test was considered positive when a pink color was observed within 5 minutes.

2.2.8. Bacterial kill curves in Urine

Phage therapy experiments in urine were done with phage ECA2 and also with a phage cocktail with the phage ECA2 and other specific phage of *E. coli*, the phage cocktail phT4A/ECA2. Urine samples were provided by the laboratory of clinical analysis Avelab Aveiro (Portugal). Early urine samples were collected, using the Avelab Laboratory protocol, by midstream clean-catch technique after patient daily hygiene. The middle jact was collected directly into the sterile recipient. The urine samples presented a pH of 6, a density of 1.021 and did not contain proteins, epithelial cells or bacteria. The urine was previously centrifuged (13 000 x g, 10 minutes) and filtered through a 0.45 μm-pore-size polycarbonate membrane. Phage inactivation experiments were done at MOI of 100 for both phage ECA2 and phage cocktail phT4A/ECA2. Bacteria and phage controls were included

in all experiments. After 0, 2, 4, 6 and 8 h of incubation aliquots of the test samples and of the controls were collected to quantify bacteria and phages as described above. Three independent assays were done in different dates.

2.2.9. Statistical analysis

The program GraphPad Prism 6.01 was used for data analysis. The significance of differences in bacterial inactivation between the use of phages and the combination of phages and antibiotics was assessed using two-way ANOVA. The comparison between rate of phage-resistant bacteria in the presence and in the absence of antibiotics was assessed using one-way ANOVA. Normal distributions were assessed by the Kolmogorov-Smirnov test. Tukey's multiple comparison test was used for a pairwise comparison of the means. A value of $p < 0.05$ was considered significant.

2.3. Results

2.3.1. Kill curves in PBS

E. coli was challenged with the phage ECA2, in order to test its potencial as a therapeutic agent. The maximum of bacterial inactivation with this phage was 4.6 log CFU/ml at 2 h of treatment and, after 8 h, the bacterial inactivation was still significantly high (2.7 log), relatively to the bacterial control (ANOVA, $p < 0.05$) **(Figure 8A).**

Bacterial density in the BC increased 3.9 log (ANOVA, $p < 0.05$) during the 8 hours of treatment.

No decrease of the phage survival (ANOVA, $p > 0.05$) was observed during the treatment, however, when the phage was incubated in the presence of its host, a significant increase in the phage concentration (1.2 log) was observed after the 8 hours of treatment, compared with the phage control (ANOVA, $p < 0.05$) **(Figure 8 B).**

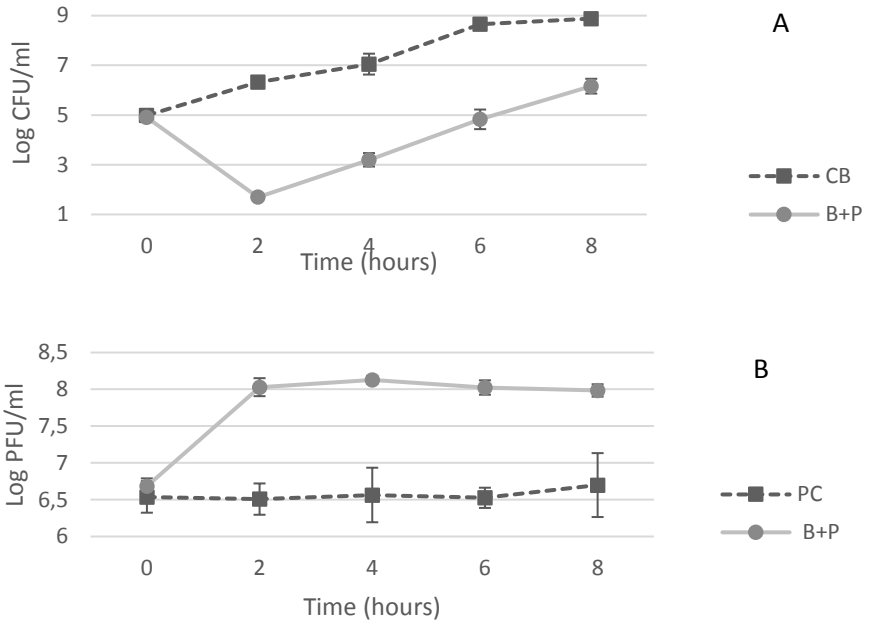


Figure 8: Inactivation of *E. coli* with phage ECA2 in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control; B+P- bacteria plus phage. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage.

2.3.2. Antibiotic test susceptibility

Table 3: Antibiotic test susceptibility to *Escherichia coli* ATCC 13706

Antibiotic	Disk content (µg)	Diameter (mm)	Result
Ampicillin	10	0	Resistant
Kanamycin	30	0	Resistant
Piperacillin	30	0	Resistant
Tetracycline	15	18	Sensitive
Chloramphenicol	30	27	Sensitive
Ciprofloxacin	5	23	Sensitive

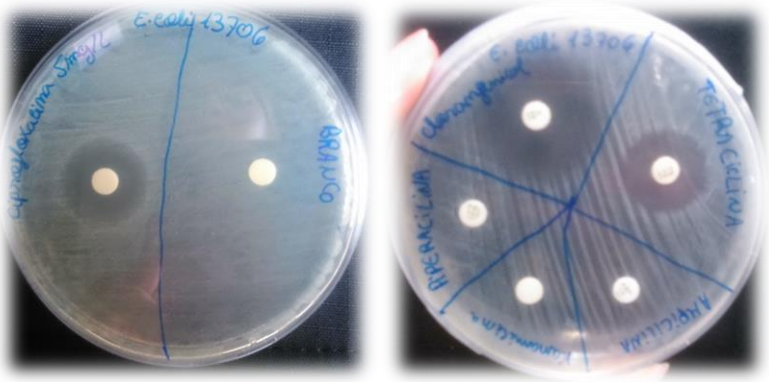


Figure 9: Results of the Antibiotic Susceptibility test of the bacteria *E. coli* ATCC 13706.

The Antibiotic susceptibility test performed with the strain of *E. coli* ATCC 13706 (**Table 3**) reveled that the bacterium is resistant to ampicillin, kanamycin and piperacillin. However, the bacterium showed susceptibility against ciprofloxacin, tetracycline and chlorampenicol.

2.3.3. Kill curves in PBS with antibiotic

2.3.3.1. Ampicillin

Ampicillin was used at a MIC of 32 µg/mL in the sample with phage and antibiotic (B+P+A) and in the antibiotic control (AC).

In the bacterial control cultures (BC and AC), *E. coli* reached a density of 8.9 log CFU/ml after 8 hours of incubation (**Figure 10 A**). However, there was no significant difference of the bacterial growth between the two bacterial controls (ANOVA, p >

0.05). In the presence of the phage, the number of viable bacteria decreased significantly, 4.5 log, at 2 h of treatment (ANOVA, $p < 0.05$). Moreover, in the presence of the phage and ampicillin, the maximum bacterial inactivation was 4.4 log at 2 h of treatment (ANOVA, $p < 0.05$). Although both samples had high bacterial inactivation, it was not observed differences between the bacterial inactivation just with the phage and in the mix of the phage and the ampicillin (ANOVA, $p > 0.05$).

No decrease in the phage survival was observed during the study period (ANOVA, $p > 0.05$). However, in the presence of its host, the phage title increased significantly (ANOVA, $p < 0.05$) for both samples (**Figure 10 B**). On the sample with bacteria and phage, the increase of the phage was 1.4 log, and on the sample with the mix of bacteria phage and antibiotic, the phage increase 1.2 log. Again there was no significantly differences between the results of the increase of the phage number (ANOVA, $p > 0.05$).

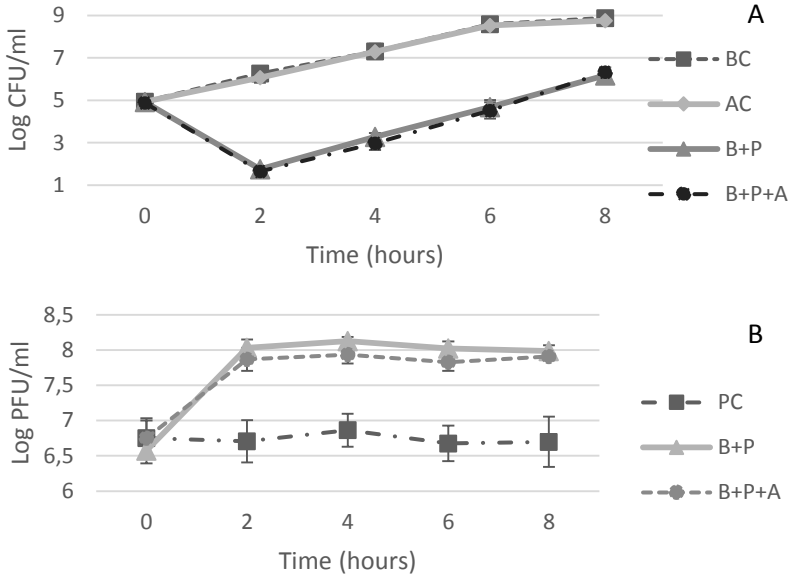


Figure 10: Inactivation of *E. coli* with phage ECA2 and Ampicillin (MIC of 32 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.3.2. Kanamycin

The strain of *E. coli* used as a model for phage therapy was challeged with the phage ECA2 in one sample (B+P) and with the phage and kanamycin at MIC 32 mg/L (B+P+A). The maximum of bacterial inactivation for the sample B+P was 4.3 log CFU/ml at 2 h of treatment and, after 8 h, the bacterial inactivation was still significantly high (≈ 3 log), relatively to the bacterial control (ANOVA, p <0.05). For the sample with antibiotic (B+P+A), the maximum of bacterial inactivation was 4.6 log at 2 h and ≈2.9 log after 8 h of incubation (ANOVA, p <0.05). There was no significantly differences between between the results of bacterial inactivation of the samples B+P and B+P+A (ANOVA, p >0.05) (**Figure 11 A).** The bacterial density increase during all the treatment for both bacterial controls (ANOVA, p <0.05), 3.8 log for the BC and 3.6 for the AC. The results of the bacterial growth of the the bacterial controls were similar (ANOVA, p >0.05).

There were no differences in the phage survival during the study period (ANOVA, p >0.05). By contrast, in the presence of its host, the phage increased 1.1 log in the sample B+P and ≈1 log for the sample B+P+A (ANOVA, p <0.05) (**Figure 11 B**).

There were no significantly differences between the results of the sample with the antibiotic kanamycin than the sample without antibiotic, just with phage (ANOVA, p > 0.05).

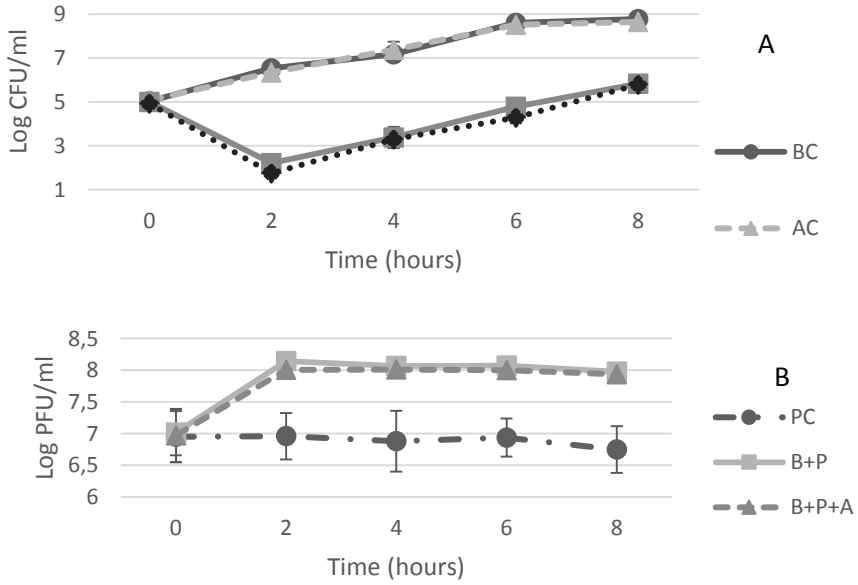


Figure 11: Inactivation of *E. coli* with phage ECA2 and Kanamycin (MIC of 32 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC- phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.3.3. Piperacillin

In the bacterial control cultures (BC and AC), the bacteria increased 3.7 log of its density after 8 hours of incubation (**Figure 12 A**). However, the results of the bacterial growth in the bacterial control without piperacillin and with the presence of antibiotic were similar (ANOVA, $p > 0.05$). In presence of the phage, the number of viable bacteria decreased significantly, ≈ 4 log, at 2 h of treatment (ANOVA, $p < 0.05$). Moreover, in the presence of the phage and piperacillin, the maximum bacterial inactivation was 3.8 log at 2 h of treatment (ANOVA, $p < 0.05$). It was not observed differences between the bacterial inactivation just with the phage and in the mix of the phage and the antibiotic (ANOVA, $p > 0.05$).

No decrease in the phage survival was observed during the study period (ANOVA, $p > 0.05$). However, in the presence of its host, the phage increased significantly (ANOVA, $p < 0.05$) for both samples (**Figure 12 B**). The phage increased ≈ 1 log for the sample B+P and 0.8 log for the sample B+P+A (ANOVA, $p < 0.05$). There were no differences in the results obtained between the samples B+P and B+P+A (ANOVA, $p > 0.05$).

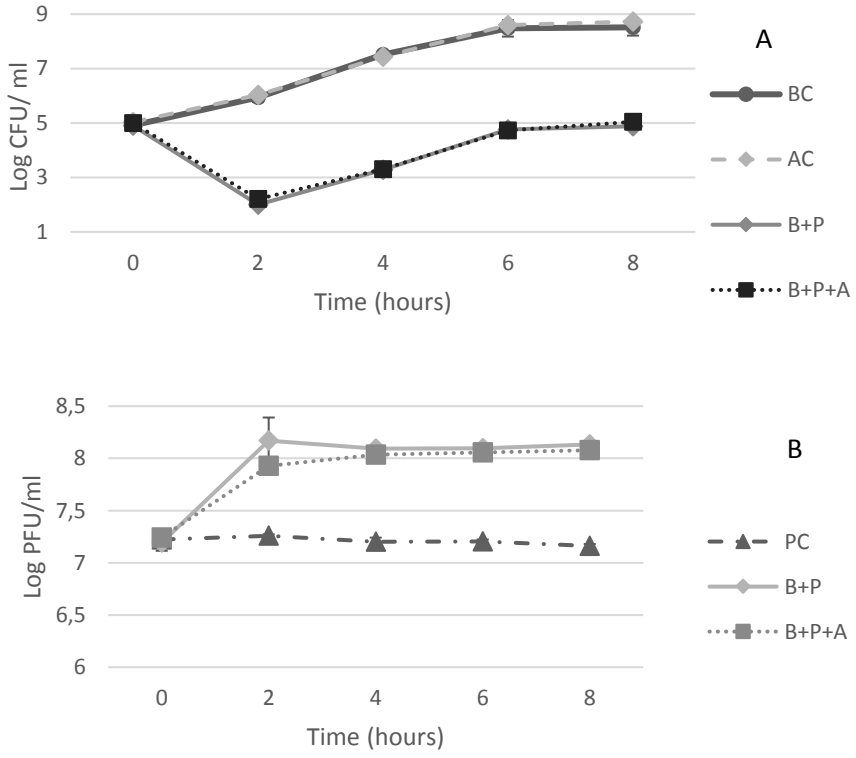


Figure 12: Inactivation of *E. coli* with phage ECA2 and Piperacillin (MIC of 16 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.3.4. Tetracycline

E. coli was challenged with the phage ECA2 (B+P) and with the mix of the phage ECA2 and the antibiotic Tetracycline at a MIC of 4 mg/ml. The maximum bacterial inactivation was observed at 2 h of treatment, for the sample B+P, with a bacterial decrease of 4 log (ANOVA, $p < 0.05$), relatively to the bacterial control. On the other hand, the sample with tetracycline (B+P+A) did not demonstrated alterations in the bacterial density during the study period (ANOVA, $p > 0.05$). Furthermore, the bacterial density of the sample B+P+A and the bacterial density of the antibiotic control had similar results during all the assay (ANOVA, $p > 0.05$) (**Figure 13 A**). The bacterial density of the BC without antibiotic shown an increase of 3.9 log at 8 h of incubation (ANOVA, $p < 0.05$). Those results were different from those obtained in AC (ANOVA, $p < 0.05$).

No decrease was verified on survival of the phage in the PC and those results were similar of those obtained with the sample B+P+A (ANOVA, $p > 0.05$). However, in the sample B+P, an increase of the phage of ≈1 log (ANOVA, $p < 0.05$) was found (**Figure 13 B**).

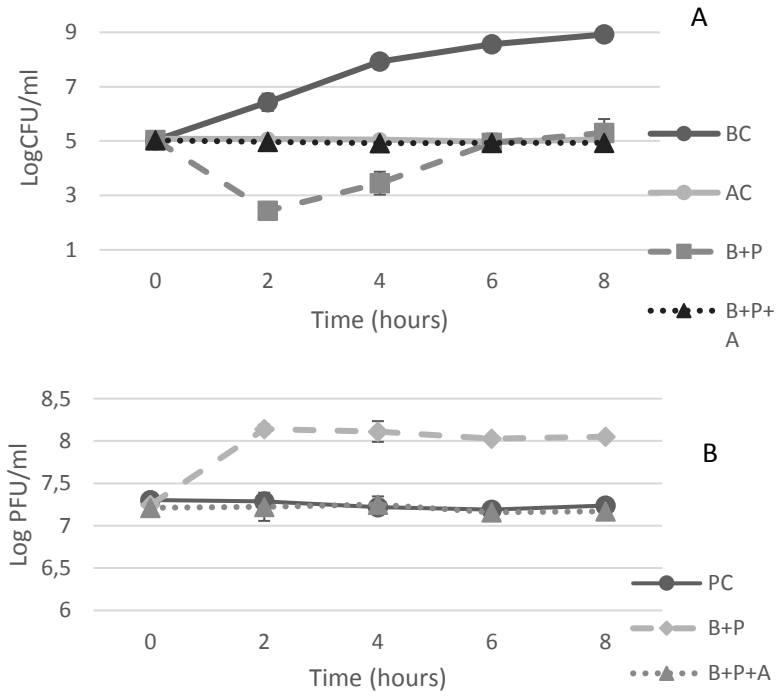


Figure 13: Inactivation of *E. coli* with phage ECA2 and Tetracycline (MIC of 4 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic

2.3.3.5. Chloramphenicol

In the bacterial control, BC, *E. coli* reached a density of 8.8 log CFU/ml after 8 hours of incubation (**Figure 14 A**) (ANOVA $p < 0.05$). That result was significantly different from the obtained from the antibiotic control (ANOVA $p < 0.05$), AC, where the bacterial density does not change during all the study period (ANOVA $p > 0.05$).

The maximum bacterial inactivation was observed at 2 h of treatment, in the sample without chloramphenicol (B+P), with a bacterial decrease of 4.1 log, comparatively to the bacterial control (ANOVA $p < 0.05$). Those bacterial inactivation was not obtained in the sample with antibiotic (B+P+A), where the bacterial density was stable during all the 8 h of treatment (ANOVA $p > 0.05$). The phage control does not demonstrate a decrease in the phage survival 8 h (ANOVA $p > 0.05$) and that result was similar to that obtained in the B+P+A (ANOVA $p > 0.05$). On the other hand, the sample B+P had an increase of ≈1 log in the phage title (ANOVA $p < 0.05$) (**Figure 14 B**). The results from both bacteria and phage concentrations were different between the two samples during all the treatment (ANOVA $p < 0.05$).

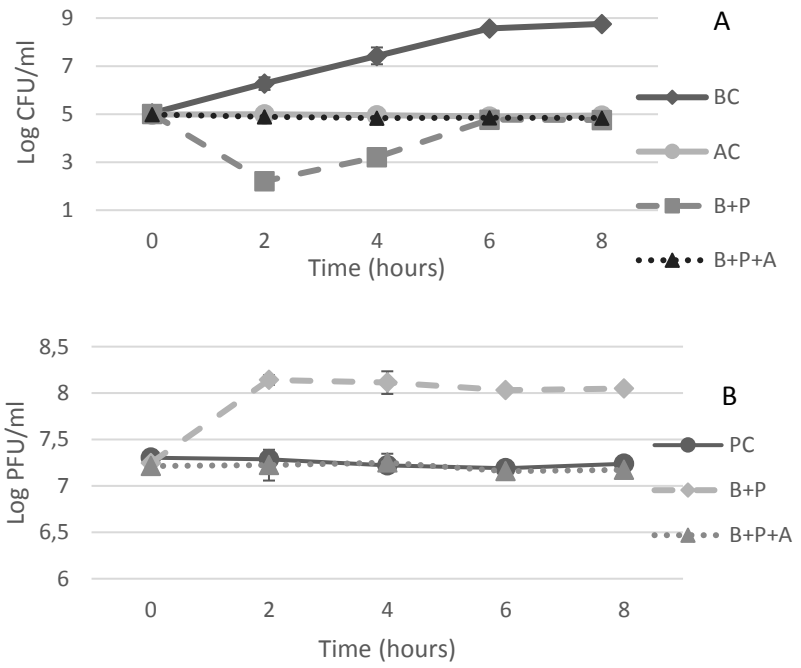
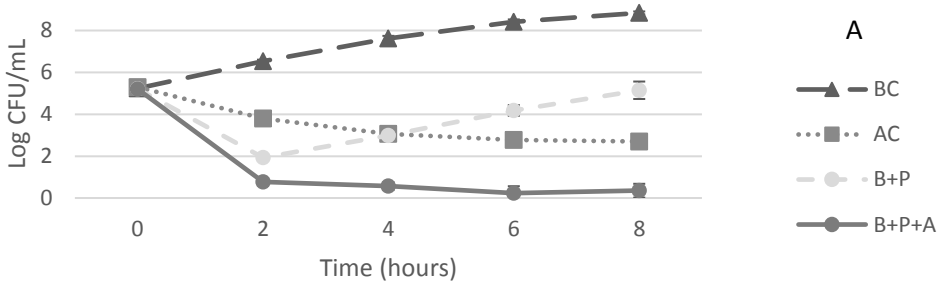


Figure 14: Inactivation of *E. coli* with phage ECA2 and Chloramphenicol (MIC of 8 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.3.6. Ciprofloxacin

In the bacterial control culture (BC), *E. coli* reached a density of 8.9 log CFU/ml after 8 hours of incubation (**Figure 15 A**) and in the antibiotic control (AC) the bacterial density decreased 2.7 log after the 8 hours of treatment. It was shown a significant diference on the bacterial growth between the two bacterial controls (ANOVA, $p < 0.05$). In presence of the phage, the number of viable bacteria decreased significantly, 4.6 log, at 2 h of treatment (ANOVA, $p < 0.05$). Moreover, in the presence of the phage and ciprofloxacin, it was verified a maximum bacterial inactivation of 8.2 log after 6 h, relatively to the bacterial control (ANOVA, $p < 0.05$). Even though, the sample B+P+A shown a maximum bacterial inactivation, after 2 h of treatment, the sample already had a bacterial inactivation of 5.7 log (ANOVA, $p < 0.05$). Although both samples had high bacterial inactivation, the conjugation of the phage with ciprofloxacin increased the antimicrobial effect against *E. coli* (ANOVA, $p < 0.05$). Additionally, there was a decrease on the bacterial survival after the treatment between the sample with phage and ciprofloxacin and the sample with the phage alone (ANOVA, $p < 0.05$).

No decrease in the phage survival was observed during the study period (ANOVA, $p > 0.05$). However, in the presence of its host, the phage title increased significantly (ANOVA, $p < 0.05$) for both samples (**Figure 15 B**). On the sample with bacteria and phage, the increase of the phage was 0.8 log, and on the sample with the mix of bacteria phage and antibiotic, the phage increase 0.7 log. There was no significant differences between the phage number values between the two samples (ANOVA, $p > 0.05$).



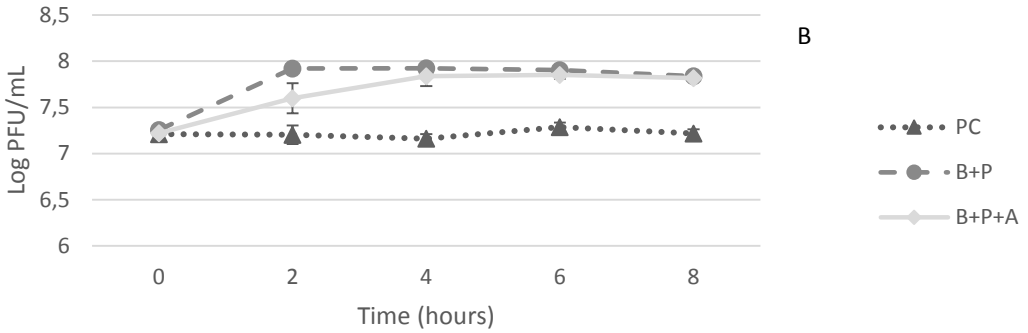


Figure 15: Inactivation of *E. coli* with phage ECA2 and Ciprofloxacin (MIC of 0.05 mg/L) in PBS during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

When the phage was tested with the antibiotic at MIC (0.5 mg/L), the bacterium was inactivated completely after 2 h, reaching an inactivation similar to that of the antibiotic alone after 2 h. Therefore, a short experiment of 2 h was done. The results show that density of the bacterial control (BC) increase during by 1.5 log CFU during the 120 min (**Figure 16 A**). For in the antibiotic control (AC) the bacterial density decreased 4.9 log after the 120 mins of treatment, relatively to the BC. A significant increase in the bacterial growth between the two bacterial controls (ANOVA, $p < 0.05$). In the presence of the phage, the bacterial density decreased 4.7 log after 120 min, but a significant decrease was observed after 90 min. The control of antibiotic and the sample with the mix of phage and ciprofloxacin did not show significant differences in the bacterial density among the assay (ANOVA, $p > 0.05$).

No decrease in the phage survival was observed during the study period (ANOVA, $p > 0.05$) (**Figure 16** B). However, in the sample B+P, the phage increased 0.9 log after the 2 h. The increase was already observed at 90 min, the phage density was os 0.6 log. These results were significantly different from those obtained in the sample B+P+A (ANOVA, $p > 0.05$), which was stable during the 120 min period assay.

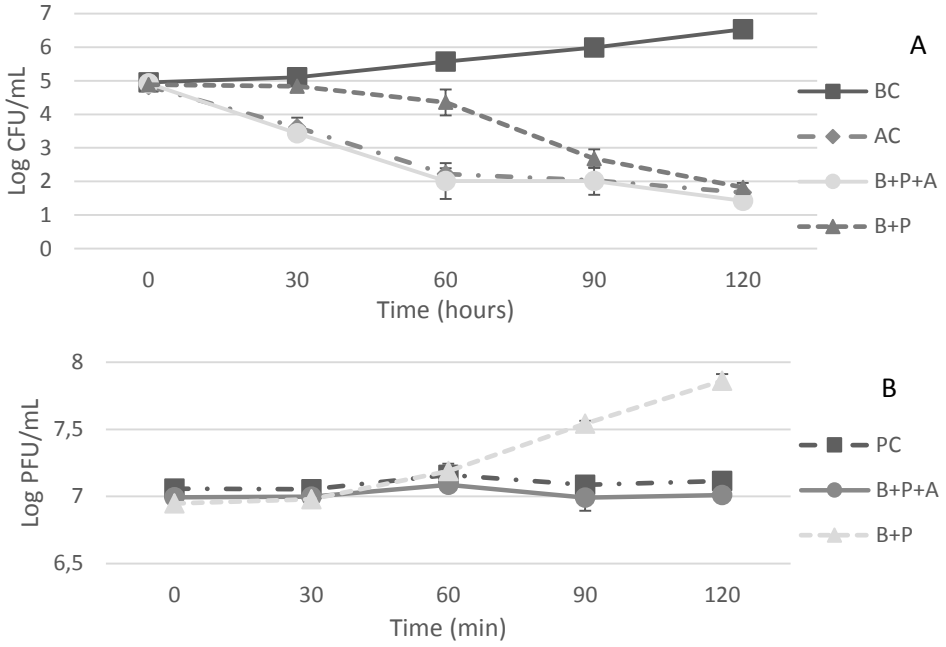


Figure 16: Inactivation of *E. coli* with phage ECA2 and Ciprofloxacin (MIC of 0.5 mg/L) in PBS during 120 min. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.4. Kill curves in Urine

In order to test the properties of phages in the clinically relevant setting, the killing assays were repeated in urine. It was also tested a cocktail with two phages (ECA2/phT4A) to verify if the bacterial inactivation increase when compared with the inactivation using the single phage ECA2 suspension. The results of bacterial inactivation obtained with the phage ECA2 were significantly higher than the results obtained with the phage cocktail (ANOVA, $p < 0.05$). The maximum bacterial inactivation was 4.3 log for the phage ECA2 and 3.5 log for the phage cocktail, both after 4 hours of treatment (ANOVA, $p < 0.05$) Although, after 2 hours of incubation, the results of the bacterial inactivation between the phage ECA2 and cocktail were similar ($\approx$1.4 log) (**Figure 17 A**). These results were different from those obtained in PBS, because in PBS the maximum amount of bacterial inactivation occurred at 2 hours of treatment with about 4.6 log (ANOVA, $p < 0.05$). Also, the bacterial density in the BC increased 2.3 log (ANOVA, $p < 0.05$) during the 8 hours of treatment.

The phage survival was constant during study period for both phage controls, the single phage and the cocktail and the results obtained between them were similar (ANOVA, $p > 0.05$). In the presence of the host, the phage increase significantly, by 1 log and 0.7 log to the phage ECA2 and the phage cocktail respectively (ANOVA, $p < 0.05$). These results were not different between phages throughout the entire treatment (ANOVA, $p > 0.05$) (**Figure 17 B**).

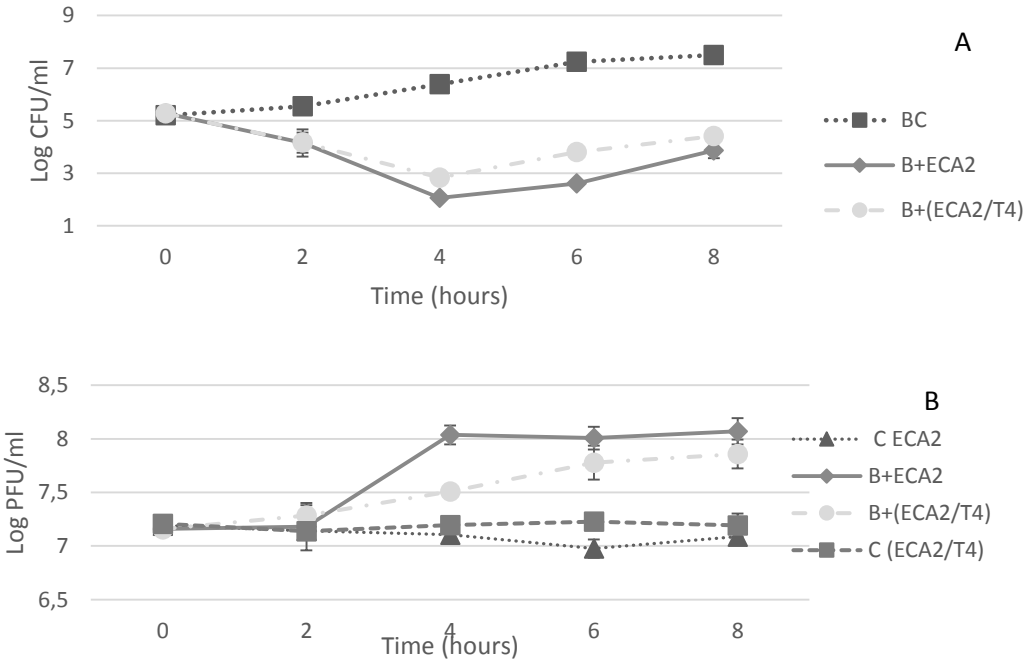


Figure 17: Inactivation of *E. coli* with phage ECA2 and the cocktail of two phages ECA2/T4 in human urine during 8 hours. (A)- Bacterial concentration: BC- bacterial control; B+ECA2- bacteria plus phage ECA2, B+(ECA2/T4)- bacteria plus cocktail (ECA27T4). (B)- Phage concentration: C ECA2-phage control; C(ECA2/T4)- cocktail control; B+ECA2 bacteria plus phage ECA2; B+(ECA2/T4)- bacteria plus phage cocktail

2.3.5. Kill curves with ciprofloxacin in Urine

It was selected the antibiotic ciprofloxacin to be used in conjugation with the phage ECA2 in urine, due to the best results obtained in PBS. The best results of bacterial inactivation were obtained with the sample B+P+A, for which a bacterial inactivation of ≈8 log was observed after 6 h of treatment. Even though, after 4 h of treatment, the bacterial density was already low (0.15 log). The reduction was significantly different between the samples B+P and B+P+A (ANOVA, $p < 0.05$) (**Figure 18 A**). For the sample with the phage

(B+P) a bacterial decrease of 5.2 log, relatively to the bacterial control (ANOVA, $p < 0.05$) was observed. These results were similar to those obtained in PBS (ANOVA, $p > 0.05$).

The bacterial control (BC) showed a bacterial increase of 2.9 log and the antibiotic control had a bacterial increase of 1.4 log during the 8 hours assay. Those results of the bacterial controls were significantly different (ANOVA, $p < 0.05$). However, the results obtained in the antibiotic control were significantly different from those obtained in PBS (ANOVA, $p > 0.05$).

The phage survival was constant during study period for phage control (ANOVA, $p > 0.05$). In the presence of the host, the phage increase significantly, by 0.6 log and 0.7 log for the sample B+P+A and the sample B+P, respectively (ANOVA, $p < 0.05$). These results were not different throughout the entire treatment (ANOVA, $p > 0.05$) (**Figure 18 B**).

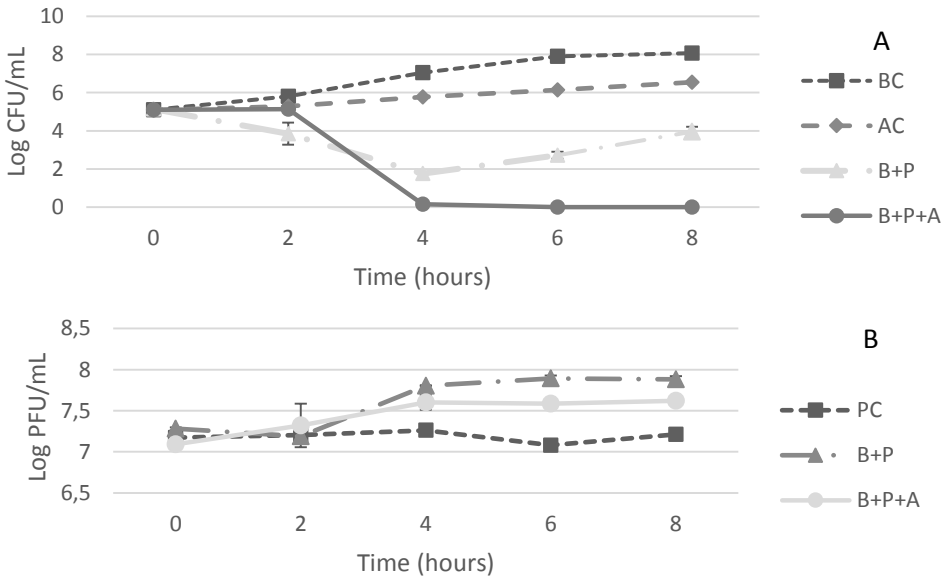


Figure 18: Inactivation of E. coli with phage ECA2 and Ciprofloxacin (MIC of 0.05 mg/L) in Urine during 8 hours. (A)- Bacterial concentration: BC- bacterial control AC- Antibiotic control; B+P- bacteria plus phage, B+P+A- bacteria plus phage plus antibiotic. (B)- Phage concentration: PC-phage control; B+P bacteria plus phage; B+P+A- bacteria plus phage plus antibiotic.

2.3.6. Determination of the emergence of bacterial mutants

It was tested the development of phage resistant mutants of *E. coli* against the phage ECA2, using antibiotics for which the bacterium was initially resistant (ampicillin, kanamycin and piperacillin) and for the antibiotic for which the bacterium was sensible, ciprofloxacin.

The bacterium showed different rates of phage-resistant mutants for the phage ECA2 and for the mixture of the phage with antibiotics (**Table 4**), but these differences were not significant (ANOVA, $p > 0.05$) for the antibiotics ampicillin, kanamycin and piperacillin.

However, for the mixture of ECA2 and ciprofloxacin a significant decrease in the phage-resistant mutants, 0.9 log, relatively to the sample with ECA2 (ANOVA, $p < 0.05$) was observed.

The samples with ampicillin, kanamycin and piperacillin without phage contact, showed a frequency of mutants against those antibiotics of ≈1 (ANOVA, $p > 0.05$). In contrast, the results of the bacterial resistance to ciprofloxacin were significant lower to those of the bacteria resistance to the phage. That difference was about 0.8 log (ANOVA, $p < 0.05$).

Also the phage-resistant bacterial colonies were smaller than those of the control without added phage and have grown slower, during 4 days of incubation. In the control, colonies were visible after 24 h of incubation in similar conditions. The samples were tested in TSB medium and urine and the results obtained were similar (ANOVA, $p > 0.05$).

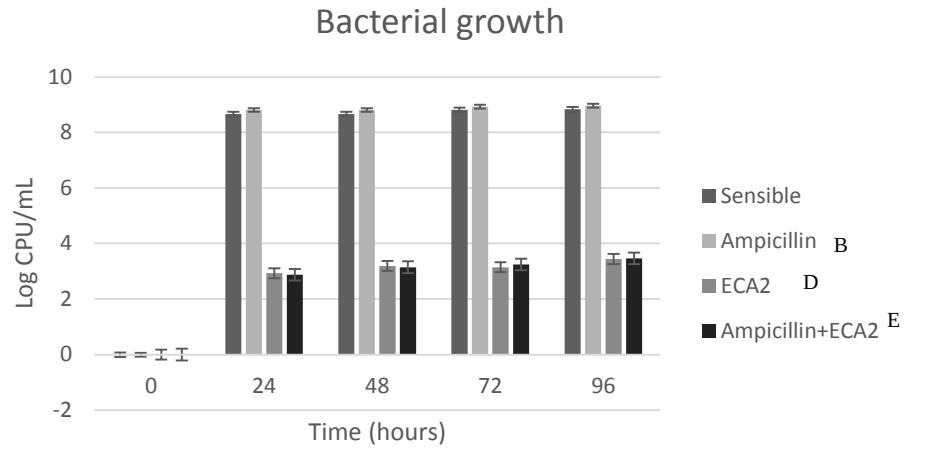
Table 4: Frequency of mutants of *Escherichia coli* ATCC 13706 to the phage ECA2, antibibiotics and to the conjugation of both.

Sample	Frequency of antibiotic-mutans (CFU/ml)	Sample	Frequency of phage-mutans (CFU/ml)	Sample	Frequency of phage and antibiotic mutans (CFU/ml)
Ampicillin	1.023158±0.24	Phage ECA2	$5.02402±1.47x\ 10^{-6}$	Ampicillin + ECA2	$1.17±1.03x\ 10^{-5}$
Kanamycin	1.012104±0.27	Phage ECA2	$5.02402±1.47x\ 10^{-6}$	Kanamycin +EC2	$1.4±1.13x\ 10^{-5}$
Piperacillin	1.181003±0.39	Phage ECA2	$5.02402±1.47x\ 10^{-6}$	Piperacillin +EC2	$2.3±1.1x\ 10^{-5}$
Ciprofloxacin	$2.745± 0.16x10^{-4}$	Phage ECA2	$5.02402±1.47x\ 10^{-6}$	Ciprofloxacin +ECA2	$2.4±1.5x10^{-7}$

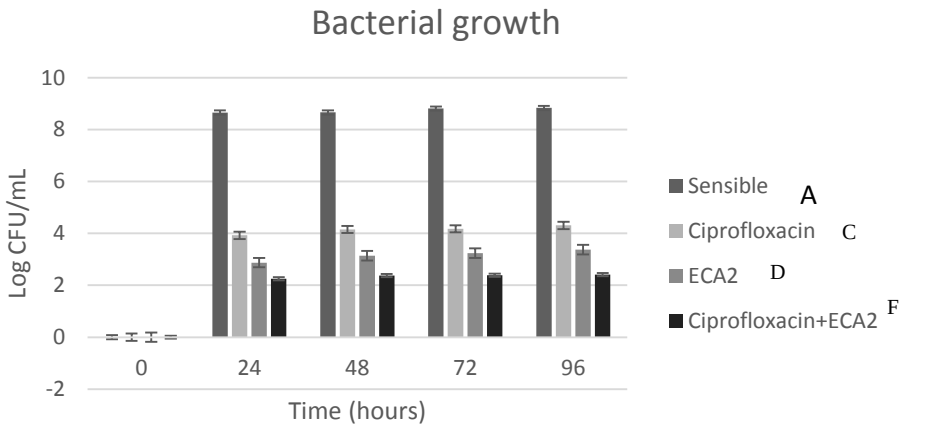
2.3.7. Growth of bacterial resistant mutants

The results obtained for bacterial growth without phage and ampicillin (sensible bacteria), bacteria resistant to ampicillin, bacteria resistant to the phage ECA2 and bacterial resistant to the mixture of ampicillin and ECA2 was determined during 96 hours of incubation (**Figure 19 I)**. In the samples with the bacteria without the phage and antibiotic and bacteria resistant to ampicillin the number after 24 h was similar , ≈9 log, and was stable during the 4 days. On the other hand, the samples with phage showed a lower number, ≈3.5log, which grew slower then those observed in samples without phages. For kanamycin and piperacillin, a similar profile of variation was observed (data not shown).

When, ciprofloxacin was tested, the sample with antibiotic showed a higher number of bacterial resistant mutants than sample with the phage ECA2 after 24 h. After 24 h, the number of bacterial resistant mutants to ciprofloxacin does not changed during the next 3 days, the number of bacterial resistant to the phage changed during the following 3 days (**Figure 19 II**). For samples with ciprofloxacin and phage ECA2 also an increase of the CFU/mL was observed during the incubation period but the increase was significantly lower than that of the bacteria which was grown in the presence of the phage and of the bacteria alone.



(I)



(II)

Figure 19: Bacterial concentration of resistant mutants to phage, antibiotics and the misture of phage and antibiotic and of sensitive bacteria during 96 h of incubation.
(A)- sensitive bacteria; (B)-bacteria resistant to ampicillin 32 µg/mL; (C)-bacteria resistant to ciprofloxacin 0.05 µg/mL; (D)- bacteria resistant to phage ECA2; (E)- bacteria resistant to phage ECA2 plus ampicillin 32 µg/mL; (F)-bacteria resistant to phage ECA2 plus ciprofloxacin 0.05 µg/mL.

2.3.8. Physiological characteristics of *E. coli* before and after phage treatment.

The results of the catalase and indol tests after phage treatment were both positive as the results for the cultures without phage treatment.

E. coli before treatment with the phage was resistant ampicillin (0 mm), kanamycin (0 mm) and piperacillin (0 mm) and sensitive to ciprofloxacin (23 mm). This pattern of variation does not changed after phage addition both in PBS and in urine.

2.4. Discussion

The emergence of antibiotic resistant bacterial pathogens is a serious concern worldwide and has directed more research interest to alternative therapies. A promising alternative to antibiotics is the use of bacteriophages. Nowadays, several controlled studies conducted *in vitro* [94,95,101,102], *ex vivo* [104,105] and in animal models [96,106–110]; have demonstrated that phage therapy has great potential to control infections caused by bacteria [95,110]. However, although these studies show support for the efficacy of phage therapy, the development of phage-resistant mutants is a general shortcoming [95,97,97,111,112].

This shortcoming can be, however, overcome by the use of phage cocktails [14,45,48,105,113–116]. Moreover, recent works suggest that positive interactions have been observed between lytic phages and antibiotics in controlling bacterial pathogens both *in vitro* and *in vivo* [87–89,117,118]. However, little is known about the interactions process of phages and antibiotics in combined therapies namely when 1) bacteria is resistant to antibiotics, 2) the antibiotic is bacteriostatic, 3) the effects of antibiotic doses and 4) how compare the emergence of phage-resistant and antibiotic-resistant mutants, aspects that we have tried to lighten in this study.

The results of this study showed that 1) phage and antibiotic combinations could result in high synergistic effect in the inactivation of bacteria, even when the antibiotic is used at sublethal doses (below MIC), but 2) the efficacy of the combination depends on the antibiotic resistance status of the targeted bacteria to the employed antibiotic and of the antibiotic type (bactericide or bacteriostatic), 3) causing the same or less resistance than phages and antibiotics applied alone and that 4) the development of resistance to phages is lower than that observed relatively to antibiotics.

Combination therapies are already used in the treatment of diverse microbial illnesses such as tuberculosis, malaria or AIDS. For the successful of these combined therapies it is essential that each agent has different bacterial targets, such as in the case of phages and antibiotics associations, contributing thus to an increased inactivation. In this study, bacterial density reduction by the combined action of the tested phage and antibiotic was higher than that caused either by phage and antibiotic applied individually but only when the bacterium was sensitive to a bactericidal antibiotic, the ciprofloxacin. The maximal inactivation of *E. coli* by the combined action of the phages and the ciprofloxacin at a concentration 10 times

lower than MIC (0.05 µg mL^{-1}) was of 8.2 log after 6 h, but the bacterial reduction by the phage and antibiotic applied individually was 4.6 and 2.7 log, respectively, after 2 and 8 h of treatment. The *E. coli* inactivation by the mixture after 2 h was already of 5.7 log. When the phage was tested with the antibiotic at MIC, the bacterium was inactivated efficiently after 1 h, reaching an inactivation similar to that of the antibiotic alone after the same time. However, when phages are used alone, after 1.5 h of treatment it was already possible to detect phage particles in the sample (phage concentration at 3.3 x 10^7 PFU/mL at 1.5h), but when the phage is used with ciprofloxacin, the increase in phage particles occurred only after 2 h (phage concentration: 7.8 x 10^6 PFU/mL at 1.5 h). At MIC, the ciprofloxacin affected the protein synthesis, which avoid to the replication of the phage by the host bacteria. Consequently, no synergistic effect can occurred at MIC. Our *in vitro* experimental results with ciprofloxacin and phages confirm previous findings [90,119] showing that sublethal doses of antibiotics produce a phage-antibiotic synergistic effect, reducing the bacterial number but only when bacteria are sensitive to the used antibiotics. The same results were observed when the ciprofloxacin at MIC was tested. Besides, the combination of antibiotics and phages could also reduce the doses of phages. It has been shown that low multiplicities of phage infection (MOI) can be effective to inactivate bacteria *in vitro* and *in vivo* [120,121]. So, it would be important to test the effect of the use of the combined effect of antibiotics and phages at different MOI. Decreasing both antibiotic and phage concentrations would decrease the release of bacterial toxins after lysis, namely in case of Gram negative bacterial inactivation, reducing the frequency of septic shock situations.

The increase in bacterial inactivation was not observed for the combined therapy when the tested antibiotic was bacteriostatic or when the bacterium was resistant to the tested antibiotics independently of their mechanisms of action/bacterial target sites. Thus, our results are not in accordance with previous studies in which is mentioned that the synergistic effects of phages and antibiotics are relatively insensitive to antibiotic type [122]. This profile was only observed when the bacterium was resistant to the tested antibiotic.

The *E. coli* strain tested in this study was also sensitive to the tetracycline and chloramphenicol, which are bacteriostatic antibiotics. However, with these two antibiotics no increase in bacterial inactivation was observed when they were used in combination with phages. These antibiotics only avoid bacterial growth, not causing bacterial reduction, avoiding also phage replication. Both antibiotics inhibit the bacterial protein synthesis

preventing viral replication by the host. However, a study with tetracycline and a phage showed a synergistic effect in the inactivation of *Burkholderia cepacia* [123]. Tetracycline was used at 2.5 mg/L to 40 mg/L, showing synergistic effect in bacterial inactivation at ½ MIC and an increase in the number of produced phages at 2X and 4X MIC (MIC of 10 mg/L for *Burkholderi*a strain K56-2). In our study, tetracycline was tested at MIC concentration for *E. coli* (4 mg/L). These differences suggest that the synergistic effect can vary with the bacteria. Further studies using different bacteria are necessary to confirm this hypothesis. Similarly, linezolid applied with phage caused also a higher reduction than the antibacterial alone on *Staphylococcus aureus* density [124]. The linezolid was applied at 25 mg/kg, a concentration higher [125] than MIC (4 mg/L) and in different conditions than those used in our study, in vivo inactivation of a Gram positive bacterium.

When phages were tested in the presence of antibiotics for which the *E. coli* was resistant, no synergistic effect of the combined therapy was observed. For the bactericidal antibiotics piperacillin and ampicillin, drugs that affect the cell wall synthesis, and for the bactericidal antibiotic kanamycin, which inhibits the protein synthesis, the inactivation was similar to that observed when the phage was used alone. Also, for the three antibiotics the combined therapy the number of produced phages was also similar to that when phages were tested individually. This means that when bacteria are resistant to antibiotics, independently of the mechanism of action of the antibiotic, and consequently, independently of the affected targeted bacterial site, no synergistic effect of the two treatments decrease the bacterial density. To the best of our knowledge there are no other studies using the combination of phages with antibiotics for which bacteria are resistant. However, there are some studies showing that some bacteria, such as the *Burkholderia cepacia* complex, which possess high levels of innate antimicrobial resistance, when treated with the combination of phages and antibiotics for which these bacterial species are sensitive, increase the reduction of bacterial density when compared with that using phages or antibiotics alone [123]. Overall, although antibiotics belonging to different classes and having different mechanisms of action cause a synergistic effect on bacterial inactivation when combined with phages [126], if the bacteria are resistant to the used antibiotics no synergistic effect is observed.

Several studies have reported that the synergistic effect of phages and antibiotics may be due to the stimulation of bacterial production of phages by the antibiotic [90,117,119,123,127]. However, in this study, when the combination of phages and

antibiotic ciprofloxacin, at a concentration 10 x lower than MIC, was used to inactivate the *E. coli*, the number of phages produced was similar to that observed when phages were tested without antibiotic. Further studies, using different antibiotic concentrations and different bacteria and even other phages, are necessary to clarify the reason for this different behavior.

It has been shown that the combination of antibiotics with phages is an alternative that cannot only be effective at reducing bacterial numbers but also to contribute to managing resistance levels. However, this view has only been discussed with regard to antibiotic resistance and not to control phage-mutant emergence. In our study we compared not only the resistance of the bacteria to the four antibiotics tested with and without phages, but also the resistance to the phages in the presence and in the absence of antibiotics. Relatively to the antibiotics, and as already indicated by some authors the results of this study showed that for the four antibiotics tested, independently of their type and mechanism of action, the phages limit the emergence of antibiotic resistant variants in combined treatments. The resistance to the four antibiotics was lower than that observed for the combination of phage and antibiotic (range 1.023158 to 2.745 x 10^{-4} CFU/mL without phages and range 2.3 x 10^{-5} to 2.4 x 10^{-7} with phages). Although, some authors detected that phage-antibiotic combinations can cause the same resistance than either antimicrobial introduced in isolation to our knowledge none study indicated that resistance to antibiotics in the presence of phages was higher than that developed without phages[87,88,122,128]. Thus, contrary to the suggestion that double-resistant bacteria would be strongly selected when antibiotic cocktails is used [129], antibiotic and phage combinations indicate the opposite effect.

Additionally, in this study, the resistance to the antibiotics alone was higher than that observed when the phages were used alone (1.023158 to 2.745 x 10^{-4} CFU/mL for antibiotic and range 5.02402 x 10^{-6} CFU/mL for phages). This means that although some phage-mutants can emerge after phage treatment, the frequency of resistance would be lower than that caused by the conventional antibiotic worldwide used. However, more studies using other bacteria and phages are needed to confirm this pattern of resistance development. These results confirm the findings of other authors, like Verma et al (2009), which compared the resistance to antibiotics in the presence of phages with that observed with phages and without antibiotics[87].

Our findings showed also that the addition of antibiotics during phage treatment can also control the phage-mutant emergence. When ciprofloxacin was added at a sublethal

concentration (concentration 10x lower than MIC) together with the phage, the rate of emergence of phage-mutants was lower than that observed when the antibiotic was not used. However, this reduction in the phage-mutants development was not detected for the other three antibiotics tested with the phage. For these, the emergency of phage-mutants was similar to that observed without antibiotic addition. Overall, the resistance of phage-mutants in the presence of antibiotics was the same or less than when phages were tested alone. This conclusion was confirmed by the antibiogram results obtained after treatment with the combination of phages and antibiotics and with phages alone. After treatment with the combination and with the phage alone, the bacterium was sensitive to the ciprofloxaxin, tetraciclin and chlorophenicol but continue resistant to the ampicillin, kanamycin and piperacillin.

Our results are in accordance with the suggestion that simultaneous resistance to antibiotics and phages entails larger costs than to either antibacterial agent separately. In fact, previous results of our group have shown that phage-resistant mutants after successive cultivation in culture medium change the pattern of resistance to the phage. After 3-5 successive streak-plating steps of phage-mutants isolates on solid medium, a clear lysis plaques can be observed after the spot test for different sets of bacteria and phages [95–97,110] However, when the efficiency of infection is determined, using the double agar layer method, for these phage-resistant mutants no increase in the efficiency of infection was detected. Similarly, when infrared spectroscopy was used to study the surface of the these phage-resistant mutants relatively to sensitive cells to the phages, spectral differences between resistant mutants and the sensitive cells were clear, being the differences more relevant for peaks associated to amide I and amide II from proteins [95]. It is a possibility that the proteins present in the external surface of bacterial resistant cells, which can be used as receptors for phages, are someway modified, thus leading to the observed resistance. Nonetheless, colonies of phage-resistant mutants were smaller than colonies formed by the non-phage added control and were visible only after 3 days of incubation. In the non-phage added control, bacterial colonies were visible after 24 hour of incubation. These results suggest that the remaining bacterial mutants (forming small size colonies and showing slow growth) maintained their viability in the presence of phages but their phenotypes were affected. Similar results were already observed in other studies [91,92,97]. These decrease in the bacterial growth after phage exposure could be fitness cost which can contribute to

their elimination from the environment faster than their wild-type parents. However, in this study the two physiological bacterial characteristics, catalase and indole after phage treatment were similar to those of non-treated bacteria. Further studies using other bacterial characteristics are needed to understand the interaction phage-bacteria and, consequently, to evaluate the fitness of the phage-resistant mutants.

In order to test if phage therapy could be used to control UTI caused by *E. coli*, a bacterium responsible for more than half of the UTI worldwide [10], the phage ECA2 and a phage cocktails with other phage specific for *E. coli* (the phage phT4A), the effectiveness of phage treatment was also tested in urine samples. The phage ECA2 alone and in the cocktail was effective to inactivate *E. coli* in urine. The phages survived in the urine, maintained their concentration in the absence of the host, and significantly increased their titer in the presence of the bacterium during the study period. Phages effectiveness to inactivate the *E. coli* in urine was similar to that observed in the experiment in PBS, 5 log against to 4.6 log after, but the maximal bacterial inactivation occurred later, after 4 h, than in PBS (maximal inactivation at 2 h of treatment). When phage therapy was done in the presence of ciprofloxacin at sublethal concentration, a synergistic effect, as observed in PBS, was detected, and the bacterium was inactivated to the detection limit of the method (reduction of 8 log) after 6 h of treatment. Also, after 4 h of treatment, almost all bacteria were inactivated. When the antibiotic was tested alone, the bacterial inactivation was lower than in PBS. This can be due to the degradation of the ciprofloxacin at low pH values. In fact, the FDA (2014) [130] showed the ciprofloxacin is not stable at low values of pH. However, in the presence of the phages, this effect was not observed, which suggest that ciprofloxacin when used in combination with phages could be used at lower concentrations.

The high bacterial inactivation efficiency with phages combined with a higher inactivation in the presence of antibiotic at a sublethal concentration (inactivation to the detection limit of the method) and the long periods of phage survival, even in urine samples, paves the way for depth studies, especially *in vivo* studies, to control urinary tract infection and to overcome the development of resistances by *E. coli*, the bacterium most frequently isolated in UTI at the community level and at hospital settings. Although the viability of the phages was not affected by the low pH of urine, the stability of the antibiotic was depressed, even though the efficiency of the combined phage-antibiotic therapy has not been affected,

the results of this study highlight the importance of testing the efficacy of new approaches to inactivate bacteria in clinically relevant setting.

2.5. Future perpectives

After this work, it will be interesting to evaluate:

- The synergistic effect between the phage and other antibiotics which the bacteria is sensible (test MIC and subletal doses of antibiotic);
- Isolate bacteria from UTI patients and apply phage therapy;
- Test the synergism phage-antibiotics for other bacteria and phages;
- Evaluate the synergistic mechanisms between phages and antibiotics;
- Understand the resistance mechanisms of phages;
- Evaluate the effect of the mix of phage therapy with antibiotics on the virulence factors of bacteria;
- Apply phage therapy with antibiotics in animal models, like mice and *Galleri mellonella*.

Chapter 3 - References

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