Universidade de Aveiro Departamento de Biologia
2018

Ana Sofia Correia Lopes

Combined inativation of *Escherichia coli* by phages and antibiotics

Inativação combinada de *Escherichia coli* por fagos e antibióticos

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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 a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, e Doutora Carla Sofia Gomes Pereira (coorientadora), investigadora de Pós-doutoramento do Departamento de Química da Universidade de Aveiro.

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palavras-chave Terapia fágica, resistência a antibióticos, *Escherichia coli*, ciprofloxacina

resumo

Escherichia coli, é uma bactéria comensal não patogénica, mas que pode desenvolver variantes mais virulentas que podem colonizar outros locais que não o sistema gastrointestinal. Esta bactéria é uma das principais responsáveis por infeções moderadas e graves no ambiente hospitalar e na comunidade, estando envolvida na emergência e disseminação de resistência às fluoroquinolonas e cefalosporinas de terceira geração. Estima-se que nos próximos anos o número de mortes causadas pela resistência, mesmo em bactérias comensais, será muito alto, fazendo com que seja muito urgente encontrar alternativas para combater as bactérias patogénicas. Uma abordagem alternativa ou suplementar aos antibióticos é a terapia fágica, que utiliza bacteriófagos (fagos) líticos capazes de infetar apenas células procariotas. Vários estudos já mostraram efeitos encorajadores ao usar a terapia fágica, no entanto, mutantes resistentes a fagos têm sido considerados uma grande preocupação, quando se pretende usar este método para inativar bactérias patogénicas. A resistência pode, no entanto, ser superada com a combinação de fagos e antibióticos, o que impede o crescimento de bactérias resistentes e pode ter melhores resultados do que quando estas terapias são usadas separadamente. Neste estudo, avaliou-se o efeito do tratamento combinado de fagos e antibióticos na inativação de *E. coli*. Para tal utilizou-se o fago Ec-Bio e uma estirpe bioluminescente de *E. coli* testada. Foi testado o antibiótico ciprofloxacina em concentrações letais e subletais adicionadas em diferentes tempos (0, 6, 12 e 18 h) em combinação com o fago que foi testado a duas multiplicidades de infeção (MOI), 1 e 100. Em geral, a eficiência do tratamento não aumentou com o aumento da MOI. Quando a ciprofloxacina foi adicionada em diferentes tempos (6, 12 e 18 h), a produção de fagos foi semelhante à obtida com o fago sozinho, mas superior à observada quando a ciprofloxacina foi adicionada ao mesmo tempo do fago (tempo 0). A adição do fago na ausência de antibiótico causou uma redução de ~ 2,8 log de *E. coli*, após 12 h de tratamento. Os resultados obtidos com as misturas de fagos com ciprofloxacina, nas concentrações subinibitórias (1/5 e 1/10 da MIC), não causaram diferenças significativas quando comparados com os resultados obtidos apenas com o fago, entretanto, após o mesmo período de tempo, à MIC e 2xMIC, a inativação bacteriana foi inferior do que a obtida somente com o fago. A combinação de fago e ciprofloxacina à MIC e 2xMIC, atingiu a taxa máxima de inativação após 18 e 36 h, respetivamente, altura em que já se observava recrescimento de *E. coli* quando o tratamento foi feito apenas com o fago. A *E. coli* foi mais eficazmente inativada quando o antibiótico foi adicionado após o fago. A eficácia do tratamento combinado variou com a concentração do antibiótico e o tempo de adição do antibiótico, evitando mais eficazmente o recrescimento bacteriano quando o antibiótico foi utilizado à MIC e adicionado após 6 h da adição do fago. Neste caso, a resistência bacteriana foi menor que a observada quando o fago e antibiótico foram usados separadamente (4.0×10^{-7} para o tratamento combinado, 3.9×10^{-6} para o antibiótico sozinho e 3.4×10^{-5} para os fagos sozinhos). O tratamento combinado com fagos e antibióticos pode ser eficaz na redução da densidade bacteriana, mas também para prevenir o surgimento de variantes resistentes. No entanto, a concentração do antibiótico e o seu tempo de aplicação são fatores essenciais a serem considerados no tratamento combinado.

keywords Phage therapy, antibiotic resistance, *Escherichia coli*, ciprofloxacin

abstract

Escherichia coli, a non-pathogenic commensal bacterium, can, however, developed more virulent variants that colonize outside the gastrointestinal system. This bacterium is one of the main responsible for moderate and serious infections in the hospital and community environments, being involved in the rapid evolution of fluoroquinolones and third generation cephalosporin resistance. It is estimated that in the coming years the number of deaths caused by resistant bacteria, including even commensal strains, will be very high, leading to an increased need to find alternatives to fight against pathogenic bacteria. An alternative or supplementary approach to antibiotics is phage therapy, which uses lytic bacteriophages (phages) that are able to infect only prokaryotes cells. Several studies have already shown encouraging effects when using phage therapy, however, phage-resistant mutants have been considered a major concern when this method is employed to inactivate pathogenic bacteria. Resistance can be overcome with the combination of phages and antibiotics, which prevents the re-growth of resistant bacteria and reduce more efficiently bacterial density than when used separately. In this study, the effect of combined treatments of phages and antibiotics in the inactivation of *E. coli* was evaluated. For that, it was used the phage Ec-Bio and the bioluminescent-transformed strain of *E. coli*. It was tested the antibiotic ciprofloxacin at lethal and sublethal concentrations added at different times (0, 6, 12 and 18 h) in combination with the phage to inactivate *E. coli* at two multiplicity of infection (MOI), 1 and 100. In general, bacterial inactivation did not increase with the increase of MOI. When the ciprofloxacin was added at different times (6, 12 and 18 h), phage production was similar to the obtained with phage alone, but higher than that observed when ciprofloxacin was added at the same time of the phage. The phage alone caused a reduction of ~2.8 log of *E. coli* after 12 h of treatment. The mixtures of phage with ciprofloxacin, at sublethal concentrations (1/5 and 1/10 of MIC), did not cause significant differences when compared with the results obtained just with the phage alone, however, after the same time period, at MIC and 2xMIC, the inactivation of *E. coli* was lower than that obtained with phage alone. Otherwise, the combination of phage and ciprofloxacin at MIC and 2xMIC, reached the maximum rate of inactivation, after 18 and 36 h, respectively, but at these times, re-growth of *E. coli* was observed when the phage was used alone. A higher difference was observed between the treatment with the mixture phage and antibiotic at MIC and the antibiotic alone than when the antibiotic was used 2xMIC. *E. coli* was most effectively inactivated when the antibiotic was added after the phage. The efficacy of the combined treatment varied with the antibiotic concentration and the time of antibiotic addition, avoiding bacterial regrowth when the antibiotic was used at MIC and added after 6 h of phage addition. In this case, bacterial resistance was lower than when phages and antibiotics were applied alone (4.0×10^{-7} for the combined treatment, 3.9×10^{-6} for the antibiotics alone and 3.4×10^{-5} for the phages alone). The combined treatment with phages and antibiotics can be effective in reducing the bacterial density, but also to prevent the emergence of resistant variants. However, the antibiotic concentration and the time of antibiotic application are essential factors to be considered in the combined treatment.

Contents

List of abbreviations and acronyms

MIC	Minimum inhibitory concentration
CFU	Colonies forming units
PBS	Phosphate buffer system
ANOVA	Analysis of variance
PFU	Plaque forming units
TSB	Tryptone Soy Broth
TSA	Tryptone soy agar
TSB	Tryptic Soy Broth
RLU	Relative luminescence unit
EOP	Efficiency of plating
EUCAST	European Committee on Antimicrobial Susceptibility Testing

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Objectives, thesis outline and publications

Objectives

The objective of this study was to evaluate the potential effect of single and combined use of phages and antibiotics in the inactivation of *E. coli* in order to control infections and to reduce the development of bacterial resistance to phages and antibiotics.

Thesis Outline

This document is divided into three chapters. Chapter 1 includes a literature review, serving as a basis for the studies carried out in Chapter 2. This Chapter describes the experimental work done in order to test the combination of a phage and an antibiotic, using different concentrations of antibiotic and phages (two MOIs), to control the emergence of resistant bacteria. Additionally, it was tested the influence of time of addition of ciprofloxacin in the inactivation of *E. coli*. In these assays a bioluminescent *E. coli* was selected as a model microorganism to monitor real-time phage therapy kinetics, through the measurement of bioluminescence. It was also tested the emergence of resistances to ciprofloxacin or phages alone and to the combination of phages and ciprofloxacin. Chapter 3 includes the references used in the present document.

Publications

During the dissertation period, I participated in the experimental work of an ongoing project, which results were published in this manuscript:

New insights on phage efficacy to control *Aeromonas salmonicida* in aquaculture systems: An *in vitro* preliminary study.

A copy of this manuscript is presented as Annex 1 of this document.

Chapter 1 – General Introduction

1.1. Antibacterial therapy

Antibiotic is defined as an agent produced naturally by an organism or prepared synthetically, used in the treatment and prevention of bacterial infections (Ahmed *et al.*, 2017; Nicolaou and Rigol, 2017). In 1928, Alexander Fleming made accidentally an important discovery for humanity, by observing the inhibition of a bacterial culture of *Staphylococcus aureus*, by the action of a fungus, *Penicillium* genus (Gaynes, 2017). After many years, Ernst Chain, Norman Heatley and Howard Florey were able to develop a way to isolate penicillin which has been used to treat bacterial infections during the World War II. Penicillin was introduced to clinical use in 1946 where it made a significant effect on public health (Nicolaou and Rigol 2017). During the golden age of discovery, 150 types of antibiotics were developed. Since then, the spread of resistance has greatly outpaced the rate of drug development (Lobanovska and Pilla, 2017) (Figure 1.1).

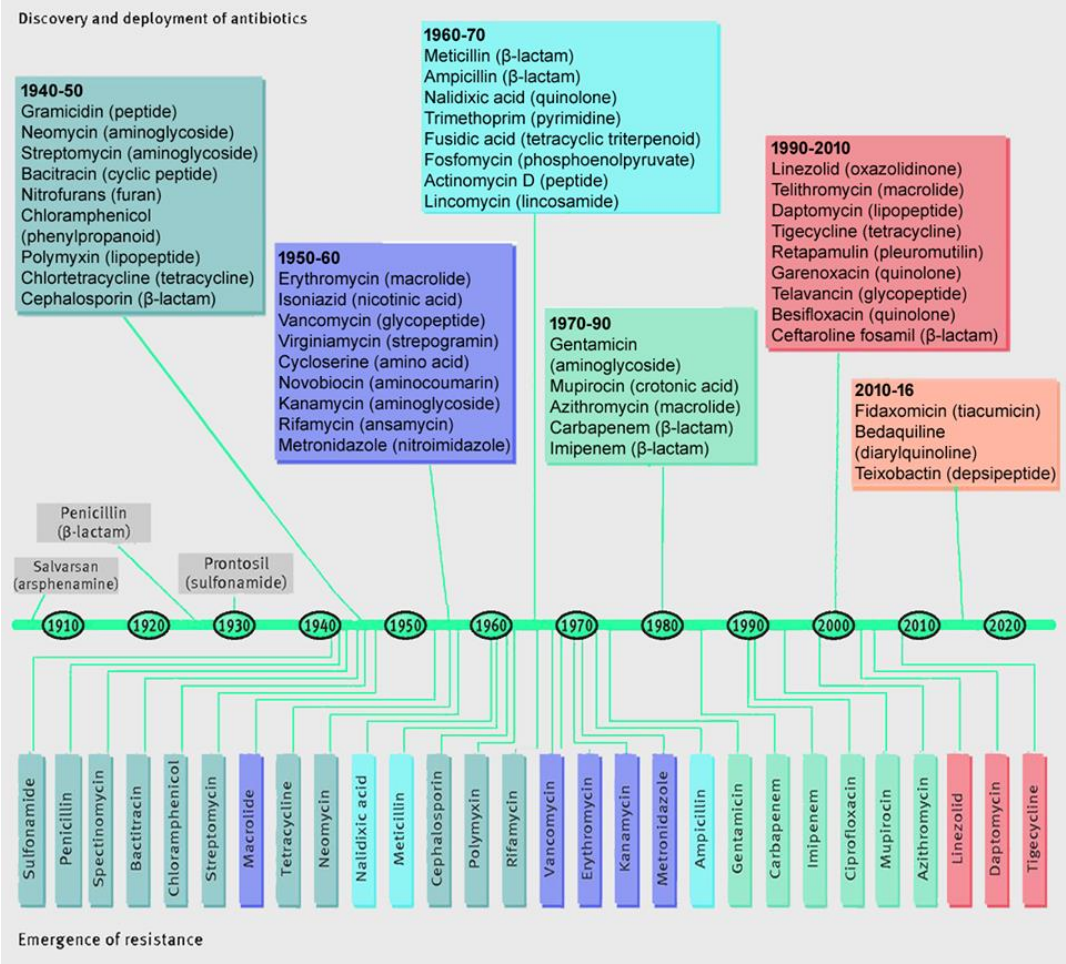

Figure 1.1: Discovery of antibiotics and emergence of antimicrobial resistance (Das *et al.*, 2017).

The antibiotics can be classified based on the cellular components or system they affect, such as: interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of a metabolic pathway and disruption of bacterial membrane structure (Tenover, 2006; Tortora, Funke and Case, 2010; Li *et* al., 2015). Also, they can be classified into bactericidal (if induce cell death) and bacteriostatic (if only inhibit cell) (Nicolaou and Rigol 2017).

1.1.1. Mechanisms of action of antibiotics

Antibiotics are classified into five major groups (Marinho *et al.*, 2016) based on its intracellular target and its mechanism of action (Li *et al*., 2015) (Figure 1.2):

a) **Cell wall Synthesis inhibition** (e.g. β-lactams, such as penicillin and derivatives, cephalosporins, carbapenems and glycopeptides). These compounds are more effective against infection by Gram positive bacteria. That kind of antibiotics have the peptidoglycan present on the cell wall as target. The successful treatment with these drugs can result in changes to cell shape and size, induce cellular stress responses and culminate in cell lysis (Tenover, 2006; Kohanski *et al*., 2010; *Liet* al., 2015).

b) **Cell membrane disruption** (e.g. the family of polycationic peptide antibiotics, such as polymyxins). Drugs that interfere with the cytoplasmic membrane are bactericidal. Some antimicrobial agents bind to cellular envelope components, such as phospholipids and liposaccharides (LPS), leading to membrane rupture and loss of cellular contents, killing the bacterium. (American Society for Microbiology, 2005; Mendes and Burdmann, 2010; Li *et* al., 2015).

c) **Nucleic acid synthesis inhibition** (e.g. quinolones and rifampicin). This is caused by two classes of drugs, fluoroquinolones and rifampicin. The first one interfere with DNA synthesis by blocking the enzyme DNA gyrase and the second binds to DNA-dependent RNA polymerase, which blocks the synthesis of RNA (American Society for Microbiology, 2005; Li *et al*., 2015).

d) **Protein synthesis inhibition** (e.g. tetracycline, aminoglycosides, chloramphenicol and macrolides). The procedure of mRNA translation involves the ribosome,

allowing protein synthesis, and is composed of two subunits, the 50S and 30S. Drugs that inhibit protein synthesis bind to ribosomal subunits and are divided according to these subunits, interfering with a phase of the protein synthesis (Kohanski *et al.*, 2010; Li *et al*., 2015).

e) Metabolic process inhibition (e.g. sulfonamides and trimethoprim). Antimicrobial agents inhibit cytoplasmic enzymes involved in the biosynthesis of essential bacterial metabolites, like para-aminobenzoic acid (PABA). PABA is involved in the synthesis of folic acid, precursor to the synthesis of nucleic acids. Antibiotics with this mechanism can compete with PABA or act on the folic acid synthesis pathway, inhibiting the enzyme dihyrofolate reductase (American Society for Microbiology, 2005; Rosenthal and Tan, 2011).

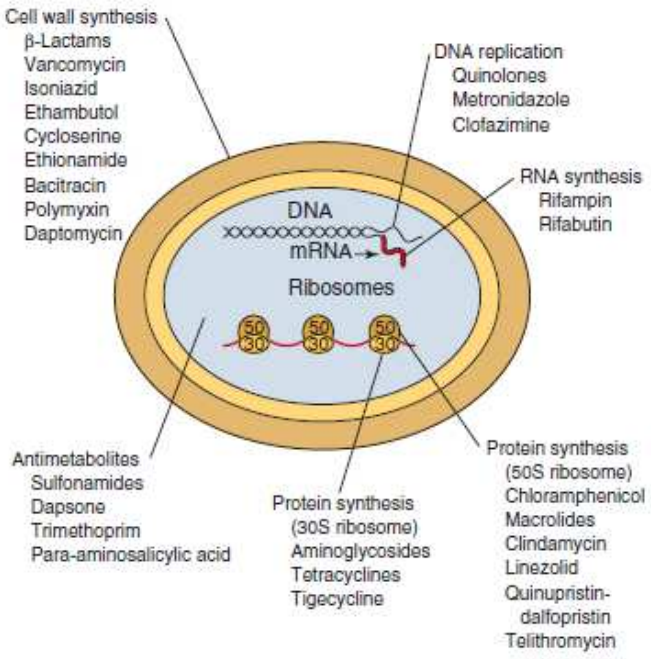

Figure 1.2: Basic sites of antibiotic activity (Murray *et al.*, 2016).

1.1.2. Fluoroquinolones

Fluoroquinolones are synthetic and one of the most broadly used classes of antibiotics. They are bactericidal and inhibit the bacterial enzyme DNA gyrase (topoisomerase type II) or topoisomerase type IV, which are essential for DNA replication, recombination and repair. The

topoisomerase type II helps to roll up and unroll DNA throughout DNA replication. The enzyme binds to DNA and introduces double stranded breaks that let the DNA to unroll. These antimicrobial agents bind to the DNA gyrase-DNA complex and permit the fragmented DNA strands to be released into the cell, which results in cell death (Mohr, 2016; Murray *et al.*, 2016).

Ciprofloxacin (Figure 1.3) is a second-generation fluoroquinolone (Scoper, 2008) and has a bactericidal activity (Mohr, 2016). This antibiotic has a broad spectrum and an excellent activity against Gram-positive and Gram-negative bacteria (Murray *et al.*, 2016). It is used to treat several infections, including UTI, osteomyelitis, gonococcal infections and chronic bacterial prostatitis (Emmerson, 2003).

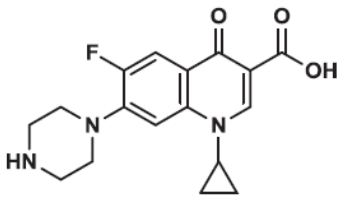

Figure 1.3: Ciprofloxacin. Antibiotic widely used (adapted from Nicolaou and Rigol 2017).

1.1.3. Antibiotics resistance

The increasing spread of bacterial resistance to antibiotics (Figure 1.4) at the community setting is promoted by several factors. The excessive and misuse of antibiotics in human medicine, in veterinary and in agriculture represent some of the behaviors responsible for selective pressure which enables the selection and spread of clones that carry antibiotic-resistance genes (Furuya and Lowy, 2006). Bacterial resistance is closely associated with excessive and prolonged use of antimicrobial agents in clinical practice. Prolonged therapy with antibiotics may lead to the development of resistance in microorganisms that initially are sensitive to antibiotics, but later can adapt gradually and develop resistance to these and consequently increasing the risk of mortality, increasing the costs and days of hospitalization (Giedraitienė *et al.*, 2011). Threatening microbes causing infections in hospitals and in the community are overcoming 50% resistance rate over the world (Fair and Tor, 2014a; WHO, 2014b; Renwick *et al.*, 2016). It was estimated that in 2050 the resistance to antibiotics will be responsible for 10 million deaths and for the loss of 100 trillion USD of the world's economic outputs (O'Neill, 2016). Also, the use in agriculture and veterinary for growth promotion, feed efficiency, and routine disease prevention purposes in animal agriculture is another important reason for the spread of resistant bacteria (Giedraitienė *et al.*, 2011). Over the years, the antibiotics have been used abusively and incorrectly. Annually,

large amounts of antibiotics are released in the environment for not being completely metabolized when used in humans, animals (veterinary medicine) or in other applications (Marinho *et al.*, 2016).

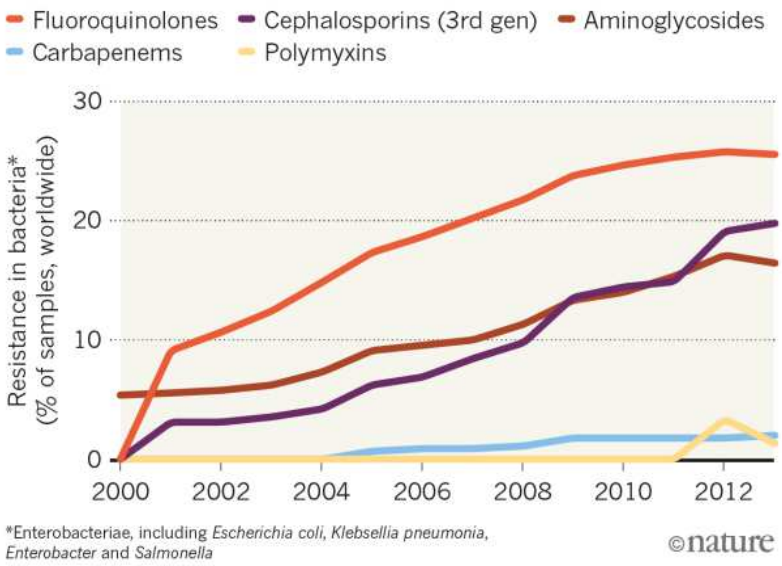

Figure 1.4: The spread of antibiotic resistance (Reardon, 2015).

Antimicrobial resistance can be intrinsic or acquired. The intrinsic resistance is the innate ability of all bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class (Cox and Wright, 2013). Acquired resistance occurs when a microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. Unlike intrinsic resistance, traits associated with acquired resistance are found only in some strains or subpopulations of each particular bacterial species. The acquired resistance can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms. The most common way to acquired resistance is through horizontal gene transfer between strains and species (Bockstael and Van Aerschot, 2009). There are three main different mechanisms of horizontal gene transfer in bacteria (Figure 1.5), these being:

a) **Transformation** is the process by which bacteria capture and incorporate segment of DNA (exogenous or foreign) from bacterial lysates that carry

antimicrobial resistance genes. The gene resistance may be incorporate into chromosome or plasmid in the recipient cell, giving rise to a resistant cell (American Society for Microbiology, 2005; Murray *et* al., 2016).

b) **Conjugation** occurs when two bacteria cells are close. DNA transfer is unidirectional, from the donor cell to the receptor cell, and occurs through a bridge-like structure known as pilus. This mechanism occurs typically between members of the same or related species. Gene transfer is performed through plasmids or integrative conjugative elements. Many of these carry genes for antimicrobial resistance (American Society for Microbiology, 2005; Heuer and Smalla, 2007; Murray *et al.*, 2016)

c) **Transduction** is the transfer of genetic information by which non-viral DNA can be transfer mediated by phages. Many phages infect only a narrow range of hosts. This mechanism has the advantage of not requiring cell-cell contact (American Society for Microbiology, 2005; Heuer and Smalla, 2007; Murray *et al.*, 2016).

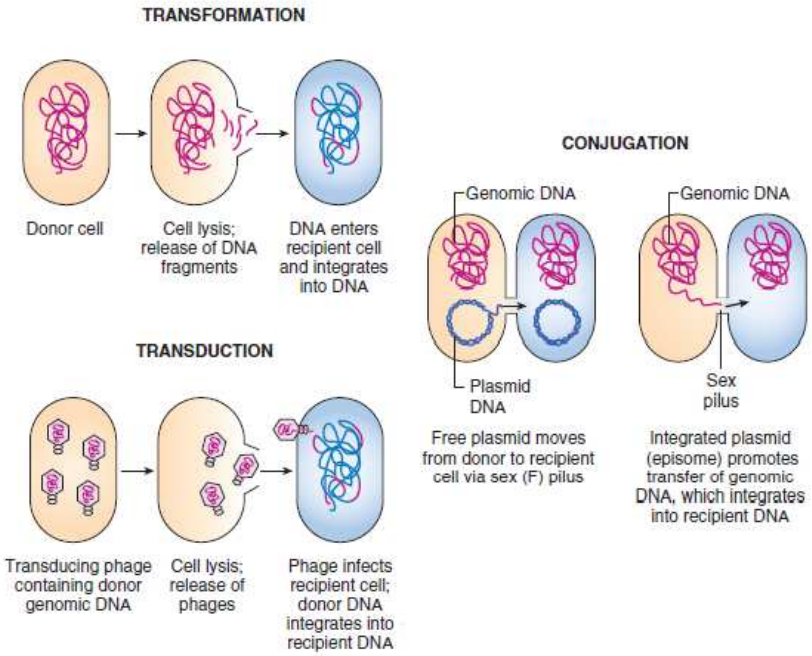

Figure 1.5: Mechanisms of bacterial gene transfer (Murray et al., 2016).

Bacteria can protect themselves through mutation in the target site, enzymatic modification or degradation of the antibiotic, efflux of antibiotics, reduced permeability to antibiotics and acquisition of alternative metabolic pathways (Vranakis *et al.*, 2014).

In last decades, the development of new antibiotics has lagged behind the acquisition of antibiotic resistance in bacteria (Hede K, 2014). Therefore, new antimicrobial strategies are essential to effectively control antibiotic resistance. Recently, phages have received much attention as an alternative and/or supplementary approach to antibiotics (Weber-Dabrowska *et al.*, 2016; Anne-sophie and Benoit, 2017) because of their efficacy of bacterial inactivation, host specificity, safety and low costs (Parisien *et al.*, 2008).

1.2. Phage therapy in the inactivation of pathogenic bacteria

Phage therapy consists in the utilization of phages to inactivate pathogenic bacteria. Phages were discovered by the early 1920s and their infectious cycles were understood by that time, but after antibiotic advent the interest in phage therapy declined in the western world (Sulakvelidze *et al.*, 2001). Nevertheless, in former Soviet Union and Eastern Europe, this method of treatment was continuously used to treat and prevent bacterial infection diseases (Sulakvelidze *et al.*, 2001). Recently, the emergence of pathogenic bacteria resistant to antibiotics, has motivated the western scientific community to reevaluate phage therapy as an alternative option for the treatment of bacterial infections (Wittebole *et al*., 2014). In a time when bacterial resistance to antibiotics is increasing, the use of phages has different advantages, along with relatively few disadvantages.

Phages production is simple, but in the past when this therapy was first applied clinically side effects were difficult to control. There are some requirements, not taken into account in the past, in the production of phages that are: phages must be free from microorganisms (purified), lytic and can not transduce virulence factor genes of the host; stability over storage and during application must be tested; the host range need to be well known; to avoid resistance and toxin transmission to bacteria, genome sequencing should be done; the receptor of the phage must be known. In a population of 10^6 to 10^8 there is a great possibility of spontaneous mutants resistant to phages displaying an altered receptor; and amenability to scale up (Skurnik and Strauch, 2006).

The difficulties of acceptance and implementation of phage therapy as an alternative to combating of infections are due to (a) differences in biological, physical, and pharmacological properties of phages compared to conventional antimicrobials, (b) the need to employ phage cocktails due to high phage specificity and (c) current approval processes for antimicrobial agents

that are based on chemically derived drugs and which are therefore less suitable for phages (Cooper *et al*., 2016). Phage-derived enzybiotics are already suitable for the current approval processes as therapeutic proteins, however, phage therapy may require other pathways of approval (Cooper *et al*., 2016; Abedon *et al.*, 2017; Aminov *et al.*, 2017).

1.2.1. Bacteriophages

Phages were discovered independently by Frederick W. Twort in 1915 and by Felix d'Herelle in 1917 (Sulakvelidze *et al.*, 2001). Felix d'Herelle named them phages (formed from "bacteria" and "phagein" that means "to eat", in Greek) and developed the method of quantification of viruses and some theories, including the replication cycle of the phage (Sulakvelidze *et al.*, 2001). After the discovery of phages, d'Herelle introduced the term phage therapy, that was regarded as a possible method of treatment against bacterial infectious diseases (Sulakvelidze *et al.*, 2001). Soon after its discovery, phages began to be exploited and used to control infections by pathogenic bacteria (Abedon *et al.*, 2011).

Phages are viruses that infect only prokaryotes (bacteria and archaea) (Skurnik and Strauch, 2006; Ceyssens and Lavigne., 2010), resulting usually in propagative lyses (lytic cycle) or lysogenization (lysogenic cycle) of the infected cell (Azizian *et al.*, 2013; Borie *et al.*, 2014; Teng-hern, Kok-gan and Han, 2014). Lytic phages may be candidates for phage therapy, because they replicate fast within their hosts and lyse them (Azizian *et al.*, 2013). Phages can be defined as a capsid-encoding organism that is composed by proteins and nucleic acids, self-assembles in a nucleocapsid that uses a ribosome-encoding prokaryotic organism for the completion of its life cycle (Raoult and Forterre, 2008).

Phages are the most abundant organisms in the biosphere and they are a ubiquitous feature of prokaryotic existence (total number estimated to be 10^{30}-10^{32}) (Fuhrman, 1999; Clokie *et al.*, 2011; Bhardwaj *et al.*, 2015). The presence of the phages in the biosphere results in phages coevolving with their host bacteria and provide the earth's ecological equilibrium in several environmental or ecological niches (Vos and Pirnay, 2015).

The phages present a variety of different morphological types, but the majority displays a capsid, collar and tail (Figure 1.6) (Hanlon, 2007). Phages contain a core nucleic acid encapsulated with a protein or lipoprotein capsid which is connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers. The capsid is a protein shell often in the shape of an icosahedron and usually comprises double-strand (dsDNA), but there are phages with single stranded DNA (ssDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) and

can vary between 17 kb and 500 kb (Melo *et al.*, 2017). The capsid is organized into capsomeres, whose main function is to protect the genetic material (Sharma *et al.*, 2017). The tail may or may not be a contractile structure to which six fibers are usually connected, containing receptors on their ends that recognize binding sites on the surface of the bacterial cell. Phage tails are very varied in DNA content and composition, dimensions, structure and physiology (Ackermann, 2003). However, not all phages have tails and tail fibers and in this situation other attachment mechanisms are present (Goodridge and Abedon, 2003; Hanlon, 2007).

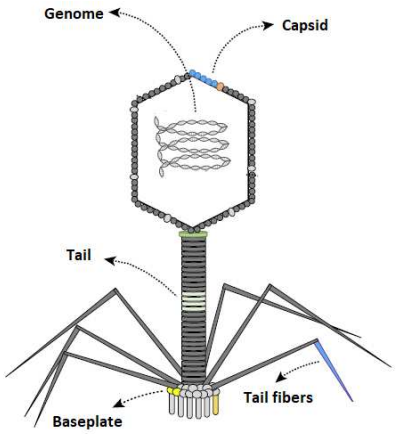

Figure 1.6: Representation of a typical bacteriophage (adapted from Nobrega et al. 2015).

1.2.2. Taxonomy of bacteriophages

The International Committee on Taxonomy of Viruses (ICTV) is responsible for the classification of the viruses (Aiewsakun and Simmonds, 2018). Bacteriophage taxonomy is based on morphological (size and shape) and molecular characteristics (type of nucleic acid) (Table 1.1). Phages are tailed, polyhedral, filamentous, or pleomorphic (Ackermann, 2003, 2007).

Nowadays, it can be found around 4 families, 22 subfamilies and 164 genera, belonging to the *Caudovirales* order. Over 96% of phages are tailed and belong to *Caudovirales* order, and are divided into four families: (1) *Myoviridae*, in which viruses have a contractile tail constituted by hem, a central tube and a big capsid head (~150 nm), (2) *Siphoviridae*, in which viruses have long not contractible tails, and a relatively small capsid head (~50-60 nm), (3) *Podoviridae*, in which viruses have no contractile tail and have short tails and a small capsid head (~50-60 nm) and (4) *Ackermannviridae* (Ackermann, 2007; Comeau *et al.*, 2012; Rossmann, 2013; Cruz-Flores and Cáceres-Martínez, 2016; ICTV, 2017; King *et al.*, 2018). *Ackermannviridae* was recently approved, taking into account the DNA sequencing (ICTV, 2017). The other non-tailed phages (represent less

than 4%), are classified into ten families, and are cubic, filamentous, or pleomorphic and contain double-stranded or single-stranded DNA or RNA as the genome.

Table 1.1: Taxonomy and properties of phage families (adapted from Ackermann and Prangishvili, 2012; Mäntynen *et al.*, 2015; Pereira, 2016; ICTV, 2017).

Order	Shape	Families	Nucleic acid	Characteristics
Caudovirales	Tailed	*Myoviridae*	dsDNA, Linear	Contractile tail
		Siphoviridae		Long, non-contractile tail
		Podoviridae		Short, non-contractile tail
		Ackermannviridae		*
Unassigned	Polyhedral	*Microviridae*	ssDNA, circular	Icosahedral capsid
		Corticoviridae	dsDNA, circular, superhelical	Icosahedral capsid with lipid layer
		Tectiviridae	dsDNA, linear	Icosahedral capsid with inner lipoprotein vesicle
		Leviviridae	ssRNA, linear	Quasi-icosahedral capsid
		Cystoviridae	dsRNA, linear, segmented	Enveloped, icosahedral capsid, lipids
	Pleomorphic	*Plasmaviridae*	ssDNA, circular, superhelical	Pleomorphic, envelope, lipids, no capsid
		Fuselloviridae	dsDNA, circular, superhelical	Envelope, lipids, no capsid
	Filamentous	*Inoviridae*	ssDNA, circular	Rod-shaped with helical symmetry
Ligamenvirales		*Lipothrixviridae*	dsDNA, linear	Enveloped filaments, lipids
		Rudiviridae	dsDNA, linear	Helical rods

*Not found

1.2.3. Life cycle

The life cycles of phages typically, can be classified broadly into categories lytic (virulent) and lysogenic (temperate) cycles (Sharma *et al.*, 2017). There is a third way by which phages can also interact with their hosts, the pseudolysogenic cycle (Figure 1.7).

A prokaryotic virus has various steps throughout the life cycle and these are common to all virus. The steps are adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release and transmission (Weinbauer, 2004).

In the lytic cycle, the host cell is injected with the genome of the phage (Hogg, 2013). These need to multiply themselves to cause lysis of the host cell to release the newly formed phages (Skurnik and Strauch, 2006). On the surface of the host cell, phages adsorb to specific receptor sites on the surface of the host cell and subsequently form an irreversible attachment. Capacity to recognize and attach to receptor molecules on the cell surface mainly dictates the host range of a phage (Hanlon, 2007).

The penetration of tail occur through cell walls degraded enzymatically driving to the insertion of phage nucleic acid into the cytoplasm of the host (Weinbauer, 2004). For DNA phages, once inside the cell, the bases of the phage nucleic acid are often modified to protect against the attack by restriction enzymes and cellular nucleases. The viral genome is transcribed by RNA polymerases of the host cell, producing premature mRNA, that will take over the metabolic machinery of the host and redirect the metabolic processes to the production of new viral components, such as nucleic acid and proteins. After the replication and assembly of new phage particles within the host cell, the new phages are released to the environment (Hanlon, 2007; Wittebole *et al.*, 2014). Most of the dsDNA develop lytic enzymes that attack the bacterial peptidoglycan, for the dissemination of its offspring phages (Fischetti, 2005). These phages produce a lytic enzyme, usually called endolysin or muralytic enzymes, that are produced within the cytoplasm but require another enzyme, capable of permeabilize the membrane, designated holin. Endolysin need this protein, because most of these enzymes lack a secretory signal sequence and, in addition, this protein gives access to murein (Young *et* al., 2000). The holin rupture the membrane, allowing the lysin to degrade the peptidoglycan (Young *et al*., 2000; Fischetti, 2005). It also controls the timing of the cell lysis which leads to a disruption of the membrane and the release of the phage progeny (Fischetti, 2005). The filamentous phage can escape the host cell by extrusion through the cell wall without causing the destruction of the host,

however these phages did not present relevance for phage therapy (Hanlon, 2007). The period of time between the attachment of a phage particle to the cell surface and the release of the newly synthesized phages is called the latent period, sometimes also known as the burst time (Hogg, 2013).

In the lysogenic cycle, the phage integrates its own DNA into the genome of the host (Wang and Goldenfeld, 2010). A copy of the phage genome is maintained in a suppressed state within the host genome and replicates with the host chromosome (Skurnik and Strauch, 2006; Hogg, 2013). The temperate phages induce a state of lysogeny in the bacterial host (Hanlon, 2007) and they do not enter automatically on a lytic cycle (Skurnik and Strauch, 2006; Hogg, 2013). Cells may divide multiple times but, occasionally, one will spontaneously lyse and release progeny phage (Gill and Hyman, 2010). The prophage enters the lytic cycle when forced by a given stimuli, such as pollution, starvation and others. These phages can transfer DNA host fragments, which may contain antibiotic resistance and therefore the only phages used in phage therapy are the lytic ones (Wang and Goldenfeld, 2010; Rossitto *et al.*, 2018).

The pseudolysogeny cycle, or false lysogeny, is defined as a phenomenon where there is a continuous production of phage in the presence of high host cell abundance (Ackermann and DuBow, 1987). In this cycle the bacteriophage lysis does not outcome in total host death and the abundance of phage coexists with exponential host growth. This may be the outcome of a mixture of resistant and sensitive host cells and/or a mixture of virulent and temperate phages. Thus, the phage may proceed with lytic infection or enter a dormant intracellular phase, with no integration of the phage genome into host cell replicons (Wommack and Colwell, 2000). Pseudolysogeny is an environmental condition in which the bacterial cell coexists in an unbalanced relationship with infective viruses (Ripp and Miller, 1997, 1998). Under these conditions, host cells do not offer enough energy in order to phage incoming into a true lysogenic or lytic condition (Williamson *et al.*, 2001).

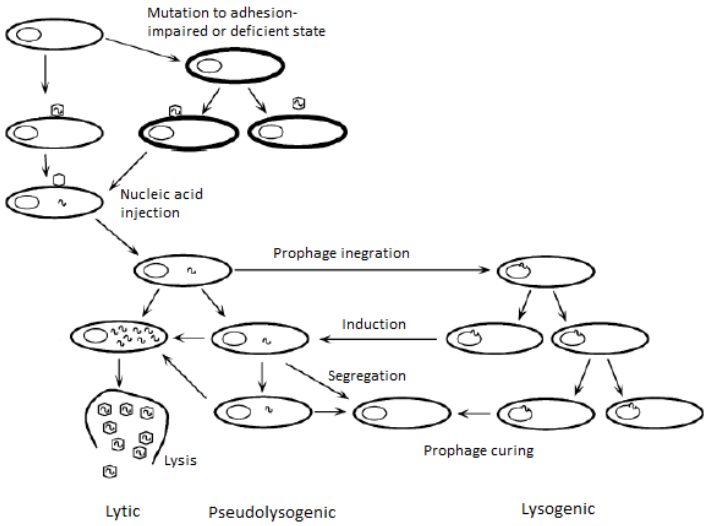

Figure 1.7: Schematic representation of phage life cycles (adapted from Weinbauer, 2004).

1.2.4. Phage therapy applications

Phage therapy has been studied to be applied or is even already applied in several areas, such as medicine (Sunagar *et al.*, 2010; Kumari *et al.*, 2011; Rahmani *et al.*, 2015; Zhang *et al.*, 2017), food safety (Pereira *et al.*, 2017a; Sharma *et al.*, 2017), veterinary (Hawkins *et al.*, 2010; Suresh *et al.*, 2017) and aquaculture (Silva *et al.*, 2014a, 2016; Kalatzis *et al.*, 2016; Wang *et al.*, 2017). Also, it has been used to treat wastewater (Withey *et al.*, 2005; Beheshti *et al.*, 2015).

1.2.4.1. Clinical applications of phage therapy

Through the summer of 1919, d'Herelle applied phages in five children with bacillary dysentery admitted at the hospital *Necker-Enfants-Malades* in France, achieving therapeutic success in all cases (Dublanchet and Bourne, 2007).

Clinical phage therapy is allowed for routine use in a limited number of countries, however, the corresponding data from these efforts is limited. Formerly, in phage therapy, clinical use was performed prior to animal testing, but in the modern era, this therapy has to adopt current standards of drug development, that is, in which animal testing by need precedes clinical use (Abedon *et al.*, 2017).

The Republic of Georgia is the one place in the world where phage therapy is regularly used in a number of hospitals and clinics for both prophylactic and treatment purposes. Most of the available phages have been associated with the Eliava Institute. The main focus of this institute has been on therapeutic phage cocktail formulation, characterization, production and

implementation. Hirszfeld Institute, in Poland, is another important source of information. In this Institute, researchers have developed individual therapeutic phages and reinforced their use by local physicians for a diversity of applications where antibiotics were unsuccessful (Kutter *et al.*, 2010).

Researchers are evaluating the use of phage in clinical trials in humans (Table 1.2), however, only a limited number of clinical trials have been conducted so far, which is surprising given the growing concern around antibiotic resistance. The reason for the lack of data is varied and concern about the safety of phage use in humans has been an obstacle to the development of phage therapy in the western world, although there are already commercially available phage preparations available in Russia and Georgia for decades (Vandenheuvel, Lavigne and Brüssow, 2015).

Table 1.2: Overview of safety and clinical phage trials (Adapted from Vandenheuvel et al., 2015).

Trial phase	Experimental details	Target bacterium	Observations	Refs
Safety trials	Exposure to phages (e.g., environment, skin, gut, food consumption)	NA	No indication of health risks was found.	Brüssow, 2001; Desiere *et al.*, 2001; Suttle, 2005; Reyes *et al.*, 2010; Oh *et al.*, 2014
	Animal experiments	*E. coli*	Oral administration of T4 did not affect the microbiota of mice; no phage was found in the blood or organs.	Chibani-Chennoufi *et al.*, 2004a; Weiss *et al.*, 2009
	Human experiments	NA	Oral administration of T4 did not result in anti-T4 antibodies or liver damage.	Bruttin and Brüssow, 2005

Phase I/II clinical trials	Oral administration of phage cocktails to healthy adults and children	NA	No adverse effects were observed.	Sarker et al., 2012
	Intravenous administration of φX174 to HIV patients	NA	No adverse effects were observed.	Ochs *et al*., 1971; Fogelman *et al.*, 2000; Rubinstein *et al.*, 2000
	The Intralytix trial: phage therapy on venous leg ulcers	*E. coli; Pseudomonas aeruginosa; S. aureus*	No adverse effects were observed.	Rhoads et al., 2009
	The Belgian Military Hospital trial: phage therapy on burn wounds	*P. aeruginosa; S. aureus*	No adverse effects were observed; the bacterial load remained unchanged.	Merabishvili et al., 2009; Rose et al., 2014
	The Polish case studies	Suppurative multidrug-resistant infections	No serious adverse effects observed; the success rate was high.	Weber-Dabrowska et al., 2000
	The Biocontrol trial: phage therapy against ear infections	*P. aeruginosa*	No adverse effects were observed; 12 of 42 patients showed significant improvements.	Wright et al., 2009
	The Nestlé trial: phage therapy against diarrhea	*E. coli*	The trial was stopped because of the absence of a therapeutic effect; no adverse effects were	Chibani-Chennoufi et al., 2004b

			observed.	
Phase III clinical trials	The Eliava trial: phage therapy against *Shigella* dysentery	*Shigella* spp.	A significant decrease in disease incidence was observed.	Babalova et al., 1968; Sulakvelidze et al., 2001

NA - not applicable

1.2.4.2. Commercialization of phage products

D´Herelle developed, in the commercial laboratory in Paris (later known as the company L'Oréal), products based on phages. These products were called Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage and Bacté-staphy-phage. In the 1940, Eli Lilly Company (Indianapolis, Ind.) prepared seven phage products to treat abscesses, septic wounds and vaginitis, mastoid infections and respiratory tract infections caused by staphylococci, streptococci, *E. coli* and others microorganisms (Sulakvelidze *et al.*, 2001). Phages may be versatile in terms of formulation development. They can be applicated in many forms, like as liquids, creams, impregnated into solids and others, and are suitable for most routes of administration (Loc-Carrillo and Abedon, 2011). Presently, some bacteriophage products have been licensed and approved for human application (Guang-Han *et al.*, 2016) and others are in development (Table 1.3).

Some phage therapeutic products, such as Phage BioDerm, use the combination of phage and antibiotics. Phage BioDerm is used in humans and is a biodegradable, non-toxic polymer impregnated with phages and the antibiotics ciprofloxacin and benzocaine. Conventional treatment was used to treat patients with ulcers and wounds, but it was not successful. After that, patients were treated with Phage BioDerm and a success rate of 70% was observed (Guang-Han *et al.*, 2016).

Table 1.3: Some phage based products developed or in development for treatment of human disease (Adapted from Cooper et al., 2016).

Company	Product	Target	Application	Type	Company website
Micreos	Staphefekt	*S. aureus*	Topical	Endolysin	https://www.staphefekt.com

AmpliPhi	AB-SA01	*S. aureus*	Intravenous, intrasinal and topical	Phage	http://www.ampliphibio.com
	AB-PA01	*P. aeruginosa*	Intravenous and inhaled		
Technophage	TP-102	*	Ulcers	Phage	http://www.technophage.pt
	TP-122		Respiratory		
	TP-164		Infection		
	TA-111		Neuroscience		
	TZ-113				
	TZ-161				
	TA-111/16				
	TA-101/16		Immunology		
	TA-141				
Pherecydes Pharma	PP021	*E. coli*	Burn and skin	Phage	http://www.pherecydes-pharma.com
	PP1131 PP1231	*Pseudomonas*	Burn, skin and respiratory Tract infection		
	PP2351	*Staphylococcus*	Bone, Joint and Prosthesis		
	PhagoBurn	*P. aeruginosa* and *E. coli*	Skin infections		
	PneumoPhage	*P. aeruginosa*	Respiratory tract infections		
	Phosa	*S. aureus*	Bone and joint or diabetic foot ulcer infections		

Avid Biotics	Avidocin	*Clostridium difficile*	Infections	Phage derived	http://www.avidbiotics.com
ContraFect	CF-301	*S. aureus*	Bloodstream infections	Phage derived lysins	http://www.contrafect.com
	CF-296	*S. aureus*	*		
	GN lysins	*P. aeruginosa*	*		
	BP lysins	*Streptococcus pneumoniae, Enterococcus faecalis, Bacillus anthracis* and Group B *Streptococcus*	*		
	CF-404	*Influenza*	Seasonal and pandemic strains of human influenza		
Pharmacy Eliava	INTESTI Bacteriophage	*Shigella, Salmonella, E. coli, Proteus, S. aureus, P. aeruginosa* and *E. faecalis*	Inflammatory and enteric infectious diseases	Phage	http://bacteriophagepharmacy.com
	PYO Bacteriophage	*S. aureus, Streptococcu, E. coli, P. aeruginosa* and *Proteus*			
	Fersis Bacteriophage	*Staphylococcus* and *Streptococcus*			
	SES bacteriophage	*Staphylococcus, E. coli* and *Streptococcus*			

	Enko Bacteriophage	*Shigella, Salmonella, E. coli* and *Staphylococcus*			

*Information not available

1.2.4.3. Other applications of phage therapy

When it comes to the use of phage therapy in a clinical context, the approval timelines and long product developments in Western regulatory frameworks are obstacles. Because of this, many companies and researchers have turned to the area of food safety, agriculture and others. Environmental Protection Agency (EPA), United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) have approved successful phage-based products that have been developed by several of these companies. Some of these products, which are used as sterilizing agents for processed foods are, for example, ListShield™ and LISTEX™ P100 (Lu and Koeris, 2011). In food safety, phage therapy can be applicated to restrict bacterial contamination on several foods such as chicken flesh, meat, fruits, and vegetables (Sharma *et al.*, 2017).

1.2.5. Advantages and disadvantages of phage therapy

Phage therapy has several advantages over antibiotics. Antibiotics have a low specificity (greater spectrum of action), compared to phages, and may cause side effects to the drug recipient, such as secondary infections, the appearance of resistant bacteria and allergies. However, phage specificity also may be a disadvantage, since detecting the causative agent of the infection may be delayed and may aggravate the condition of the patient. This obstacle can be overcome using a mixture of different phages (El-Shibiny e El-Sahhar 2017; Pereira et al. 2016a)

Phages do not affect the cells of mammals because they target only bacterial cells (Golkar *et al.*, 2014), with phage therapy being safe and relatively free of side effects (Weber-Dabrowska *et al.*, 2016).

Antibiotics may not be effective and can be metabolized and excreted from the body without the drug being able to reach the site that is being infected. On the other hand, the phages are widely spread throughout the body after being administered systemically, reaching the infected site (El-Shibiny and El-Sahhar 2017) and replicating in that location (Golkar *et al*., 2014). The very small size of phages allows them to go to body sites that are not reached by antibiotics (Wittebole *et al*., 2014; El-Shibiny and El-Sahhar, 2017). The exponential multiplication of phages at the site

of infection makes treatment less frequent and at lower doses compared to treatment using antibiotics (El-Shibiny and El-Sahhar 2017). One dose is enough (Vieira *et al.*, 2012). Phages can be used to treat infections caused by bacterial strains resistant to antibiotics, including multidrug resistant strains (Weber-Dabrowska *et al.*, 2016).

Phages are prevalent in nature and their acquisition and selection is very fast compared to the development of antibiotics, which can take several years until they have an effective drug. In addition, the cost of producing antibiotics is much higher (El-Shibiny and El-Sahhar 2017).

Despite the many advantages of phage therapy, it also has several disadvantages, such as the lack of established protocols for dose, frequency, duration and route of administration of the treatment, lack of properly documented clinical research, and poor knowledge of phage behavior *in vivo*. The side effects in the long run are unknown, and the purity and stability of phage preparations are doubtful, if sufficient quality control information is not available (El-Shibiny and El-Sahhar 2017). The lack of legislation is another limitation to wide use of phages to combat antibiotic-resistant bacteria (Weber-Dabrowska *et al.*, 2016). An important disadvantage, when using phage therapy, is the development of phage-resistance by some bacteria (Gill and Hyman, 2010; Silva *et al.*, 2014a; Pereira *et al.*, 2016a, 2017b).

The most frequent cause of bacterial phage resistance appear to be genetic mutations affecting phage receptors, restriction modification or abortive infection associated with the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) in the bacterial genome (Heller, 1992; Labrie *et al.*, 2010). However, currently, the emergence of phage-resistant mutants also can be due to phenotypic resistance (Laanto *et al.*, 2012; Vieira *et al.*, 2012; Bull *et al.*, 2014). This resistance may be: i) induced, the products of phage-lysed bacteria result in a change in uninfected bacterial gene expression, thus reducing adsorption; ii) intrinsic, reduced adsorption is due to a physiological or gene expression state that happens prior to the phage introduction; and iii) dynamic, degradation or blocking of bacterial receptors by phage proteins released during cell lysis (Bull *et al.*, 2014).

Phages co-evolve with their hosts allowing them to overcome the resistance acquired by the bacteria (Wittebole *et al.*, 2014; El-Shibiny and El-Sahhar, 2017). The acquisition of resistance to the phages can reduce the fitness of the host with consequences in growth and pathogenicity, mostly if the phage receptor is required for virulence. Phage resistance can be associated to lower virulence, making the resistant bacteria less virulent than the susceptible ones. However, possible alterations in the virulence of phages depend on the specific phage-host system (León and Bastías, 2015; Oechslin *et al.*, 2017; Hill *et al.*, 2018). Phage resistance may contribute to bacterial

elimination from the environment quicker than their wild-type parents (Bohannan *et al.*, 1999; Brockhurst *et al.,* 2005; Duarte *et al.*, 2018). Resistance triggered by phages is a less important problem than that caused by antibiotics. Although bacteria can develop resistance to a determined phage, specific to them, there are always different phages with a similar target range (Wittebole *et al*., 2014; El-Shibiny and El-Sahhar, 2017). Contrarily to phages, antibiotics do not have the ability to evolve and escape the mechanisms of defense of the host. Moreover, phage resistance can be overcome more easily than resistance to antibiotics by using phage cocktails (Samson *et al.*, 2013; Hill *et al*., 2018).

1.3. Combination of antibiotics and bacteriophages in the inactivation of pathogenic bacteria

While there are limitations to the use of phage and antibiotics as the sole agent for treating bacterial infections (Bull *et al.*, 2002; Ul Haq *et al.*, 2012), these bacterial viruses may be an effective adjunct to antibiotic treatment (Knezevic *et al.*, 2013; Torres-Barceló *et al.*, 2014; Torres-Barceló and Hochberg, 2016; Valério *et al.*, 2017) (Table 1.4).

The combination antibiotic-phage may have the advantage of reducing the development of resistance, particularly in multiple infections caused by bacteria (Kutateladze and Adamia, 2010; Kamal and Dennis, 2015; Torres-Barceló and Hochberg, 2016; Weber-Dabrowska *et al.*, 2016), as well as stimulate the increase of the production of phage and/or their activity and increases the efficacy of killing bacteria (Kamal and Dennis, 2015). This evolution can be explained by the fact that (Figure 1.8):

(i) Bacteria resistant to one or both can emerge, but grow slowly due to costs and/or are fewer pathogenic than sensitive bacteria

(ii) Double-resistant bacteria do not emerge due to trade-offs between resistance mechanisms. A direct negative interaction between mechanisms of resistance can be a powerful limitation on their evolution.

(iii) If they are applied sequentially then double mutants (resistant to both antibiotic and phages) are extremely rare or absent. Synergy may result from low bacterial densities dropping the probability of the emergence of resistance mutations (Torres-Barceló and Hochberg, 2016).

Antibiotics and phages could be applied sequentially, which might limit the emergence of double-resistant mutants (Imamovic and Sommer, 2013; Moulton-Brown and Friman, 2018). Torres-Barceló et al. (2014) suggest the existence of an optimal window of opportunity in the implementation of combined therapies to restrain pathogens. They observed that when a specific

time delay in antibiotic introduction is applied, regardless of antibiotic dose and resistance to either antibiotics or phages, bacterial density is reduced. This delay allows the phages to have their strongest impact on bacterial population density prior to the introduction of antibiotic (Torres-Barceló et al., 2014).

Some studies demonstrated that phage-antibiotic combinations can cause the same resistance than phage and antibiotic introduced individually (Verma et al., 2009; Zhang and Buckling, 2012; Torres-Barceló et al., 2014). The interaction between phages and antibiotics during extensive periods of time may result in the emergence of phage-resistant and growth of bacterial density (Kumari et al., 2010; Nouraldin et al., 2016). However, it was also found that bacterial density does not increase in vivo, since the combination of phage and host defenses, after phage therapy, is enough to keep the bacterial density under the lethal limit (Levin and Bull, 2004).

Although there are studies using the combination antibiotic and phages to control problematic bacteria, these are still few and the existing ones determine only resistance to antibiotics and not to phages. Valério et al. (2017) tested not only the resistance to four antibiotics, but also studied the resistance to phages with and without antibiotics. They found that phages limit the emergence of antibiotic resistant variants in combined treatment, independently of the type and mechanisms of action of the four antibiotics. However, further studies are needed, in particular with addition of phage and antibiotics sequentially.

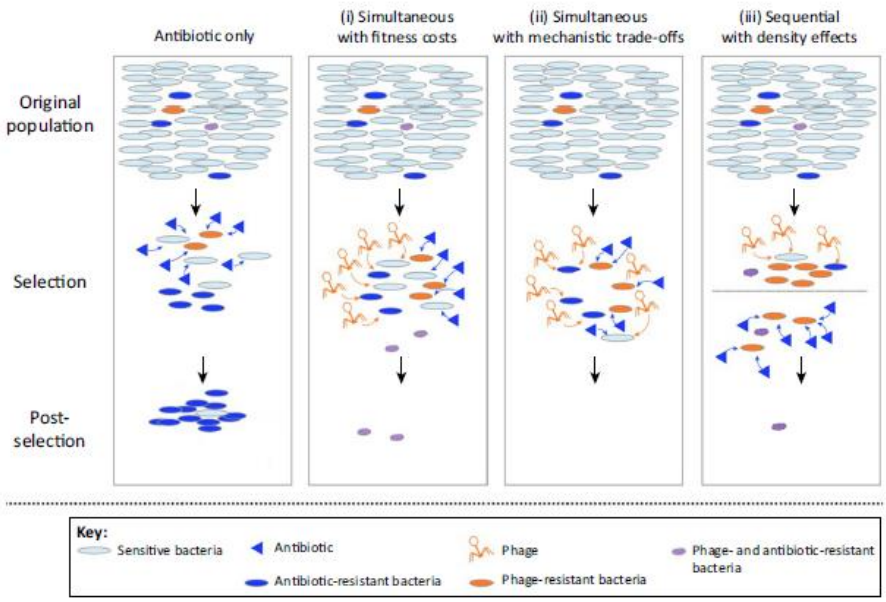

Figure 1.8: Phage-antibiotic synergy mechanisms (Torres-Barceló and Hochberg, 2016).

There are some terms used to describe the effects of combined treatment (Piggott *et al.*, 2015; Baeder *et al.*, 2016) (Table 1.4). According to the literature, combined effect can be the sum (additive), smaller than the sum (antagonistic) and greater than the sum (synergic), of the individual effects working in the same direction. Successful treatment is most likely for synergy, however simple additives may also get it, particularly when the host immune system is involved as a third member in the control (Crain *et al*., 2008; Torres-Barceló and Hochberg, 2016). Phage-antibiotic synergy is defined as the phenomenon whereby sub-lethal concentrations of certain antibiotics can substantially stimulate the host bacteria's production of virulent phage (Comeau *et al.*, 2007; Kaur *et al.*, 2012; Ryan *et al.*, 2012; Torres-Barceló and Hochberg, 2016). The synergism effect of the antibiotic-phage combination has no direct association with the type of antibiotic (Torres-Barceló *et al.*, 2016) and is not affected by the antibiotic resistance status of the target cell (Kamal and Dennis, 2015). Contrarily to that, Valério *et al.* (2017) observed that the efficacy of the combination of phages and antibiotics depends on the antibiotic resistance status of the bacteria to the used antibiotic and of the antibiotic type (bactericide or bacteriostatic). From an evolutionary understanding, the logic of combining phages and antibiotics is that two different selective pressures are most likely to kill both nonresistant (susceptible to antibiotics) and antibiotic-resistant (susceptible to phages) pathogen genotypes, and are probably better than when used separately (Torres-Barceló and Hochberg, 2016).

Table 1.4: Some studies showing antibiotic-phage encouraging effects to control problematic or model bacteria (Adapted from Torres-Barceló & Hochberg, 2016).

Bacteria	**Phage Family**	**Antibiotic**	**Effects**	**Refs**
P. aeruginosa	Not described	Ciprofloxacin, meropenem	-Highly synergistic effect - Inhibited regrowth of phage-resistant mutants	Oechslin et al., 2017
E. coli	Not described	Ciprofloxacin, ampicillin, piperacillin, kamanycin,	-Decreased the bacterial counts - Decrease of emergence resistance	Valério et al., 2017

		tetracycline, chloramphenicol	to phages	
P. aeruginosa	Not described	Amikacin	-Showed biofilm eradication	Nouraldin et al., 2016
P. aeruginosa	*Podoviridae*	Carbenicillin, gentamicin, and trimethoprim	- Limited bacterial density recovery - Reduction of antibiotic resistance levels	Torres-Barceló *et al.*, 2016
Burkholderia cepacia	*Myoviridae*	Meropenem, ciprofloxacin, tetracycline	- Phage-antibiotic synergy -Increased survival of larvae	Kamal and Dennis, 2015
E. coli and *P. aeruginosa*	Not described	Tobramycin	-Reduce of emergence resistance to antibiotic	Coulter et al., 2014
P. aeruginosa	*Podoviridae*	Streptomycin	-Decreased of bacterial density - Limited resistance to antibiotic and phages	Torres-Barceló et al., 2014
P. aeruginosa	*Siphoviridae*	Cefotaxime	-Synergistic reduction of bacterial growth	Knezevic et al., 2013
S. aureus	*Myoviridae*	Linezolid	-Stopped MRSA hindpaw foot infection -Decreased bacterial density	Chhibber et al., 2013
Pseudomonas Fluorescens	*Podoviridae*	Kanamycin	-Decreased bacterial survival -Limited resistance to antibiotic	Zhang and Buckling, 2012
S. aureus	*Myoviridae*	Gentamicin	-Decreased bacterial density	Kirby, 2012

			-Prevention of phage-resistant variants	
E. coli	Not descibed	Cefotaxime	-Enhanced eradication of bacterial biofilms	Ryan et al., 2012
Klebsiella pneumoniae	*Podoviridae* (T7-like)	Ciprofloxacin	-Eradication of bacteria -Prevention of resistant variants to antibiotic	Verma et al., 2009
E. coli	Not descibed	Ofloxacin, gentamicin, ampicillin	- Reduced number of antibiotic-resistant variants	Lu and Collins, 2009
E. coli	*Myoviridae*	Cefotaxime	- Phage-antibiotic synergy -Eradication of bacterial biofilms	Comeau et al., 2007
E. coli	Not described	Enrofloxacin	-Total protection of birds	Huff et al., 2004

Chapter 2 – Sequential combined effect of phages and antibiotics on the inactivation of *E. coli*

2.1. Abstract

The emergence of antibiotic resistance in bacteria is a global concern and the use of bacteriophages alone or combined with antibiotics is attracting increasing attention as an alternative approach to inactivate resistant bacteria. However, phage-resistant mutants have been considered a major concern when phage treatment is employed. The aim of this study was to evaluate the effect of combined treatments of phages and antibiotics in the inactivation of *Escherichia coli*. *E. coli* is one of the main responsible for moderate and serious infections in the hospital and community environments, being involved in the rapid evolution of fluoroquinolones and third generation cephalosporin resistance. For this, ciprofloxacin at lethal and sublethal concentrations, added at different times (0, 6, 12 and 18 h), was tested in combination with the phage Ec-Bio to inactivate *E. coli*. The efficacy of the combined treatment varied with the antibiotic concentration and with the time of antibiotic addition, avoiding bacterial regrowth when the antibiotic was used at MIC and added after 6 h of phage addition, and causing less bacterial resistance than phages and antibiotics applied alone (4.0×10^{-7} for the combined treatment, 3.9×10^{-6} for the antibiotics alone and 3.4×10^{-5} for the phages alone). The combined treatment with phages and antibiotics can be effective in reducing the bacterial density, but also to prevent the emergence of resistant variants. However, the antibiotic concentration and the time of antibiotic application are essential factors to be considered in the combined treatment.

Keywords: Bacteriophages, antibiotics, combination therapies, *Escherichia coli*, resistance

2.2. Introduction

Escherichia coli is a non-pathogenic commensal bacterium characterized by its diversity and versatility since it is able to colonize human and other animal intestines. However, this species developed some variants that colonize outside the gastrointestinal system. These strains harbor more virulence factors, causing severe diseases such as diarrhea, urinal tract infections, septicemia, pneumonia and meningitis (Cabal *et al.*, 2016). Additionally, the development of resistance to antibiotics within intestinal and extra-intestinal *E. coli* strains is currently increasing, especially against beta-lactam antibiotics and to quinolones (Allocati *et al.*, 2013; Iredell *et* al., 2016). According to the World Health Organization (WHO), one of the most problematic areas of drug resistance is the rapid evolution of fluoroquinolones and third generation cephalosporin

resistance in Enterobacteriaceae, namely in *E. coli*, which is the main representative species of this family (Fair and Tor, 2014b; WHO, 2014). Actually, *E. coli* strains, namely antibiotic resistant strains, are among the main responsible for moderate and serious infections in the hospital and community environment (Allocati *et al.*, 2013; Cabal *et al.*, 2016).

Infections with resistant bacteria, namely those caused by Gram-negative bacteria, are difficult to treat, causing severe illness and requiring costly and sometimes toxic alternatives, such as last resort antibiotics, such as colistin which is used against Gram-negative bacteria. However, bacterial strains resistant to last resort antibiotics have been isolated worldwide (Levine, 2006; Wang *et al.*, 2018). The development of novel, but still conventional, antibiotics is not likely to solve the problem and it is probably only a matter of time until they will also be ineffective. Bacteria will inevitably find ways of resisting the conventional antibiotics, which is why new approaches are urgent to be used as alternative to antibiotics.

The use of phages as antibacterial agents can be a very promising alternative for the treatment of infections, to be used alone or in combination with antibiotics. Phage treatment is based on the use of lytic phages to combat bacterial infections, including multidrug-resistant bacteria, and has many advantages compared to antibiotics, such as: phages persist as long as the targeted bacteria are present; are specific for their target bacteria, not effecting the host natural flora; and are not pathogenic for human (Almeida *et al.*, 2009). Until the advent of antibiotics, phage therapy was widely used, especially in the Eastern Europe countries and recently, the emergence of pathogenic bacteria resistant to antibiotics, including multidrug resistant bacteria, has motivated the western scientific community to reevaluate phage therapy as a valid option for the treatment of bacterial infections (Sulakvelidze et al., 2001; Ackermann, 2003). Currently, the potential use of phage therapy in agriculture, veterinary biocontrol, food safety and in clinical treatment of human infections is also being studied (Mole and Maskell, 2001; Deresinski, 2009; Balogh et al., 2010; Gill and Hyman, 2010; Kutter et al., 2010; Mahony et al., 2011). However, a few studies have demonstrated that phages can be used to successfully prevent or control *E. coli*, even antibiotic resistant strains (Brüssow, 2005; Pererva, Miryuta and Miryuta, 2008; Rahmani *et al.*, 2015) and, to the best of our knowledge, only one study, of our group, was done using the combination of phages and antibiotics to prevent the emergence of phage-resistant *E. coli* mutants (Valério *et al.*, 2017).

The emergency of phage-resistant mutants is nowadays a major concern regarding the use of phages to control bacterial infections. It has been shown that the use of phage cocktails can reduce the development of phage-resistant mutants (Pereira *et al.*, 2016a, 2016b, 2017b; Duarte

et al., 2018) as well as the combined use of phages and antibiotics. A few studies reported a synergetic effect of the combined use of antibiotics and phages (Huff *et al.*, 2004; Comeau *et al.*, 2007; Verma *et al.*, 2009; Lu and Collins, 2009; Ryan *et al.*, 2012; Zhang and Buckling, 2012; Kirby, 2012; Chhibber *et al.*, 2013; Knezevic *et al.*, 2013; Torres-Barceló *et al.*, 2014, 2016; Kamal and Dennis, 2015; Nouraldin *et al.*, 2016; Chaudhry *et al.*, 2017; Valério *et al.*, 2017; Oechslin *et al.*, 2017), but only four of these showed that the combination of use of antibiotics and phages reduced the emergence of phage-resistant mutants (Verma *et al.*, 2009; Kirby, 2012; Viertel *et al*., 2014; Torres-Barceló *et al.*, 2016; Oechslin *et al.*, 2017). Oechslin et al. (2017) showed that the phages cocktail PP 1131 was active against *Pseudomonas aeruginosa* infection in endocarditis and highly synergistic with ciprofloxacin. In this study, phage-resistant mutants regrew after 24 h but were prevented by the combination with ciprofloxacin. Viertel et al. (2014) also observed a reduction of the combined therapy in the emergence of phage-resistant bacteria. These authors stated that the decrease in bacterial resistance to phages and/or antibiotics in dual therapy is due to the fact that a strain that is non susceptible to one antimicrobial agent can be eliminated by the second one. Torres-Barceló et al. (2016), showed a strong synergism effect of the combination of antibiotics and phages on *Pseudomonas aeruginosa* PAO1 population density and in limiting its recovery rate. Another study, Kirby, (2012) observed the that after the treatment with the combination of gentamicin and phage SA5, the phage-resistant isolates were extinct.

In our previous study, we demonstrated the efficacy of the combination of the two therapies depends on the antibiotic resistance status of the targeted bacteria to the employed antibiotic and of the antibiotic type (bactericide or bacteriostatic), causing the same or less bacterial resistance than phages and antibiotics applied alone (Valério *et al.*, 2017). So, in the present study, we extended our research to the use of a new phage and we studied the efficiency of the combination of the two therapies using different concentrations of antibiotic and phages to control the emergence of phage-resistant bacteria. Additionally, we also consider the influence of time of antibiotic addition in the inactivation of *E. coli*. For this, a bioluminescent *E. coli* (Alves *et al.*, 2008) was selected as a model microorganism to evaluate in real-time phage therapy kinetics through the measurement of bioluminescence, thus avoiding the laborious and time-consuming conventional method of counting colony-forming units (CFU). The bioluminescent *E. coli* is resistant to ampicillin, chloramphenicol, kanamycin and piperacillin.

2.3. Material and methods

2.3.1. Bacterial strains and growth conditions

The bioluminescent *E. coli* used as phage host in this study was a genetically transformed *E.coli* Top10 (Alves *et al.*, 2008). Other bacterial strains were used to determine the phage host range: *Salmonella enterica* serovar Typhimurium (ATCC 13311 and ATCC14028), *Escherichia coli* (ATCC 25922 and ATCC 13706), *Aeromonas hydrophila* (ATCC 7966), *Vibrio fischeri* (ATCC 49387), *Vibrio parahaemolyticus* (DSM 27657), *Vibrio anguillarum* (DSM 21597), *Photobacterium damselae damselae* (DSM 7482), *Shigella flexneri* (DSM 4782), *Listeria innocua* (NCTC 11288), *Listeria monocytogenes* (NCTC1194) and *Aeromonas salmonicida* (CECT 894), five strains of *Salmonella enterica* serovar. Enteriditis isolated from food (gently provided by Controlvet Laboratory), *E. coli* (AE11, AN19, AD6, AF15, BC30, AC5, AJ23, BN65, BM62), *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus mirabilis* and *Providencia* sp and *Pseudomonas aeruginosa* isolated in previous works from water samples collected in Ria de Aveiro (Louvado et al., 2012; Pereira et al., 2016b).

The bioluminescent *E. coli*, used as phage host in the phage treatment experiments was grown in Tryptic Soy Broth (TSB, Liofilchem, Italy). A stock culture was stored at - 80 °C in 10% glycerol. Before each assay, stock culture of bioluminescent bacteria was aseptically inoculated into of 30 mL of TSB and was grown overnight at 25 °C at 120 rpm stirring. Then, an aliquot (300 µL) of this culture was transferred to 30 mL of fresh TSB and was grown overnight at 25 °C under stirring (120 rpm), to reach 10^8 luminescence units (URLs), corresponding approximately 8 log colony forming units (CFU)/mL.

The other bacterial strains used in this study, were maintained in solid Tryptic Soy Agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 30 mL of TSB and were grown and incubated as described above. Then an aliquot of this culture was subcultured in 30 mL of fresh TSB, as described above, to reach an optical density (O.D. 600 nm = 0.8), corresponding to about 10^{8-9} cells per mL.

2.3.2. Correlation between bioluminescence and CFU

An overnight culture of the bioluminescente *E. coli* (10^8 CFU/mL) was serially diluted (10^{-1}–10^{-8}) in phosphate buffered saline [PBS, 137 mM NaCl (Sigma), 2.7 mM KCl (Sigma), 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4 (Sigma), pH 7.4]. The non-diluted (10^0) and diluted aliquots were read on a luminometer (1 mL) (TD-20/20 Luminometer, Turner Designs, Inc., Madison, WI, USA) to determine the bioluminescence signal. Simultaneously, 1 mL of each dilution was pour plated in TSA and incubated 25 °C for 24 h. Three independent experiments were performed, and the results were averaged.

2.3.3. Phage preparation

The phage was isolated in a previous work from water samples collected from the Corte das Freiras aquaculture (Silva *et al.*, 2014b). The phage was identified as a T4-like phage with 95% of homology with the Enterobacteriaceae phage Ec-Bio (accession number HM563683) (Silva *et al.*, 2014b). Silva et al. (2014b) demonstrated that the phage was effective in reducing the bioluminescent *E. coli*.

The phage suspension was prepared from the phage stock isolated in previous works in SM buffer [0.1 M NaCl (Sigma), 8 mM $MgSO_4$ (Sigma), 20 mM Tris-HCl (Sigma), 2% (w/v) gelatin, pH 7.5]. Three hundred microliters of the phage stock were added to thirty milliliters of TSB with double concentration and one milliliter of the *E. coli* in exponential growth phase. Suspension was grown overnight and incubated at 25 ˚C at 50 rpm. The lysate was centrifuged at 13.000 rpm for 30 min at 4 ˚C, to remove intact bacteria or bacterial debris. Phage suspensions were stored at 4 °C. Phage suspension titer was determined by the double-layer agar method (Adams, 1959). Successive dilutions of the phage suspension were performed in PBS and 500 µL of each dilution together with 200 µL of fresh bacterial culture were mixed with 5 mL of TSB 0.6% top agar layer [30 g/L TSB (Liofilchem), 6 g/L agar (Liofilchem), 0.05 g/L $CaCl_2$ (Sigma), 0.12 g/L $MgSO_4$ (Sigma), pH 7.4], and placed over a TSA plate. The plates were incubated at 25 ºC for 12 h. After incubation, the number of plaques was counted and the results expressed as plaque-forming units (PFU)/mL.

2.3.4. Phage host range determination and efficiency of plating analysis

Phage host range was determined by spot test according Vieira *et al.* (2012) The plates were incubated at 25 °C and examined for plaques after 6 - 12 h. Bacterial sensitivity to a phage was established by a lysis cleared zone at the spot. According to the clarity of the spot, bacteria were differentiated into two categories: clear lysis zone (+) and not lysis zone (-). Then, efficiency of plating (EOP) was determined for bacteria with positive spot tests (occurrence of a clear lysis zone), using the double-layer agar method (Adams, 1959). The EOP was calculated (average PFU on target bacteria / average PFU on host bacteria) according to (Kutter, 2009). For each phage, three independent experiments were done.

2.3.5. Antibiotic preparation

The antimicrobial agent used in this study was a ciprofloxacin (Cip, Sigma–Aldrich, St. Louis). Stock solutions were prepared following the manufacture instructions and used for preparation of the dilutions and stored in the freezer at - 80 °C.

2.3.6. Determination of Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) for the antibiotic under study was determined in triplicate by the microdilution method in Mueller Hinton broth according to Clinical Laboratory Standardization Institute (CLSI) and European Committee on Antimicrobial (EUCAST) (CLSI, 2013; EUCAST, 2015). To determine the MIC of ciprofloxacin, different concentrations of the antibiotic (0.10 - 0.50 μg/mL) were prepared by serial dilution in Mueller Hinton broth. The tubes were inoculated with 100 μL of the bioluminescent *E. coli.* Control for bacterial growth without antibiotic were also included. The MIC was defined as the lowest concentration that showed no growth in the Mueller Hinton broth. Three independent experiments were done.

2.3.7. Kill curves with phage and ciprofloxacin in Tryptic Soy Broth (TSB)

Bioluminescent *E. coli* inactivation was determined using the phage and ciprofloxacin at MIC, 2xMIC, 1/5 MIC and 1/10 MIC, at a MOIs of 1 and 100 in TSB. In order to obtain a MOI of 1 and 100, 6.5 μL of 10^8 URLs of the overnight bioluminescent *E. coli* culture (final concentration of 10^5 URLs) and 12 μL for MOI of 1 and 300 μL for MOI of 100, of 10^8 PFU/mL of the phage suspension (final concentration of 10^5 and 10^7 PFU/mL, respectively) were inoculated in sterilized glass erlenmeyers with 30 mL of TSB and incubated at 25 °C at 50 rpm stirring (B+P). When the assays were performed with ciprofloxacin (B+P+Cip), the the same phage and bacteria concentrations were used. For these assays, three control samples were included: the bacterial control (BC), the phage control (PC) and antibiotic control (CipC). The bacterial control was inoculated with bioluminescent *E. coli* but not with phage, the phage control was inoculated with phage and without bacteria and the antibiotic control was inoculated with the bioluminescent *E. coli* and antibiotic. The controls and test samples were incubated exactly in the same conditions. Aliquots of test sample and bacterial and phage controls were collected at time zero and after 6, 12, 18, 24 and 36 h of incubation for bioluminescence signal measurement (BC, B+P, CipC, B+P+Cip) and phage quantification (PC, B+P, B+P+Cip). The bioluminescence signal was measured in the luminometer (TD-20/20 Luminometer, Turner Designs, Inc., Madison, WI, USA) in triplicate. The phage titer was determined, in duplicate, through the double agar layer method and plates

were incubated for 12 h at 25 °C and expressed in PFU/mL. Three independent experiments were performed.

2.3.8. kill curves with different ciprofloxacin addition times

E. coli inactivation was determined using phage and ciprofloxacin at MIC and at MOI of 100 in TSB. The bacterial and phage suspension was inoculated as described (see section 2.3.7), with a final concentration of 10^5 CFU/mL and 10^7 PFU/mL, respectively. When the assays were performed with ciprofloxacin (B+P+Cip), the concentrations of phage and bacteria described above (see section 2.3.7) were used. The ciprofloxacin was added at time zero and 6, 12 or 18 h of incubation. Three control samples were included: the bacterial control (BC), the phage control (PC) and antibiotic control (CipC). The bacteria control, phage control, antibiotic control and test samples were prepared as described above (see section 2.3.7). All controls were incubated exactly in the same conditions as the test samples. Aliquots of test samples, bacterial and phage control were collected at time zero and after 6, 12, 18, 24 and 36 h of incubation. The phage titer and bioluminescence signal were determined as described above (see section 2.3.7). Three independent experiments were performed.

2.3.9. Determination of the rate of emergence of bacterial mutants

The development of resistant mutants of *E. coli* to phages, to ciprofloxacin at 1/5 MIC and MIC, to phage and ciprofloxacin at 1/5 MIC and MIC was evaluated. To determinate the frequency of phage-resistant bacteria, ten isolated colonies from a plate with sensitive bacteria were selected and inoculated into ten tubes with 5 mL of TSB, grown at 25 °C for 18 h. The previously prepared ten TSB cultures of bacteria were also used to determine the development of phage-resistant bacteria in the presence of ciprofloxacin. To determine the phage resistant mutants, aliquots of 100 µL from the 10^0 to10^{-2} dilutions of the bacterial culture aliquots of 100 µL of the phage from a stock solution of 10^9 PFU/mL were inoculated in tubes with TSB 0.6%, plated on TSA plates and incubated at 25 °C for 48 h (because some of the phage-resistant mutants grow very slowly). Simultaneously, 100 µL aliquots of 10^{-5} to 10^{-7} dilutions of the bacterial culture were plated by incorporation on TSA plates without phage or without phage and antibiotic, and incubated at 25 °C for 24 h. The previous prepared ten TSB cultures of bacteria were also used to determine the phage-resistant mutants in the presence of ciprofloxacin. The same procedure was used, but the cultures were added of antibiotic. To determinate the frequency of *E. coli* mutants resistant to the antibiotic (without phage), aliquots of 100 µL from the 10^{-1} to 10^{-5} dilutions of the

bacterial culture were plated on TSA plates and incubated at 25 °C for 48 h. To determinate the frequency of *E. coli* mutants resistant to the antibiotic (with phage) aliquots of 100 µL from the 10^0 to 10^{-2} dilutions of the bacterial culture were plated on TSA plates by double agar layer method and incubated at 25 °C for 48 h. The calculation of the frequency of mutants was done by dividing the number of resistant bacteria (obtained from the ten isolated colonies) by the total 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.

2.3.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.04. Normal distributions were assessed by Kolmogorov-Smirnov test and homogeneity of variances were assessed by Levene's test. A value of $p < 0.05$ was considered statistically significant and in these cases Tukey's multiple comparison test was used for a pairwise comparison of the means. The existence of significant differences on bacterial concentration in killing curves assays was analyzed using a two-way ANOVA with repeated measures. The significance of the differences was evaluated by comparing the results obtained in the test samples and control samples for the different times between treatments of each of the three independent assays, along different time. One-way ANOVA was used to examine differences between the concentration of resistant bacteria in presence of the antibiotics, in the presence of the phage alone and in presence of both simultaneously.

2.4. Results

2.4.1. Phage Host Range and Efficiency of Plating (EOP) Analysis

Spot tests indicated that phage had the capacity to form completely cleared zones on 5 (*E. coli* bioluminescent, *E. coli* BC30, *E. coli* ATCC 25922, *S.* Typhimurium ATCC 13311 and *S.* Enteriditis CVD) of the 32 strains (Table 2.1). However, EOP results indicated that the phage formed phage lysis plaques in only 2 strains (*E. coli* ATCC 25922 and *S. Typhimurium* ATCC 13311) of the 32 strains tested. Phage infected *E. coli* ATCC 25922 and *S. Typhimurium* ATCC 13311 with an efficacy of 2.27×10^3 and 3.45×10^{-3}, respectively (Table 2.1).

Table 2.1: Host range and efficiency of plating of *E. coli* phage determined on 32 bacterial strains. Clear lysis zone (+) and not lysis zone (-).

Species	Infectivity of phage	Efficacy of plating (%)
Escherichia coli bioluminescent (host)	+	100
Escherichia coli AE11	-	0
Escherichia coli AN19	-	0
Escherichia coli AD6	-	0
Escherichia coli AF15	-	0
Escherichia coli BC30	+	0
Escherichia coli AC5	-	0
Escherichia coli AJ23	-	0
Escherichia coli BN65	-	0
Escherichia coli BM62	-	0
Escherichia coli ATCC 25922	+	2.27×10^{3}
Escherichia coli ATCC 13706	-	0
Enterobacter cloacae	-	0
Citrobacter freundii 6F	-	0
Proteus mirabilis	-	0
Providencia sp.	-	0
Salmonella Typhimurium ATCC 13311	+	3.45×10^{-3}
Salmonella Enteriditis CVA	-	0
Salmonella Enteriditis CVB	-	0
Salmonella Enteriditis CVC	-	0
Salmonella Enteriditis CVD	+	0
Salmonella Enteriditis CVE	-	0
Salmonella Typhimurium ATCC 14028	-	0
Shigella flexneri DSM 4782	-	0
Vibrio parahaemolyticus DSM 27657	-	0
Vibrio anguillarum DSM 21597	-	0
Aeromonas salmonicida CECT 894	-	0
Aeromonas hydrophilla ATCC 7966	-	0
Listeria innocua NCTC 11288	-	0
Listeria monocytogenes NCTC 1194	-	0
Photobacterium damselae damselae DSM 7482	-	0
Pseudomonas aeruginosa	-	0

2.4.2. Determination of Minimum inhibitory concentration (MIC)

The assessed MIC of the bioluminescent *E. coli* to ciprofloxacin was 0.25 mg/L.

2.4.3. Correlation between bioluminescence and CFU

A linear correlation between viable counts and the bioluminescence signal of overnight cultures of the bioluminescent *E. coli* was observed (Figure 2.1).

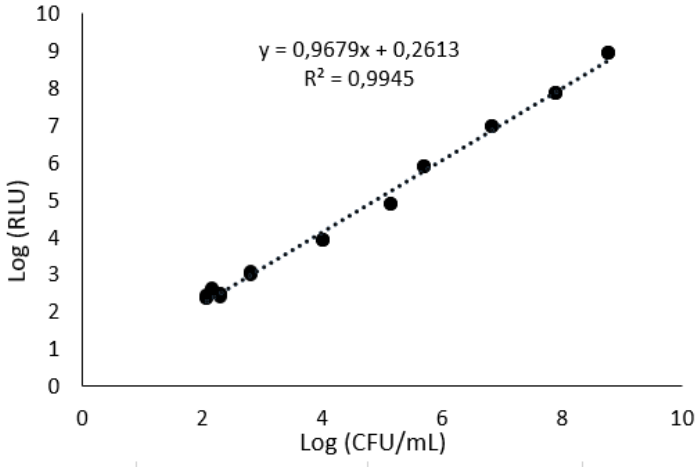

Figure 2.1: Association between the bioluminescence signal and viable counts of an overnight culture of a transformed bioluminescent *E. coli*. Bioluminescence is expressed in RLUs and viable counts in CFU/mL.

2.4.4 Kill curves with phage and ciprofloxacin in TSB

The results of the experiments show that the phage Ec-Bio was able to cause a decrease in the bioluminescence signal (ANOVA, $p < 0.05$) after 12 h of phage treatment (B+P), relatively to the bacterial control (BC) (Figure 2.2 AI-DI). The profile of variation was similar for both MOI (by 2.5 log units for MOI of 1 and 2.8 log units after 12 h for MOI of 100). However, after this period, *E. coli* regrowth was observed, reaching values similar (ANOVA, $p > 0.05$) to that obtained in bacterial control after 36 h of incubation (Figure 2.2 AI-DI).

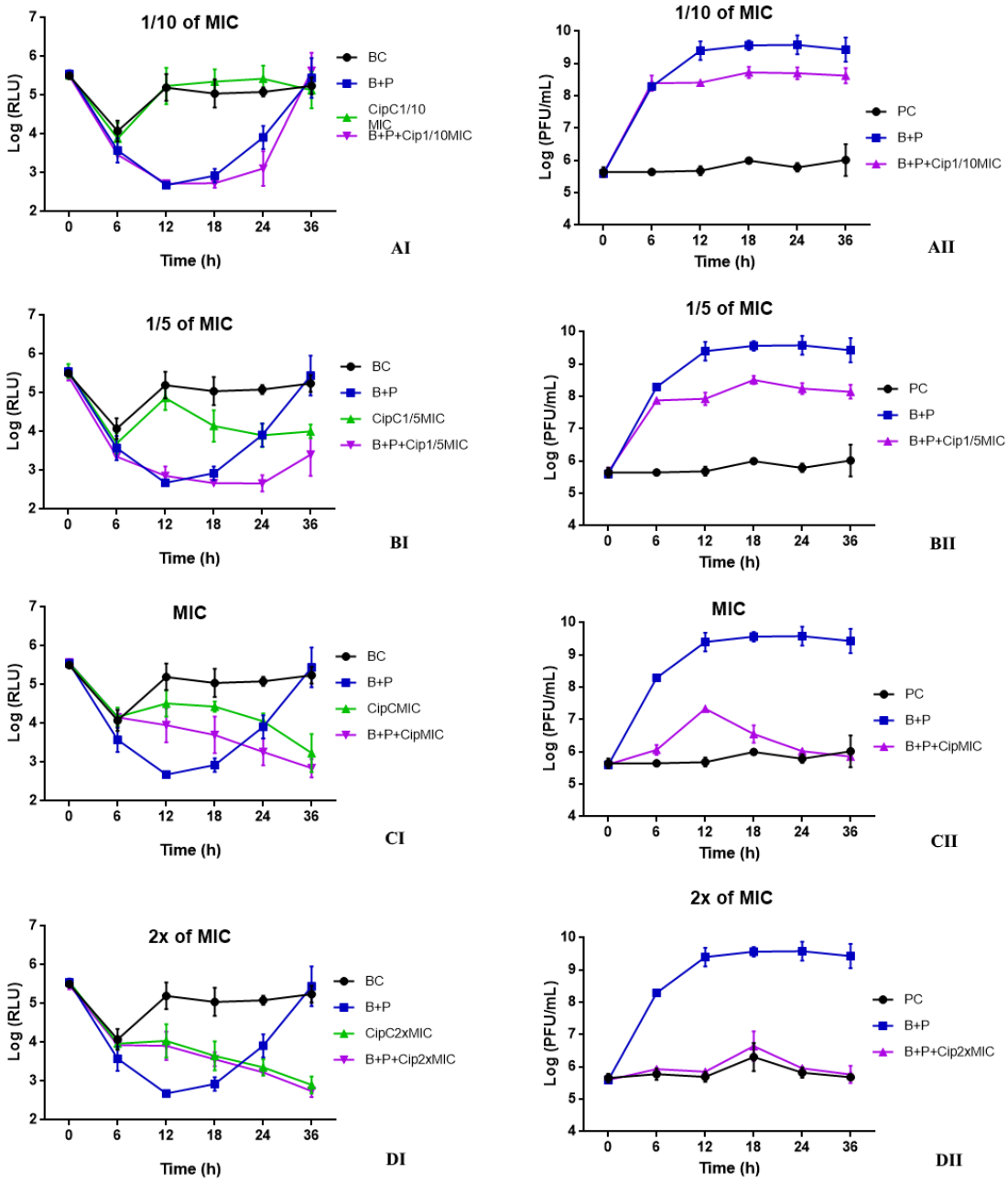

Figure 2.2: Effect of phage alone (MOI of 1) and combined phage and ciprofloxacin treatments. at different concentrations (A - 1/10 of MIC, B - 1/5 of MIC, C – MIC and D - 2x of MIC) on the inactivation of bioluminescent *E. coli* (I) and the phage concentration (II) in TSB during 36 h. BC – bacterial control, PC – Phage control, B+P – bacteria plus phage, CipC – bacteria plus ciprofloxacin, B+P+Cip – bacteria plus phage plus antibiotic. Values represent the mean of three independent experiments.

When the phage was combined with antibiotic at a sublethal concentrations (1/10 and 1/5 of MIC) at both MOIs, the *E. coli* inactivation was similar in both MOIs (ANOVA, $p > 0.05$) (Figure 2.2AI and BI and Figure 2.3AI and BI). The maximum rate of bacterial inactivation when phage was combined with ciprofloxacin at 1/10 (B+P+Cip1/10MIC) and 1/5 of MIC (B+P+Cip1/5MIC),

relatively to the bacterial control, was ~2.7 and 2.4 log RLU, respectively, achieved after 12 h of treatment (Figure 2.3AI and 2.3BI). However, after 36 h of incubation for the both sublethal concentrations and for both MOIs, *E. coli* regrowth was observed, reaching bacterial densities similar (ANOVA, $p > 0.05$) to that obtained in bacterial control after 36 h of incubation (Figure 2.2AI and BI and Figure 2.3AI and BI). For this treatment, significant differences (ANOVA, $p < 0.05$) relatively to the treatment with antibiotic alone (CipC1/10MIC and CipC1/5MIC) were observed during 36 h of incubation. The bacterial inactivation in the treatment with antibiotic (CipC1/10MIC and CipC1/5MIC) was significantly lower than that observed in the treatment with phage, with or without the antibiotic (ANOVA, $p < 0.05$). The bacterial inactivation in the treatment with phage and antibiotic (B+P+Cip1/10MIC and B+P+Cip1/5MIC) was significantly higher (ANOVA, $p < 0.05$) than that observed when the phage was used alone (B+P) after 24 and 36 h of incubation. No decrease in bacterial inactivation was observed for the treatment with ciprofloxacin at these two sublethal concentrations (B+P+Cip1/5MIC and B+P+Cip1/10MIC) when compared with the bacterial control (BC).

When the phage was combined with ciprofloxacin at lethal concentrations, MIC (B+P+CipMIC) and at 2xMIC (B+P+Cip2xMIC), the increase in the MOI from 1 to 100 promoted a decrease in *E. coli* bioluminescence after 12, 18 and 24 h of incubation (Figure 2.2CI and 2.2DI and 2.3CII and 2.3 DII, ANOVA, $p < 0.05$), but the pattern of variation between treatments was similar (ANOVA, $p > 0.05$) for both MOIs.

The maximum rate of bacterial inactivation for the mix of the phage and ciprofloxacin at MIC (B+P+CipMIC) was ~2.5 log RLU achieved after 18 h of treatment at MOI of 100. In this treatment, as well as in the treatment with the antibiotic alone (CipCMIC), no regrowth of bacteria was observed until the end of the treatment (Figure 2.2CI and 2.3CII). When the phage was used alone, a significant regrowth, after 12 h of incubation, was observed (ANOVA, $p < 0.05$).

The maximum rate of *E. coli* inactivation when the phage was combined with ciprofloxacin at 2xMIC (B+P+Cip2xMIC), relatively to bacteria control, was ~2.7 log RLU (Figure 2.2DII, ANOVA, $p < 0.05$), achieved after 18 h at MOI of 100, which was similar to that obtained in antibiotic control (Cip2xMIC). For this treatment, no significant differences (ANOVA, $p > 0.05$) relatively to the treatment with antibiotic alone (CipC2xMIC) were observed during 36 h of incubation (Figure 2.2DI and 2.3DII). However, the bacterial inactivation in the treatment with antibiotic, with or without the phage, was significantly higher than that observed when the phage was used alone (ANOVA, $p < 0.05$). When the phage was combined with antibiotic at 2xMIC (B+P+Cip2xMIC) or

the antibiotic was used alone (CipC2xMIC), no regrowth of bacteria was observed until the end of the treatment.

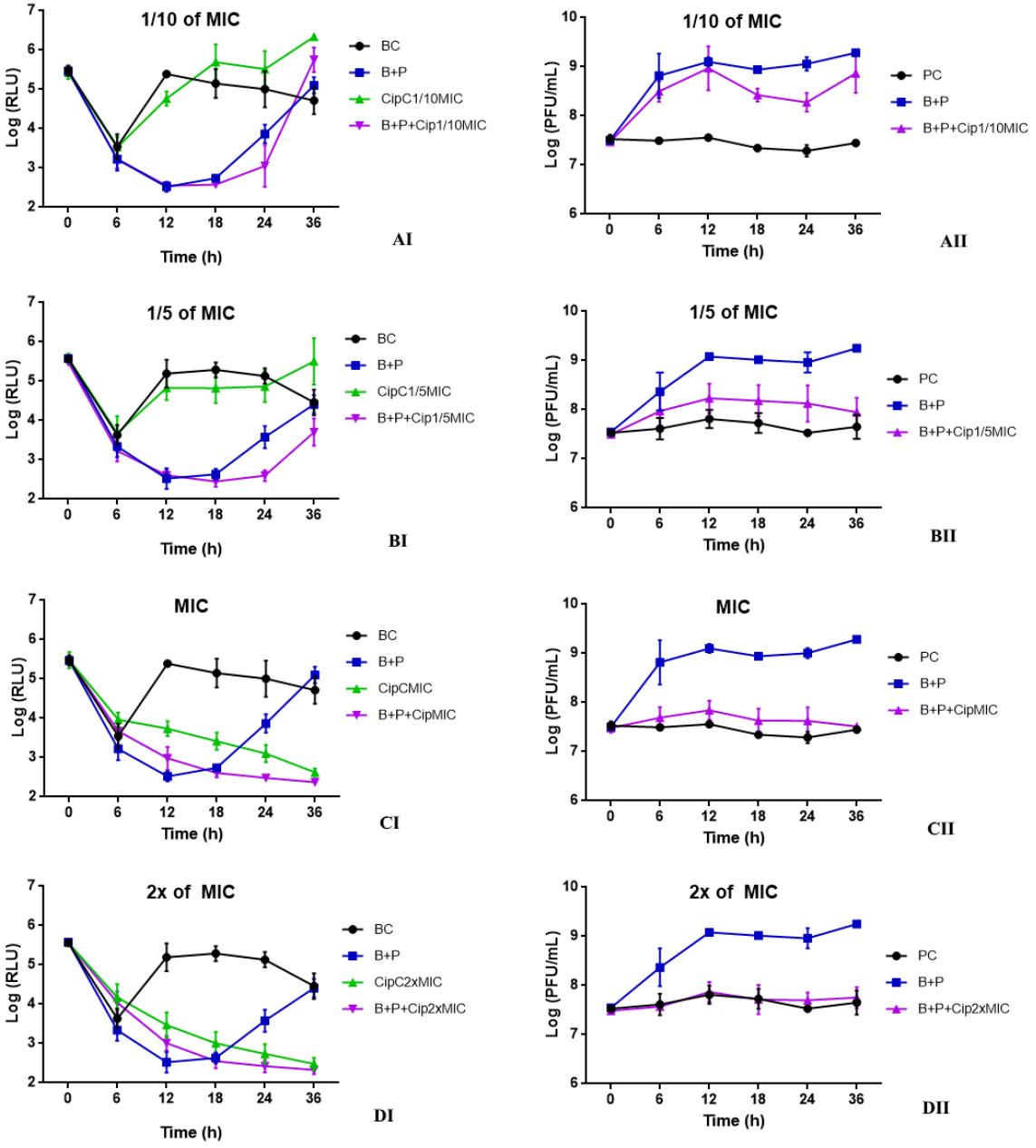

Figure 2.3: Effect of phage alone (MOI of 100) and combined phage and ciprofloxacin treatments. at different concentrations (A - 1/10 of MIC, B - 1/5 of MIC, C – MIC and D - 2x of MIC) on the inactivation of bioluminescent *E. coli* (I) and the phage concentration (II) in TSB during 36 h. BC – bacterial control,PC – Phage control, B+P – bacteria plus phage, CipC – bacteria plus ciprofloxacin, B+P+Cip – bacteria plus phage plus antibiotic. Values represent the mean of three independent experiments.

For both MOIs, the phage control (PC) remained constant throughout the experiment (ANOVA, $p > 0.05$), but when the phage was incubated in the presence of the host without antibiotic, a significant increase (~3.4 log PFU/mL for MOI of 1 and ~1.8 log PFU/mL for MOI of 100, ANOVA, $p < 0.05$) was observed (ANOVA, $p < 0.05$) (Figure 2.2 and 2.3).

When the phage was incubated in the presence of the host and antibiotic at sublethal concentrations, the phage concentration was significantly higher (ANOVA, $p < 0.05$) than those observed in the phage control (PC) (Figure 2.2 AII and BII and 2.3AII and2.2BII). Increases of ~ 2.0 and 0.5 log PFU/mL, respectively for MOIs of 1 and 100 with ciprofloxacin at 1/10, and increases of ~ 2.0 and 1 log PFU/mL, respectively, for MOIs 1 and 100 with ciprofloxacin at 1/5, however, for both MOIs, when the phage was incubated in the presence of the host and ciprofloxacin at lethal concentration, MIC (B+P+CipMIC) and 2xMIC (B+P+Cip2xMIC), the phage concentration remained constant (ANOVA, $p > 0.05$) throughout the experiment (Figure 2.2 CI and DI and 2.3CII and DII). In these cases, the phage concentrations were similar to that of the phage control (PC) (ANOVA, $p > 0.05$).

2.4.5. Influence of time of addition of ciprofloxacin on the kill curves

The rate of bacterial inactivation when the antibiotic was added after 6 h of phage addition was 2.3 log RLU relatively to the bacteria control, which was significantly higher (ANOVA, $p < 0.05$) than those obtained in the other conditions tested (B+P, B+P+Cip0h, B+P+Cip12h and B+P+Cip18h) after 36 h of incubation (Figure 2.4 AI, BI and CI). When the antibiotic was added after 6 h of phage addition, no regrowth of bacteria was observed until the end of the treatment, contrarily to those observed for the other two conditions.

When the antibiotic was added after 12 h of phage addition, the bacterial inactivation, after 36 h of incubation, decrease ~1.9 log RLU relatively to the bacteria control and was similar (ANOVA, $p > 0.05$) to that obtained when the antibiotic was added same time of the phage (decrease ~2.1 log RLU, relatively to the bacteria control). However, the bacterial inactivation in this treatment was significantly higher (ANOVA, $p < 0.05$) than those obtained in treatment with phage without antibiotic (bacteria concentration was similar to the bacteria control) (Figure 2.4 AI, BI and CI).

When the antibiotic was added after 18 h of phage addition, after 36 h of treatment, the bacterial density was similar (ANOVA, $p > 0.05$) to that obtained in bacteria control (BC) and to that obtained in the treatment with phage without antibiotic (B+P) (Figure 2.4 CI). In this

condition, until 18 h of incubation, bacterial inactivation was yet similar to that obtained in B+P (ANOVA, $p > 0.05$) (Figure 2.4 CI).

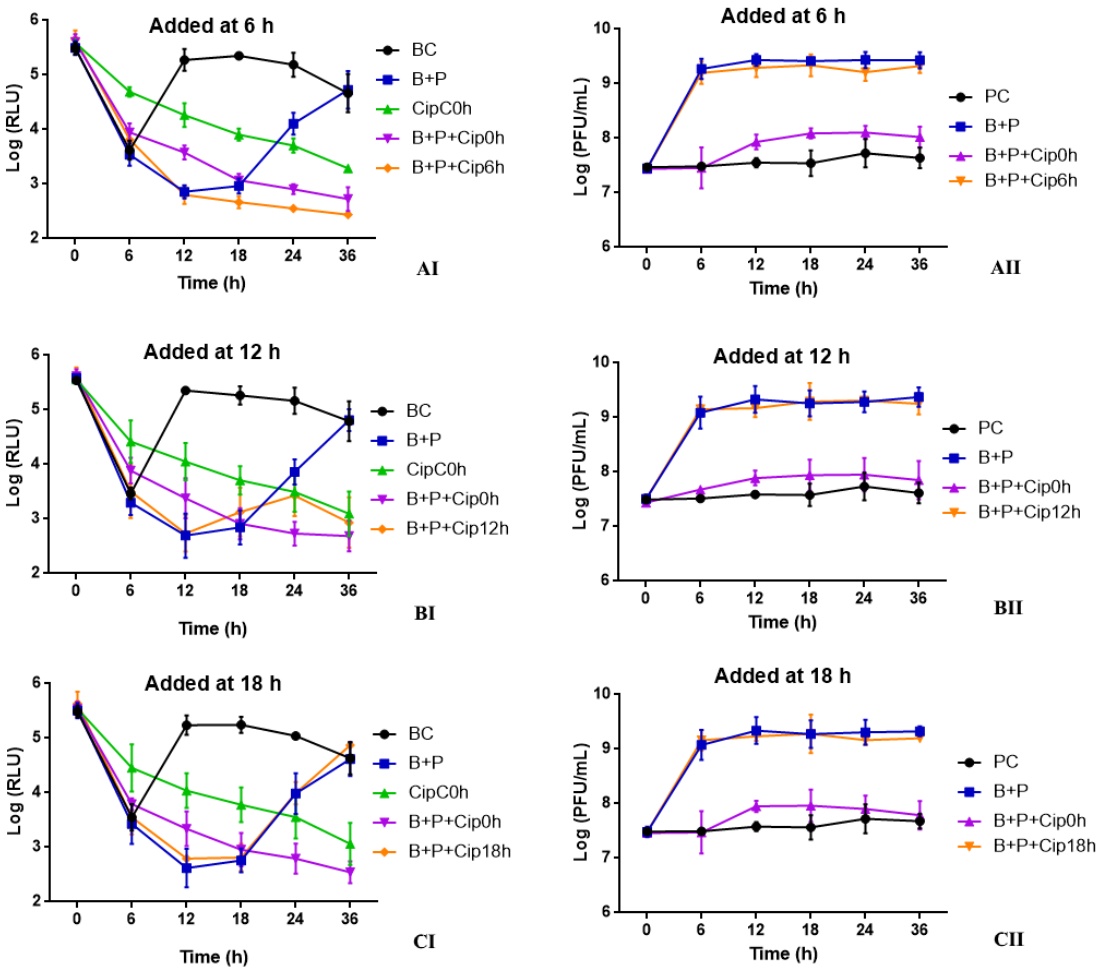

Figure 2.4: Effect of time of addition of ciprofloxacin at MIC on the inactivation of bioluminescent *E. coli* (I) and the phage concentration (II) in TSB during 36 h. A - Antibiotic added after 6 h of phage addition, B - Antibiotic added after 12 h of phage addition, C - Antibiotic added after 18 h of phage addition. BC – Bacterial control, PC – Phage control, B+P – Bacteria plus phage, CipC0h – Bacteria plus antibiotic added at the same time of the phage, B+P+Cip0h – Bacteria plus phage plus antibiotic added at the same time of the phage, B+P+Cip6h – Bacteria plus phage plus antibiotic added at 6 h of phage addition, B+P+Cip12h – Bacteria plus phage plus antibiotic added at 12 h of phage addition, B+P+Cip18h – Bacteria plus phage plus antibiotic added at 18 h of phage addition. Values represent the mean of three independent experiments.

While the phage control (PC) remained constant throughout the experiment (ANOVA, $p > 0.05$) (Figure 2.4AII, BII and CII), when the bacteria were incubated in presence of the phage without antibiotic (B+P), a significant increase (increase ~1.8 log PFU/mL, ANOVA, $p < 0.05$) was observed (Figure 2.4AII, BII and CII).

When the bacteria were treated with the phage in the presence of the ciprofloxacin added 6, 12 and 18 h after phage addition (B+P+Cip6h B+P+Cip12h and B+P+Cip18h), a significant increase was observed in the phage concentration (increase ~1.7 - 2.0 log PFU/mL, ANOVA, $p < 0.05$). The number of produced phages in the three conditions (B+P+Cip6h B+P+Cip12h and B+P+Cip18h) was similar (ANOVA, $p > 0.05$) to that obtained without the antibiotic addition (B+P) and significantly higher than that observed when the antibiotic was added at the same time of the phage (increase ~0.5 log PFU/mL, relatively phage control) (Figure 2.4AII, BII and CII).

2.4.6 Determination of the rate of emergence of bacterial mutants

The bioluminescent *E. coli* showed different rates of resistant mutants emergence when subjected to ciprofloxacin alone, phage alone, and to the mix of the phage plus ciprofloxacin (Table 2.2). The development of resistant mutants of *E. coli* against the ciprofloxacin at 1/5 MIC and at MIC was significantly higher (ANOVA, $p < 0.05$) than that obtained when phage was used alone and when the mixture of phage and ciprofloxacin was used. However, the frequency of resistant mutants of *E. coli* in the presence of the phage alone was similar (ANOVA, $p > 0.05$) to that obtained when the phage was used in combination with ciprofloxacin at 1/5 MIC, but was significantly higher than that obtained when the phage was used in combination with the ciprofloxacin at MIC (ANOVA, $p < 0.05$).

Table 2.2: Frequency of transformed *E. coli* spontaneous phage-resistant mutants.

Sample	Frequency of antibiotic-mutants (CFU/mL)	Sample	Frequency of phage and antibiotic mutants (CFU/mL)	Sample	Frequency of phage-mutants (CFU/mL)
Cip MIC (0.25 µg/mL)	3.95×10^{-6}	Phage + Cip MIC	4.04×10^{-7}	Phage	3.43×10^{-5}
Cip 1/5MIC (0.05 µg/mL)	5.24×10^{-1}	Phage + Cip 1/5MIC	4.00×10^{-5}		

2.5. Discussion

With the emergence of antibiotic resistance in common bacteria, such as *E. coli*, even in commensal strains, there is a need to develop alternative treatments. Several studies have demonstrated that phages can be used to control pathogenic bacteria (Fu et al., 2010; Rivas et al., 2010; Vieira et al., 2012; Maura and Debarbieux, 2012; Lood et al., 2015; Scanlan et al., 2015; Verstappen et al., 2016; Pereira et al., 2016a, 2016c, 2017a, 2017b), but the development of phage-resistant mutants is a general shortcoming (Bikard and Marraffini, 2012; Seed et al., 2014; Pereira et al., 2016a, 2017b). The combination of phage treatment with antibiotics is a possibility to avoid the emergence of resistance, but little is known about the interaction process of phages and antibiotics in combined therapies, particularly in regard to the emergence of phage-resistant mutants. In a previous work of our group (Valério *et al.*, 2017), we demonstrated that the phage and antibiotic combinations could result in high synergistic effects in the inactivation of bacteria, but nevertheless bacterial regrowth after treatment was observed. So, in the present study, we try to understand if development of bacterial regrowth can be circumvented by the use of different antibiotic and phage concentration combinations and also by the application of the antibiotic after different times after phage addition. We showed that 1) phage and antibiotic combinations could result in positive effects in the inactivation of bacteria, preventing bacterial regrowth, but 2) the efficacy of the combination depends greatly on the concentration of antibiotic and on the time of antibiotic addition 3) not being much influenced by the phage concentration added at the beginning of the treatment (the MOI), 4) controlling effectively the development of bacterial resistance not only against antibiotics, but also against phages.

The kinetic theory indicates that the MOI could be critical to the bacterial inactivation efficiency (Cairns *et al.*, 2009). Several studies demonstrated, that the reduction of pathogenic bacteria increases in parallel with MOI or that bacterial reduction occurs sooner at higher MOI values (ChiHsin *et al.*, 2000; Pasharawipas *et al.*, 2011; Prasad *et al.*, 2011). However, Nakai (2010) related that the initial doses of phage may not be essential, because due to its self-perpetuating nature, revealed by an increasing of phage titers along with bacteria. In this study, in general, the increase in MOI from 1 to 100 not promoted a significant increase in the efficiency of phage alone. Nevertheless, when phage was combined with ciprofloxacin at lethal concentrations, at MIC and 2xMIC, the increase of the MOI caused a decrease in bacterial density. This can be explained by the fact that in the presence of the antibiotic at high concentrations, host DNA replication inhibition avoids phage replication by the bacteria. If inhibition reduces the per-host cell output of phage, then overall phage titer may not be sufficient to cause massive reductions in bacterial cell density when the phage is added at low MOI 1. At MOI 100, even if the host DNA

replication is affected, the high number of phages already present at the beginning of the treatment is enough to more efficiently inactivate the bacteria. Contrarily, when the antibiotic was not added, the treatment with low MOI was enough to inactivate efficiently the bacteria because the host during the first hours of treatment replicates efficiently the phages, which after allows their inactivation by the new produced phages. In fact, the number of phages after 6 h of treatment at MOI of 1 increased more (by 3.4 log PFU/mL in B+P sample) than at MOI 100 (increase by 1.8 log PFU/mL). This confirms the hypothesis that due to the self-perpetuating nature of phages, precise initial doses of phage may not be essential. This is one of the major advantages of phage treatment in relation to antibiotics. On the other hand, too high MOI can be a disadvantage for the success of phage treatment because the bacteria can be inactivated before replicating the phages, which is known as "lysis from without", due to a high concentration of phage lytic enzymes (Kao and McClain, 1980; Arisaka *et al.*, 2003; Nakai, 2010; Brown and Bidle, 2014), not allowing to reach an enough number of phages to inactivate the no enzyme lysed bacteria.

Although antibiotics can affect the phage production by the host bacteria (Valério *et al.*, 2017), some studies have demonstrated improved efficacy of associating phages and antibiotics to control pathogenic bacteria (Kirby, 2012; Ryan et al., 2012; Chhibber et al., 2013; Knezevic et al., 2013; Coulter et al., 2014; Torres-Barceló et al., 2014; Valério et al., 2017), preventing even the bacterial regrowth (Kirby, 2012; Knezevic *et al.*, 2013; Torres-Barceló *et al.*, 2014; Valério *et al.*, 2017). As the combination of phages and antibiotics attack different bacterial targets, the probability of bacterial regrowth, due to the emergence of resistant mutants to both agents, is reduced (Escobar-Páramo *et al.*, 2012; Hall *et al.*, 2012).

The results of present study indicate that the combined treatment with antibiotic at sublethal and lethal concentrations when added at the same time of the phages, do not increase the efficacy of bacterial inactivation relatively to the treatment with phages alone, but effectively prevent the emergence of bacterial regrowth when used at MIC or at a concentration two times higher the MIC.

An increase in bacterial inactivation was not observed for the combined therapy when the ciprofloxacin was used at a sublethal concentration (1/10 and 1/5 of MIC). However, the combination with the antibiotic at 1/5 of MIC delayed the development of resistant bacteria. When the antibiotic was added at 1/5 of MIC, the regrowth of bacteria was observed later than in the sample treated with phages and antibiotic, after 24 h against 12 h for the treatment with the phage alone. When phage was combined with ciprofloxacin at lethal concentrations, MIC and

2xMIC, after 12 h of treatment, when the maximum of bacterial inactivation was observed, bacterial density reduction by the combined therapy (B+P+CipMIC and B+P+Cip2xMIC) was lower than that caused by the phage alone (B+P), but was higher than that obtained with ciprofloxacin alone (CipC). However, when phage was combined with ciprofloxacin at 2xMIC, the difference observed between treatment with the combination phage and antibiotic and with the antibiotic alone was not so high than that observed when the antibiotic was used at MIC. Nevertheless, the combined treatment with these high antibiotic concentrations effectively prevented the development of resistant bacteria. When the combined treatment with antibiotic at lethal concentrations was added, no regrowth of bacteria was observed until the end of the treatment, as observed for the antibiotic alone at both concentrations, but contrarily to that observed when phages were used alone (regrowth after 12 h).

In this study it was clear that the addition of the antibiotic alone or in the presence of the phages affects the phage production by the bacteria according to the used concentration, but, even in these situations, the concentration of phages does not seem to be a limiting factor for bacterial inactivation. In combined treatments with ciprofloxacin at sublethal concentrations (B+P+Cip1/10MIC and B+P+Cip1/5MIC), the number of phages in the presence of the host increased significantly (~2.0 and ~0.5 -1.0 log PFU/mL, respectively), but in treatment with ciprofloxacin at MIC and 2xMIC remained constant (phage concentration similar to that of phage control). This can be explained by the fact that as the phage and antibiotic are applied simultaneously, antibiotic is likely to constrain the phage replication by the bacteria by means of nucleic acid synthesis inhibition, increasing this constraining with the increase of antibiotic concentration. The ciprofloxacin inhibits the enzymes topoisomerase II (DNA gyrase) of the host, affecting its nucleic acid synthesis, and, consequently, interfering with the replication of the phage by the host bacteria (Wishart *et al.*, 2006). Although the antibiotic addition avoids phage replication by the bacteria, namely when used at high concentrations, the number of phage added at the beginning of the treatment was enough to allow the bacterial inactivation. However, the bacterial inactivation in these conditions is delayed relatively to the treatment with the phage alone or with the combined treatment with sublethal antibiotic concentrations.

Taking into account the results obtained in these experiments, the next step was to evaluate the efficacy of bacterial inactivation using the combined treatment but adding the antibiotic after phage addition. The results of the previous experiments indicate that efficiency of the combined therapy with antibiotics and phages depend on the ability of the host bacteria to replicate the phages, which is affected by the antibiotic concentration. Similar results were

already obtained in other studies (Levin and Bull, 1996; Ryan *et al.*, 2011). So, in these experiments, antibiotic at MIC was added after, 6, 12 and 18 h of phage addition.

The *E. coli* was most effectively inactivated when the antibiotic was added after the phage for the three tested times, but the prevention of bacterial regrowth was more effective when the antibiotic was added after 6 h of phage addition. The antibiotic addition after 12 h of the phage was also effective to prevent the bacterial regrowth but not as effective as observed after 6 h. The antibiotic addition after 18 h of phage addition was not effective to prevent bacterial regrowth. Using this sequential approach to add the phage and antibiotic in combined treatment allows the bacteria to replicate the phages efficiently. The number of produced phages in the three conditions was similar to that obtained without the antibiotic addition (increase of around 2 log in both conditions) and significantly higher than that observed when the antibiotic was added at the same time of the phage (maximum increase of around 0.5 log). Overall, these results indicate that the combined treatment with antibiotic added after 6 h of phage addition is the best option to prevent bacterial regrowth. Torres-Barceló et al. (2014) obtained similar results for *Pseudomonas aeruginosa* using the combination of phage LUZ7 and the antibiotic streptomycin when the antibiotic was added after 12 h of phage addition.

The results of this study showed that the combination of antibiotics with phages is an effective alternative to prevent bacterial regrowth, controlling the emergence of resistance to the phages but also to the antibiotic. Similar results have been obtained by other authors (Kirby, 2012; Coulter *et al.*, 2014; Torres-Barceló *et al.*, 2014, 2016; Viertel *et al*., 2014; Oechslin *et al.*, 2017; Valério *et al.*, 2017), but few of these studies discriminate between prevention of resistance to phages and resistance to antibiotics resulting from the combined treatment (Torres-Barceló *et al.*, 2014, 2016; Valério *et al.*, 2017).

In this study, we compared not only the resistance of the bacteria to the combined treatment with phages and ciprofloxacin at MIC and at a subinhibitory concentration (1/5 MIC), but also the resistance to the phages alone and to the antibiotic alone. The results of this study, as indicated by some other authors (Verma et al., 2009; Zhang and Buckling, 2012; Valério et al., 2017), showed that the combined treatment limits the emergence of antibiotic resistant variants. The overall rate of emergence of resistant bacteria was significantly lower in the combined treatment (4.0×10^{-7} and 4.0×10^{-5}) than that observed when only the antibiotic (3.95×10^{-6} and 5.24×10^{-1}) were used, for both conditions, ciprofloxacin at MIC and at 1/5 MIC. However, when the ciprofloxacin was tested at the subinhibitory concentration, the overall rate of bacterial emergence and the rate of emergence of antibiotic mutants was significantly higher. Contrarily,

other studies demonstrated that phage-antibiotic combinations cause the same resistance than phage and antibiotic introduced individually (Verma et al., 2009; Zhang and Buckling, 2012; Torres-Barceló et al., 2014). However, to the best of our knowledge, no study indicated that resistance to antibiotics in combined treatment was higher than that developed without phages.

In this study, the emergence of phage-resistant mutants was also determined. When ciprofloxacin was added at a MIC together with the phage, the frequency of emergence of phage-mutants was lower (4.0×10^{-7}) than that observed when the antibiotic was not added (3.43×10^{-5}). However, this reduction was not detected for ciprofloxacin at subinhibitory concentration (1/5 of MIC). In this case, the resistance of phage-mutants in the presence of ciprofloxacin was similar to that when phages were tested alone. However, in our previous study (Valério *et al.*, 2017), using other phage of *E. coli*, the addition of ciprofloxacin at subinhibitory concentration (1/10 MIC), during phage treatment reduced the emergence of phage-mutants. Further studies, using different antibiotic concentrations and different bacteria and phages, are necessary to clarify the reason for this different behavior.

2.6. Conclusion

In conclusion, we can state that combined treatment with phages and antibiotics is effective in reducing the bacterial density, but also to prevent the emergence of resistant variants. However, the antibiotic concentration and the time of antibiotic application are essential factors to be considered in the combined treatment. In the case of the *E. coli* stains used in this study, the combined therapy with the phage Ec-Bio and the antibiotic ciprofloxacin, the efficiency of inactivation and the prevention of resistant mutants is higher when the antibiotic is used at MIC and added after 6 h of phage addition. The combined treatment can be used to inactivate *E. coli*, including antibiotic resistant strains, which are among the main responsible for moderate and serious infections in the hospital and community environments.

Chapter 3 – References

References

Abedon, S. T., García, P., Mullany, P. and Aminov, R. (2017) 'Phage therapy: Past, present and future', *Frontiers in Microbiology*, 8(6), pp. 1–7.

Abedon, S. T., Kuhl, S. J., Blasdel, B. G. and Kutter, E. M. (2011) 'Phage treatment of human infections', *Bacteriophage*, 1(2), pp. 66–85.

Ackermann, H.-W. and DuBow, M. S. (1987) *Viruses of prokaryotes*. CRC Press, Florida, USA.

Ackermann, H.-W., Tremblay, D. and Moineau, S. (2004) 'Long-term bacteriophage preservation', *WFCC Newsletter*, 38(8), pp. 35–40.

Ackermann, H. W. (2003) 'Bacteriophage observations and evolution', *Research in Microbiology*, 154(4), pp. 245–251.

Ackermann, H. W. (2007) 'Phages examined in the electron microscope', *Archives of Virology*, 152(2), pp. 227–243.

Ackermann, H. W. and Prangishvili, D. (2012) 'Prokaryote viruses studied by electron microscopy', *Archives of Virology*, 157(10), pp. 1843–1849.

Adams, M. H. (1959) *Bacteriophages*. John Wiley and Sons Inc, New York.

Ahmed, H., Davies, F., Francis, N., Farewell, D., Butler, C. and Paranjothy, S. (2017) 'Long-term antibiotics for prevention of recurrent urinary tract infection in older adults: Systematic review and meta-analysis of randomised trials', *BMJ Open*, 7(5), pp. 1–7.

Aiewsakun, P. and Simmonds, P. (2018) 'The genomic underpinnings of eukaryotic virus taxonomy: Creating a sequence-based framework for family-level virus classification', *Microbiome*. Microbiome, 6(1), pp. 1–24.

Allocati, N., Masulli, M., Alexeyev, M. F. and Di Ilio, C. (2013) '*Escherichia coli* in Europe: An overview', *International Journal of Environmental Research and Public Health*, 10(12), pp. 6235–6254.

Almeida, A., Cunha, Â., Gomes, N. C. M., Alves, E., Costa, L. and Faustino, M. A. F. (2009) 'Phage therapy and photodynamic therapy : Low environmental impact approaches to inactivate microorganisms in fish farming plants', *Marine Drugs*, 7, pp. 268–313.

Alves, E., Carvalho, C. M. B., Tomé, J. P. C., Faustino, M. A. F., Neves, M. G. P. M. S., Tomé, A. C., Cavaleiro, J. A. S., Cunha, Â., Mendo, S., Almeida, A., Cunha, A., Mendo, S. and Almeida, A. (2008) 'Photodynamic inactivation of recombinant bioluminescent *Escherichia coli* by cationic porphyrins under artificial and solar irradiation', *Industrial Microbiology and Biotechnology*, 35(11), pp. 1447–1454.

American Society for Microbiology (2005) *Manual of antimicrobial susceptibility testing*,

Manual of antimicrobial susceptibility testing. University of Washington, Seattle, Washington

Aminov, R., Caplin, J., Chanishvili, N., Coffey, A., Cooper, I., De Vos, D., Doškař, J., Friman, V.-P., Kurtböke, I., Pantůček, R., Pirnay, J.-P., Resch, G., Rohde, C., Sybesma, W. and Wittmann, J. (2017) 'Application of bacteriophages', *Microbiology Australia*, 38(2), pp. 63–66.

Anne-sophie, B. and Benoit, G. (2017) 'Multidrug resistant (or antimicrobial-resistant) pathogens - alternatives to new antibiotics?', *Swiss Medical Weekly*, 147.

Arisaka, F., Kanamaru, S., Leiman, P. and Rossmann, M. G. (2003) 'The tail lysozyme complex of bacteriophage T4', *The International Journal of Biochemistry & Cell Biology*. Pergamon, 35(1), pp. 16–21.

Azizian, R., Dawood, S., Nasab, M. and Ahmadi, N. A. (2013) 'Bacteriophage as a novel antibacterial agent in industry and medicine', *Journal of Paramedical Sciences*, 4(4), pp. 93–101.

Babalova, E. G., Katsitadze, K. T., Sakvarelidze, L. A., Imnaishvili, N. S., Sharashidze, T. G., Badashvili, V. A., Kiknadze, G. P., Meĭpariani, A. N., Gendzekhadze, N. D., Machavariani, E. V, Gogoberidze, K. L., Gozalov, E. I. and Dekanosidze, N. G. (1968) 'Preventive value of dried dysentery bacteriophage', *Zhurnal mikrobiologii, epidemiologii, i immunobiologii*, 45(2), pp. 143–5.

Baeder, D. Y., Yu, G., Hozé, N., Rolff, J. and Regoes, R. R. (2016) 'Antimicrobial combinations: Bliss independence and loewe additivity derived from mechanistic multi-hit models', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1695).

Balogh, B., Jones, J. B., Iriarte, F. B. and Momol, M. T. (2010) 'Phage therapy for plant disease control.', *Current pharmaceutical biotechnology*, 11(1), pp. 48–57.

Beheshti Maal, K., Delfan, A. S. and Salmanizadeh, S. (2015) 'Isolation and identification of two novel *Escherichia coli* bacteriophages and their application in wastewater treatment and coliform's phage therapy', *Jundishapur Journal of Microbiology*, 8(3).

Bhardwaj, N., K. Bhardwa, S., Deep, A., Dahiya, S. and Kapoor, S. (2015) 'Lytic Bacteriophages as Biocontrol Agents of Foodborne Pathogens', *Asian Journal of Animal and Veterinary Advances*, 10(11), pp. 708–723.

Bikard, D. and Marraffini, L. A. (2012) 'Innate and adaptive immunity in bacteria: Mechanisms of programmed genetic variation to fight bacteriophages', *Current Opinion in Immunology*, 24(1), pp. 15–20.

Bockstael, K. and Van Aerschot, A. (2009) 'Antimicrobial resistance in bacteria', *Central European Journal of Medicine*, 4(2), pp. 141–155.

Bohannan, B. J. M., Travisano, M. and Lenski, R. E. (1999) 'Epistatic interactions can lower

the cost of resistance to multiple consumers', *Evolution*. Wiley, 53(1), pp. 292–295.

Borie, C., Robeson, J. and Galarce, N. (2014) 'Lytic bacteriophages in Veterinary Medicine : a therapeutic option against bacterial pathogens ?', *Arch Med Vet*, 46, pp. 167–179.

Brockhurst, M. A., Buckling, A. and Rainey, P. B. (2005) 'The effect of a bacteriophage on diversification of the opportunistic bacterial pathogen, *Pseudomonas aeruginosa*', *Proceedings Biological Sciences*, 272(1570), pp. 1385–1391.

Brown, C. M. and Bidle, K. D. (2014) 'Attenuation of virus production at high multiplicities of infection in *Aureococcus anophagefferens*', *Virology*. Elsevier, 466–467, pp. 71–81.

Brüssow, H. (2001) 'Phages of Dairy Bacteria', *Annual Review of Microbiology*, 55(1), pp. 283–303.

Brüssow, H. (2005) 'Phage therapy: The *Escherichia coli* experience', *Microbiology*, 151(7), pp. 2133–2140.

Bruttin, A. and Brüssow, H. (2005) 'Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 49(7), pp. 2874–8.

Bull, J. J., Levin, B. R., Derouin, T., Walker, N. and Bloch, C. A. (2002) 'Dynamics of success and failure in phage and antibiotic therapy in experimental infections', *BMC microbiology*, 10, pp. 1–10.

Bull, J. J., Vegge, C. S., Schmerer, M., Chaudhry, W. N. and Levin, B. R. (2014) 'Phenotypic Resistance and the Dynamics of Bacterial Escape from Phage Control', *PLoS ONE*, 9(4), p. e94690.

Cabal, A., García-Castillo, M., Cantón, R., Gortázar, C., Domínguez, L. and Álvarez, J. (2016) 'Prevalence of *Escherichia coli* virulence genes in patients with diarrhea and a subpopulation of healthy volunteers in Madrid, Spain', *Frontiers in Microbiology*, 7, pp. 1–6.

Cairns, B. J., Timms, A. R., Jansen, V. A. A., Connerton, I. F. and Payne, R. J. H. (2009) 'Quantitative Models of *In Vitro* Bacteriophage–Host Dynamics and Their Application to Phage Therapy', *PLoS Pathogens*. Edited by F. M. Ausubel. Public Library of Science, 5(1), p. e1000253.

Ceyssens, P. and Lavigne., R. (2010) 'Introduction to bacteriophage biology and diversity'. *Baccteriophages in the control of food and waterborne pathogens.* Washington, DC, USA: ASM Press, pp. 11–29.

Chan, B. K., Abedon, S. T. and Loc-Carrillo, C. (2013) 'Phage cocktails and the future of phage therapy', *Future Microbiology*, 8(6), pp. 769–783.

Chaudhry, W. N., Concepcion-Acevedo, J., Park, T., Andleeb, S., Bull, J. J. and Levin, B. R. (2017) 'Synergy and order effects of antibiotics and phages in killing *Pseudomonas aeruginosa*

biofilms', *PLoS ONE*, 12(1), pp. 1–16.

Chhibber, S., Kaur, T. and Kaur, S. (2013) 'Co-Therapy Using Lytic Bacteriophage and Linezolid: Effective Treatment in Eliminating Methicillin Resistant *Staphylococcus aureus* (MRSA) from Diabetic Foot Infections', *PLoS ONE*, 8(2), pp. 1–11.

Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Dillmann, M. L., Kutter, E., Qadri, F., Sarker, S. A. and Brüssow, H. (2004b) 'Isolation of *Escherichia coli* bacteriophages from the stool of pediatric diarrhea patients in Bangladesh', *Journal of Bacteriology*, 186(24), pp. 8287–8294.

Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Kutter, E., Sarker, S. and Brüssow, H. (2004a) '*In vitro* and *in vivo* bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 48(7), pp. 2558–69.

ChiHsin, H., ChongYi, L., JongKang, L. and ChanShing, L. (2000) 'Control of the eel (*Anguilla japonica*) pathogens, *Aeromonas hydrophila* and *Edwardsiella tarda*, by bacteriophages', *Journal of the Fisheries Society of Taiwan*, 27(1), pp. 21–31.

Clokie, M. R., Millard, A. D., Letarov, A. V and Heaphy, S. (2011) 'Phages in nature', *Bacteriophage*, 1(1), pp. 31–45.

CLSI (2013) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, *Approved StandardÐ 9th edn: Clinical and Laborratory Standards Institure*.

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M.-F. and Krisch, H. M. (2007) 'Phage-Antibiotic Synergy (PAS): β-Lactam and Quinolone Antibiotics Stimulate Virulent Phage Growth', *PLoS ONE*. Edited by D. Fox, 2(8), p. e799.

Comeau, A. M., Tremblay, D., Moineau, S., Rattei, T., Kushkina, A. I., Tovkach, F. I., Krisch, H. M. and Ackermann, H. W. (2012) 'Phage morphology recapitulates phylogeny: The comparative genomics of a new group of myoviruses', *PLoS ONE*, 7(7), pp. 1–11.

Cooper, C. J., Mirzaei, M. K. and Nilsson, A. S. (2016) 'Adapting drug approval pathways for bacteriophage-based therapeutics', *Frontiers in Microbiology*, 7(AUG), pp. 1–15.

Coulter, L. B., McLean, R. J. C., Rohde, R. E. and Aron, G. M. (2014) 'Effect of bacteriophage infection in combination with tobramycin on the emergence of resistance in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms', *Viruses*, 6(10), pp. 3778–3786.

Cox, G. and Wright, G. D. (2013) 'Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions', *International Journal of Medical Microbiology*. Elsevier GmbH., 303(6–7), pp. 287–292.

Crain, C. M., Kroeker, K. and Halpern, B. S. (2008) 'Interactive and cumulative effects of

multiple human stressors in marine systems', *Ecology Letters*, 11(12), pp. 1304–1315.

Cruz-Flores, R. and Cáceres-Martínez, J. (2016) 'The hyperparasite of the rickettsiales-like prokaryote, Candidatus Xenohaliotis californiensis has morphological characteristics of a Siphoviridae (Caudovirales)', *Journal of Invertebrate Pathology*. Elsevier Inc., 133(3918), pp. 8–11.

Das, B., Chaudhuri, S., Srivastava, R., Nair, G. B. and Ramamurthy, T. (2017) 'Fostering research into antimicrobial resistance in India', *BMJ (Online)*, 358, pp. 63–66.

Deresinski, S. (2009) 'Bacteriophage therapy: exploiting smaller fleas.', *Clinical infectious diseases*, 48(8), pp. 1096–1101.

Desiere, F., McShan, W. M., van Sinderen, D., Ferretti, J. J. and Brüssow, H. (2001) 'Comparative Genomics Reveals Close Genetic Relationships between Phages from Dairy Bacteria and Pathogenic Streptococci: Evolutionary Implications for Prophage-Host Interactions', *Virology*, 288(2), pp. 325–341.

Duarte, J., Pereira, C., Moreirinha, C., Salvio, R., Lopes, A., Wang, D. and Almeida, A. (2018) 'New insights on phage efficacy to control *Aeromonas salmonicida* in aquaculture systems: An *in vitro* preliminary study', *Aquaculture*. Elsevier, 495, pp. 970–982.

Dublanchet, A. and Bourne, S. (2007) 'The epic of phage therapy', *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale*. Hindawi Limited, pp. 15–8.

Duckworth, D. (1987) 'History and basic properties of bacterial viruses'. *Phage Ecology*. New York, USA: John Wiley & Sons, pp. 1–44.

Edgar, R., Friedman, N., Shahar, M. M. and Qimron, U. (2012) 'Reversing bacterial resistance to antibiotics by phage-mediated delivery of dominant sensitive genes', *Applied and Environmental Microbiology*, 78(3), pp. 744–751.

El-Shibiny, A. and El-Sahhar, S. (2017) 'Bacteriophages: the possible solution to treat infections caused by pathogenic bacteria', *Canadian Journal of Microbiology*, 63(11), pp. 865–879.

Emmerson, A. M. (2003) 'The quinolones: decades of development and use', *Journal of Antimicrobial Chemotherapy*, 51(90001), pp. 13–20.

Escobar-Páramo, P., Gougat-Barbera, C. and Hochberg, M. E. (2012) 'Evolutionary dynamics of separate and combined exposure of *Pseudomonas fluorescens* SBW25 to antibiotics and bacteriophage', *Evolutionary Applications*, 5(6), pp. 583–592.

EUCAST (2015) *Antimicrobial susceptibility testing EUCAST disk diffusion method - Version 5.0*, *European Society of Clinical Microbiology and Infectious Diseases*.

Fair, R. J. and Tor, Y. (2014a) 'Antibiotics and Bacterial Resistance in the 21st Century',

Perspectives in Medicinal Chemistry, pp. 25–64.

Fair, R. J. and Tor, Y. (2014b) 'Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century', *Perspectives in Medicinal Chemistry*, 6, pp. 25–64.

Fischetti, V. A. (2005) 'Bacteriophage lytic enzymes: Novel anti-infectives', *Trends in Microbiology*, 13(10), pp. 491–496.

Fogelman, I., Davey, V., Ochs, H. D., Elashoff, M., Feinberg, M. B., Mican, J., Siegel, J. P., Sneller, M. and Lane, H. C. (2000) 'Evaluation of CD4 + T Cell Function In Vivo in HIV-Infected Patients as Measured by Bacteriophage phiX174 Immunization', *The Journal of Infectious Diseases*, 182(2), pp. 435–441.

Fu, W., Forster, T., Mayer, O., Curtin, J. J., Lehman, S. M. and Donlan, R. M. (2010) 'Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an in vitro model system', *Antimicrobial Agents and Chemotherapy*, 54(1), pp. 397–404.

Fuhrman, J. (1999) 'Marine viruses and their biogeochemical and ecological effects', *Nature*, 399, pp. 541–548.

Furuya, E. Y. and Lowy, F. D. (2006) 'Antimicrobial-resistant bacteria in the community setting', *Nature Reviews Microbiology*, 4(1), pp. 36–45.

Gaynes, R. (2017) 'The discovery of penicillin—new insights after more than 75 years of clinical use', *Emerging Infectious Diseases*, 23(5), pp. 849–853.

Giedraitienė, A., Vitkauskienė, A., Naginienė, R. and Pavilonis, A. (2011) 'Medicina.', *Medicina*, 47(3), pp. 137–146.

Gill, J. J. and Hyman, P. (2010) 'Phage Choice , Isolation , and Preparation for Phage Therapy', *Current pharmaceutical biotechnology*. Edited by B. S. AckermPublishers, 11(1), pp. 2–14.

Golkar, Z., Bagasra, O. and Gene Pace, D. (2014) 'Bacteriophage therapy: A potential solution for the antibiotic resistance crisis', *Journal of Infection in Developing Countries*, 8(2), pp. 129–136.

Goodridge, L. and Abedon, S. (2003) 'Bacteriophage biocontrol and bioprocessing: Application of phage therapy to industry', *SIM News*, 53(6), pp. 254–262.

Górski, A., Jończyk-Matysiak, E., Łusiak-Szelachowska, M., Międzybrodzki, R., Weber-Dąbrowska, B. and Borysowski, J. (2017) 'The Potential of Phage Therapy in Sepsis', *Frontiers in Immunology*, 8(December), pp. 1–5.

Guang-Han, O., Leang-Chung, C., Vellasamy, K. M., Mariappan, V., Li-Yen, C. and Vadivelu, J.

(2016) ‘Experimental phage therapy for *Burkholderia pseudomallei* infection’, *PLoS ONE*, 11(7), pp. 1–17.

Hall, a. R., De Vos, D., Friman, V.-P., Pirnay, J.-P. and Buckling, A. (2012) ‘Effects of Sequential and Simultaneous Applications of Bacteriophages on Populations of *Pseudomonas aeruginosa In Vitro* and in Wax Moth Larvae’, *Applied and Environmental Microbiology*, 78(16), pp. 5646–5652.

Hanlon, G. W. (2007) ‘Bacteriophages: an appraisal of their role in the treatment of bacterial infections’, *International Journal of Antimicrobial Agents*, 30, pp. 118–128.

Hawkins, C., Harper, D., Burch, D., Änggård, E. and Soothill, J. (2010) ‘Topical treatment of *Pseudomonas aeruginosa* otitis of dogs with a bacteriophage mixture: A before/after clinical trial’, *Veterinary Microbiology*, 146(3–4), pp. 309–313.

Hede K (2014) ‘Hede K. Antibiotic resistance: An infectious arms race’, *Nature*, 509, pp. S2–S3.

Heller, K. J. (1992) ‘Molecular interaction between bacteriophage and the gram-negative cell envelope’, *Archives of Microbiology*. Springer-Verlag, 158(4), pp. 235–248.

Heuer, H. and Smalla, K. (2007) ‘Horizontal gene transfer between bacteria’, *Environmental Biosafety Research*, 6(1–2), pp. 3–13.

Hill, C., Mills, S. and Ross, R. P. (2018) ‘Phages & antibiotic resistance: are the most abundant entities on earth ready for a comeback?’, *Future Microbiology*. Future Medicine Ltd London, UK, 13.

Hogg, S. (2013) *Essential Microbiology*. 2nd edn. Wiley-Blackwell, UK.

Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M. and Donoghue, A. M. (2004) ‘Therapeutic efficacy of bacteriophage and baytril (enrofloxacin) individually and in combination to treat colibacillosis in broilers’, *Poultry Science*, 83(12), pp. 1944–1947.

ICTV (2017) *International Committee on Taxonomy of Viruses (ICTV)*. Available at: https://talk.ictvonline.org/taxonomy/ (Accessed: 31 May 2018).

Imamovic, L. and Sommer, M. O. A. (2013) ‘Use of Collateral Sensitivity Networks to Design Drug Cycling Protocols That Avoid Resistance Development’, *Science Translational Medicine*, 5(204), p. 204.

Iredell, J., Brown, J. and Tagg, K. (2016) ‘Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications’, *BMJ*, 356(6420), pp. 1–19.

Kalatzis, P. G., Bastías, R., Kokkari, C. and Katharios, P. (2016) ‘Isolation and characterization of two lytic bacteriophages, ϕst2 and ϕgrn1; Phage therapy application for biological control of

vibrio alginolyticus in aquaculture live feeds', *PLoS ONE*, 11(3), pp. 1–18.

Kamal, F. and Dennis, J. J. (2015) '*Burkholderia cepacia* complex phage-antibiotic synergy (PAS): Antibiotics stimulate lytic phage activity', *Applied and Environmental Microbiology*, 81(3), pp. 1132–1138.

Kao, S. H. and McClain, W. H. (1980) 'Baseplate protein of bacteriophage T4 with both structural and lytic functions.', *Journal of virology*, 34(1), pp. 95–103.

Kaur, S., Harjai, K. and Chhibber, S. (2012) 'Methicillin-resistant *Staphylococcus aureus* phage plaque size enhancement using sublethal concentrations of antibiotics.', *Applied and environmental microbiology*. American Society for Microbiology (ASM), 78(23), pp. 8227–33.

King, A. M. Q., Lefkowitz, E. J., Mushegian, A. R., Adams, M. J., Dutilh, B. E., Gorbalenya, A. E., Harrach, B., Harrison, R. L., Junglen, S. and Knowles, N. J. (2018) *Changes to taxonomy and the International Code of Virus Classi fi cation and Nomenclature rati fi ed by the International Committee on Taxonomy of Viruses (2018)*, *Archives of Virology*. Springer Vienna.

Kirby, A. E. (2012) 'Synergistic Action of Gentamicin and Bacteriophage in a Continuous Culture Population of *Staphylococcus aureus*', *PLoS ONE*, 7(11).

Knezevic, P., Curcin, S., Aleksic, V., Petrusic, M. and Vlaski, L. (2013) 'Phage-antibiotic synergism: A possible approach to combatting *Pseudomonas aeruginosa*', *Research in Microbiology*. Elsevier Masson SAS, 164(1), pp. 55–60.

Kohanski, M. A., Dwyer, D. J. and Collins, J. J. (2010) 'How antibiotics kill bacteria: From targets to networks', *Nature Reviews Microbiology*, 8(6), pp. 423–435.

Kumari, S., Harjai, K. and Chhibber, S. (2010) 'Evidence to support the therapeutic potential of bacteriophage Kpn5 in burn wound infection caused by *Klebsiella pneumoniae* in BALB/c mice.', *Journal of microbiology and biotechnology*, 20(5), pp. 935–41.

Kumari, S., Harjai, K. and Chhibber, S. (2011) 'Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by *Klebsiella pneumoniae* B5055', *Journal of Medical Microbiology*, 60(2), pp. 205–210.

Kutateladze, M. and Adamia, R. (2010) 'Bacteriophages as potential new therapeutics to replace or supplement antibiotics', *Trends in Biotechnology*. Elsevier Ltd, 28(12), pp. 591–595.

Kutter, E. (2009) 'Phage host range and efficiency of plating', in Clokie, M. R. J. and Kropinski, A. M. (eds) *Bacteriophages: Methods and protocols*. Humana Press, pp. 141–149.

Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S. and Abedon, S. (2010) 'Phage Therapy in Clinical Practice: Treatment of Human Infections', *Current Pharmaceutical Biotechnology*, 11(1), pp. 69–86.

Laanto, E., Bamford, J. J. K. H., Laakso, J. and Sundberg, L. L.-R. (2012) 'Phage-driven loss of virulence in a fish pathogenic bacterium', *PLoS ONE*. Edited by M. A. Brockhurst. Public Library of Science, 7(12), p. e53157.

Labrie, S. J., Samson, J. E. and Moineau, S. (2010) 'Bacteriophage resistance mechanisms', *Nature Reviews Microbiology*. Nature Publishing Group, 8(5), pp. 317–327.

León, M. and Bastías, R. (2015) 'Virulence reduction in bacteriophage resistant bacteria.', *Frontiers in microbiology*. Frontiers Media SA, 6, p. 343.

Levin, B. R. and Bull, J. J. (2004) 'Population and evolutionary dynamics of phage therapy', *Nature Reviews Microbiology*. Nature Publishing Group, 2(2), pp. 166–173.

Levine, D. P. (2006) 'Vancomycin: A History', *Clinical Infectious Diseases*, 42(Suppl 1), pp. S5-12.

Li, F., Collins, J. G. and Keene, F. R. (2015) 'Ruthenium complexes as antimicrobial agents', *Chemical Society Reviews*. Royal Society of Chemistry, 44(8), pp. 2529–2542.

Lobanovska, M. and Pilla, G. (2017) 'Penicillin's discovery and antibiotic resistance: Lessons for the future?', *Yale Journal of Biology and Medicine*, 90(1), pp. 135–145.

Loc-Carrillo, C. and Abedon, S. T. (2011) 'Pros and cons of phage therapy', *Bacteriophage*. Taylor & Francis, 1(2), pp. 111–114.

Lood, R., Winer, B. Y., Pelzek, A. J., Diez-Martinez, R., Thandar, M., Euler, C. W., Schuch, R. and Fischetti, V. A. (2015) 'Novel phage Lysin capable of killing the multidrug-resistant gram-negative bacterium *Acinetobacter Baumannii* in a mouse bacteremia model', *Antimicrobial Agents and Chemotherapy*, 59(4), pp. 1983–1991.

Louvado, A., Coelho, F. J. R. C., Domingues, P., Santos, A. L., Gomes, N. C. M., Almeida, A. and Cunha, Â. (2012) 'Isolation of surfactant-resistant Pseudomonads from the estuarine surface microlayer', *J. Microbiol. Biotechnol*, 22(3), pp. 283–291.

Lu, T. K. and Collins, J. J. (2009) 'Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy', *Proceedings of the National Academy of Sciences*, 106(12), pp. 4629–4634.

Lu, T. K. and Koeris, M. S. (2011) 'The next generation of bacteriophage therapy', *Current Opinion in Microbiology*. Elsevier Ltd, 14(5), pp. 524–531.

Mahony, J., McAuliffe, O., Ross, R. P. and van Sinderen, D. (2011) 'Bacteriophages as biocontrol agents of food pathogens', *Current Opinion in Biotechnology*, 22(2), pp. 157–163.

Mäntynen, S., Laanto, E., Kohvakka, A., Poranen, M. M., Bamford, J. K. H. and Ravantti, J. J. (2015) 'New enveloped dsRNA phage from freshwater habitat', *Journal of General Virology*.

Microbiology Society, 96(5), pp. 1180–1189.

Marinho, C. M., Santos, T., Gonçalves, A., Poeta, P. and Igrejas, G. (2016) 'A decade-long commitment to antimicrobial resistance surveillance in Portugal', *Frontiers in Microbiology*, 7, pp. 1–14.

Mateus, L., Costa, L., Silva, Y. J., Pereira, C., Cunha, A. and Almeida, A. (2014) 'Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture', *Aquaculture*. Elsevier B.V., 424–425, pp. 167–173.

Maura, D. and Debarbieux, L. (2011) 'Bacteriophages as twenty-first century antibacterial tools for food and medicine', *Applied Microbiology and Biotechnology*, 90(3), pp. 851–859.

Maura, D. and Debarbieux, L. (2012) 'On the interactions between virulent bacteriophages and bacteria in the gut.', *Bacteriophage*, 2(4), pp. 229–233.

Melo, L. D. R., Oliveira, H., Santos, S. B., Sillankorva, S. and Azeredo, J. (2017) Phages Against Infectious Diseases. *Bioprospecting.* Topics in Biodiversity and Conservation, vol 16. Springer, Cham.

Mendes, C. A. C. and Burdmann, E. A. (2010) 'Polymyxins - a review focusing on their nephrotoxicity', *Review Association Medical Brazil*, 56(6), pp. 752–758.

Merabishvili, M., Pirnay, J.-P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Van Parys, L., Lavigne, R., Volckaert, G., Mattheus, W., Verween, G., De Corte, P., Rose, T., Jennes, S., Zizi, M., De Vos, D. and Vaneechoutte, M. (2009) 'Quality-Controlled Small-Scale Production of a Well-Defined Bacteriophage Cocktail for Use in Human Clinical Trials', *PLoS ONE*. Edited by D. M. Ojcius, 4(3), p. e4944.

Mohr, K. I. (2016) 'History of Antibiotics Research', in *Current topics in microbiology and immunology*, pp. 237–272.

Mole, R. J. and Maskell, T. W. (2001) 'Phage as a diagnostic - The use of phage in TB diagnosis', *Journal of Chemical Technology and Biotechnology*, 76(7), pp. 683–688.

Moulton-Brown, C. E. and Friman, V.-P. (2018) 'Rapid evolution of generalized resistance mechanisms can constrain the efficacy of phage-antibiotic treatments', *Evolutionary Applications*. Wiley.

Murray, P. R., Rosenthal, K. S. and Pfaller, M. A. (2016) *Medical microbiology*. 8th edn. Elsevier, Canada.

Nakai, T. (2010) 'Application of bacteriophages for control of infectious diseases in aquaculture', in Sabour, P. and Griffiths, M. (eds) *Bacteriophages in the Control of Food and Waterborne Pathogens*. Washington, DC, USA: USA: ASM Press, pp. 257–272.

Nicolaou, K. C. and Rigol, S. (2017) 'A brief history of antibiotics and select advances in their synthesis', *The Journal of Antibiotics*. Nature Publishing Group, pp. 1–32.

Nobrega, F. L., Costa, A. R., Kluskens, L. D. and Azeredo, J. (2015) 'Revisiting phage therapy: New applications for old resources', *Trends in Microbiology*. Elsevier Ltd, 23(4), pp. 185–191.

Nouraldin, A. A. M., Baddour, M. M., Harfoush, R. A. H. and Essa, S. A. M. (2016) 'Bacteriophage-antibiotic synergism to control planktonic and biofilm producing clinical isolates of *Pseudomonas aeruginosa*', *Alexandria Journal of Medicine*. Alexandria University Faculty of Medicine, 52(2), pp. 99–105.

O'Neill, J. (2016) 'Tackling Drug-Resistant Infections Globally: Final Report And Recommendations'.

Ochs, H. D., Davis, S. D. and Wedgwood, R. J. (1971) 'Immunologic responses to bacteriophage φX 174 in immunodeficiency diseases', *Journal of Clinical Investigation*, 50(12), pp. 2559–2568.

Oechslin, F., Piccardi, P., Mancini, S., Gabard, J., Moreillon, P., Entenza, J. M., Resch, G. and Que, Y. A. (2017) 'Synergistic interaction between phage therapy and antibiotics clears *Pseudomonas Aeruginosa* infection in endocarditis and reduces virulence', *Journal of Infectious Diseases*, 215(5), pp. 703–712.

Oh, J., Byrd, A. L., Deming, C., Conlan, S., Kong, H. H., Segre, J. A. and Segre, J. A. (2014) 'Biogeography and individuality shape function in the human skin metagenome', *Nature*, 514(7520), pp. 59–64.

Parisien, A., Allain, B., Zhang, J., Mandeville, R. and Lan, C. Q. (2008) 'Novel alternatives to antibiotics: Bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides', *Journal of Applied Microbiology*, 104(1), pp. 1–13.

Pasharawipas, T., Manopvisetcharean, J. and Flegel, T. (2011) 'Phage Treatment of *Vibrio harveyi*: A General Concept of Protection against Bacterial Infection', *Research Journal of Microbiology*, 6(6), pp. 560–567.

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Cunha, Â., Delgadillo, I., Romalde, J. L., Nunes, M. L. and Almeida, A. (2016a) 'Bacteriophages with potential to inactivate *Salmonella* Typhimurium: Use of single phage suspensions and phage cocktails', *Virus Research*. Elsevier B.V., 220, pp. 179–192.

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Romalde, J. L., Nunes, M. L. and Almeida, A. (2017b) 'Characterization and in vitro evaluation of new bacteriophages for the biocontrol of *Escherichia coli*', *Virus Research*, 227.

Pereira, C., Moreirinha, C., Rocha, R. J. M., Calado, R., Romalde, J. L., Nunes, M. L. and Almeida, A. (2016c) 'Application of bacteriophages during depuration reduces the load of *Salmonella* Typhimurium in cockles', *Food Research International*, 90, pp. 73–84.

Pereira, C., Moreirinha, C., Teles, L., Rocha, R. J. M. R., Calado, R., Romalde, J. L., Nunes, M. L. and Almeida, A. (2017a) 'Application of phage therapy during bivalve depuration improves *Escherichia coli* decontamination', *Food Microbiology*. Elsevier Ltd, 61, pp. 102–112.

Pereira, C. S. (2016) *Phage Therapy - A New Technology for Depuration of Bivalves*. Aveiro Unviversity

Pereira, C., Salvador, S., Arrojado, C., Silva, Y., Santos, A. L., Cunha, A., Gomes, N. C. M., Gomes, N. and Almeida, A. (2011b) 'Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy.', *Journal of environmental monitoring*, 13(4), pp. 1053–8.

Pereira, C., Silva, Y. J., Santos, A. L., Cunha, Â., Gomes, N. C. M. and Almeida, A. (2011a) 'Bacteriophages with potential for inactivation of fish pathogenic bacteria : Survival , host specificity and effect on bacterial community structure', *Marine Drugs*, 9, pp. 2236–2255.

Pereira, S., Pereira, C., Santos, L., Klumpp, J. and Almeida, A. (2016b) 'Potential of phage cocktails in the inactivation of *Enterobacter cloacae*-An in vitro study in a buffer solution and in urine samples', *Virus Research*. Elsevier B.V., 211, pp. 199–208.

Pererva, T. P., Miryuta, A. Y. and Miryuta, N. Y. (2008) 'Interaction of RNA-Containing Bacteriophages with Host Cell : MS2-Induced Mutants of *E . coli* and the Occurrence of DNA-Containing Derivatives of the Bacteriophage MS2', *Cytology and Genetics*, 42(1), pp. 60–73.

Piggott, J. J., Townsend, C. R. and Matthaei, C. D. (2015) 'Reconceptualizing synergism and antagonism among multiple stressors.', *Ecology and evolution*. Wiley-Blackwell, 5(7), pp. 1538–47.

Prasad, Y., Arpana, Kumar, D. and Sharma, A. (2011) 'Lytic bacteriophages specific to *Flavobacterium columnare* rescue catfish, *Clarias batrachus* (Linn.) from columnaris disease', *J Environ Biol*, 32(2), pp. 161–168.

Rahmani, R., Zarrini, G., Sheikhzadeh, F. and Aghamohammadzadeh, N. (2015) 'Effective phages as green antimicrobial agents against antibiotic-resistant hospital *Escherichia coli*', *Jundishapur Journal of Microbiology*, 8(2), pp. 4–8.

Raoult, D. and Forterre, P. (2008) 'Redefining viruses: lessons from Mimivirus', *Nat. Rev. Microbiol*, 6, pp. 315–319.

Reardon, S. (2015) 'Spread of antibiotic-resistance gene does not spell bacterial apocalypse — yet', *Nature*, 21 December.

Reith, M. E., Singh, R. K., Curtis, B., Boyd, J. M., Bouevitch, A., Kimball, J., Munholland, J., Murphy, C., Sarty, D., Williams, J., Nash, J. H., Johnson, S. C. and Brown, L. L. (2008) 'The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen', *BMC Genomics*, 9(1), p. 427.

Renwick, M. J., Simpkin, V. and Mossialos, E. (2016) 'Targeting innovation in antibiotic drug discovery and development'. World Health organization, London, United Kingdom

Reyes, A., Haynes, M., Hanson, N., Angly, F. E., Heath, A. C., Rohwer, F. and Gordon, J. I. (2010) 'Viruses in the faecal microbiota of monozygotic twins and their mothers.', *Nature*. NIH Public Access, 466(7304), pp. 334–8.

Rhoads, D. D., Wolcott, R. D., Kuskowski, M. A., Wolcott, B. M., Ward, L. S. and Sulakvelidze, A. (2009) 'Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial', *Journal of Wound Care*, 18(6), pp. 237–243.

Ripp, S. and Miller, R. V (1997) 'The role of pseudolysogeny in bacteriophage-host interactions in a natural freshwater environment', *Microbiology*, 143, pp. 2065–2070.

Ripp, S. and Miller, R. V (1998) 'Dynamics of the pseudolysogenic response in slowly growing cells of *Pseudomonas aeruginosa*', *Microbiology*, 144, pp. 2225–2232.

Rivas, L., Coffey, B., McAuliffe, O., McDonnell, M. J., Burgess, C. M., Coffey, A., Ross, R. P. and Duffy, G. (2010) '*In vivo* and *ex vivo* evaluations of bacteriophages e11/2 and e4/1c for use in the control of *Escherichia coli* O157:H7', *Applied and Environmental Microbiology*, 76(21), pp. 7210–7216.

Rose, T., Verbeken, G., Vos, D. De, Merabishvili, M., Vaneechoutte, M., Lavigne, R., Jennes, S., Zizi, M. and Pirnay, J.-P. (2014) 'Experimental phage therapy of burn wound infection: difficult first steps.', *International journal of burns and trauma*. e-Century Publishing Corporation, 4(2), pp. 66–73.

Rosenthal, K. S. and Tan, M. J. (2011) *Microbiology and Immunology*. 3rd edn. Edited by E. F. Goljan.

Rossitto, M., Fiscarelli, E. V, Rosati, P. and Collin, M. (2018) 'Challenges and Promises for Planning Future Clinical Research Into Bacteriophage Therapy Against *Pseudomonas aeruginosa* in Cystic', 9(May), pp. 1–16.

Rossmann, M. G. (2013) 'Structure of viruses: A short history', *Quarterly Reviews of Biophysics*, 46(2), pp. 133–180.

Rubinstein, A., Mizrachi, Y., Bernstein, L., Shliozberg, J., Golodner, M., Liu, G. Q. and Ochs, H. D. (2000) 'Progressive specific immune attrition after primary, secondary and tertiary

immunizations with bacteriophage phi X174 in asymptomatic HIV-1 infected patients.', *AIDS (London, England)*, 14(4), pp. F55-62.

Ryan, E. M., Alkawareek, M. Y., Donnelly, R. F. and Gilmore, B. F. (2012) 'Synergistic phage-antibiotic combinations for the control of *Escherichia coli* biofilms in vitro', *FEMS Immunology and Medical Microbiology*, 65(2), pp. 395–398.

Samson, J. E., Magadán, A. H., Sabri, M. and Moineau, S. (2013) 'Revenge of the phages: defeating bacterial defences', *Nature Reviews Microbiology*, 11(10), pp. 675–687.

Sarker, S. A., McCallin, S., Barretto, C., Berger, B., Pittet, A.-C., Sultana, S., Krause, L., Huq, S., Bibiloni, R., Bruttin, A., Reuteler, G. and Brüssow, H. (2012) 'Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh', *Virology*, 434(2), pp. 222–232.

Scanlan, P. D., Hall, A. R., Blackshields, G., Friman, V. P., Davis, M. R., Goldberg, J. B. and Buckling, A. (2015) 'Coevolution with bacteriophages drives genome-wide host evolution and constrains the acquisition of abiotic-beneficial mutations', *Molecular Biology and Evolution*, 32(6), pp. 1425–1435.

Scoper, S. V. (2008) 'Review of third-and fourth-generation fluoroquinolones in ophthalmology: in-vitro and in-vivo efficacy', *Advances in Therapy*, 25(10), pp. 979–994.

Seed, K. D., Yen, M., Jesse Shapiro, B., Hilaire, I. J., Charles, R. C., Teng, J. E., Ivers, L. C., Boncy, J., Harris, J. B. and Camilli, A. (2014) 'Evolutionary consequences of intra-patient phage predation on microbial populations', *eLife*, 3, pp. 1–10.

Sharma, S., Chatterjee, S., Datta, S., Prasad, R., Dubey, D., Prasad, R. K. and Vairale, M. G. (2017) 'Bacteriophages and its applications: an overview', *Folia Microbiologica*. Folia Microbiologica, 62(1), pp. 17–55.

Silva, Y. J., Costa, L., Pereira, C., Cunha, A., Calado, R., Gomes, N. C. M. and Almeida, A. (2014) 'Influence of environmental variables in the efficiency of phage therapy in aquaculture', *Microbial Biotechnology*, 7(5), pp. 401–413.

Silva, Y. J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., Gomes, N. C. M., Pardo, M. A., Hernandez, I. and Almeida, A. (2014) 'Phage Therapy as an Approach to Prevent *Vibrio anguillarum* Infections in Fish Larvae Production', *PLoS ONE*, 9(12).

Silva, Y. J., Moreirinha, C., Pereira, C., Costa, L., Rocha, R. J. M. R. J. M., Cunha, Â., Gomes, N. C. M. N. C. M., Calado, R. and Almeida, A. (2016) 'Biological control of *Aeromonas salmonicida* infection in juvenile *Senegalese sole* (*Solea senegalensis*) with Phage AS-A', *Aquaculture*, 450, pp. 225–233.

Skurnik, M. and Strauch, E. (2006) 'Phage therapy : Facts and fiction', *International Journal*

of Medical Microbiology, 296, pp. 5–14.

Sulakvelidze, A., Alavidze, Z., Glenn, J. and Morris Jr, J. (2001) 'Bacteriophage Therapy', *Antimicrobial Agents and Chemotherapy*, 45(3), p. 649.

Sunagar, R., Patil, S. A. and Chandrakanth, R. K. (2010) 'Bacteriophage therapy for *Staphylococcus aureus* bacteremia in streptozotocin-induced diabetic mice', *Research in Microbiology*. Elsevier Masson SAS, 161(10), pp. 854–860.

Suresh, G., Das, R. K., Kaur Brar, S., Rouissi, T., Avalos Ramirez, A., Chorfi, Y. and Godbout, S. (2017) 'Alternatives to antibiotics in poultry feed: molecular perspectives', *Critical Reviews in Microbiology*. Informa Healthcare USA, Inc, 0(0), pp. 1–18.

Suttle, C. A. (2005) 'Viruses in the sea', *Nature*, 437(7057), pp. 356–361.

Suttle, C. A. and Chan, A. M. (1994) 'Dynamics and distribution of cyanophages and their effect on Synechococcus spp', *Applied and Environmental Microbiology*, 60(9), pp. 3167–3174.

Suttle, C. A. and Chen, F. (1992) 'Mechanisms and rates of decay of marine viruses in seawater mechanisms and rates of decay of marine viruses in seawatert', *Applied and Environmental Microbiology*, 58(11), pp. 3721–3729.

Tanji, Y., Shimada, T., Yoichi, M., Miyanaga, K., Hori, K. and Unno, H. (2004) 'Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail', *Applied Microbiology and Biotechnology*, 64(2), pp. 270–274.

Teng-hern, T. L., Kok-gan, C. and Han, L. L. (2014) 'Application of Bacteriophage in Biocontrol of Major Foodborne Bacterial Pathogens', *Journal of Molecular Biology and Molecular Imaging*, 1(1), pp. 1–9.

Tenover, F. C. (2006) 'Mechanisms of Antimicrobial Resistance in Bacteria', *American Journal of Medicine*, 119(6 SUPPL. 1).

Torres-Barceló, C., Arias-Sánchez, F. I., Vasse, M., Ramsayer, J., Kaltz, O. and Hochberg, M. E. (2014) 'A window of opportunity to control the bacterial pathogen *Pseudomonas aeruginosa* combining antibiotics and phages', *PLoS ONE*, 9(9), pp. 1–7.

Torres-Barceló, C., Franzon, B., Vasse, M. and Hochberg, M. E. (2016) 'Long-term effects of single and combined introductions of antibiotics and bacteriophages on populations of *Pseudomonas aeruginosa*', *Evolutionary Applications*, 9(4), pp. 583–595.

Torres-Barceló, C. and Hochberg, M. E. (2016) 'Evolutionary Rationale for Phages as Complements of Antibiotics', *Trends in Microbiology*. Elsevier Ltd, 24(4), pp. 249–256.

Tortora, G., Funke, B. and Case, C. (2010) *Microbiology: an introdution*. 10th edn. Pearson, San Fransisco

Ul Haq, I., Chaudhry, W. N., Akhtar, M. N., Andleeb, S. and Qadri, I. (2012) 'Bacteriophages and their implications on future biotechnology: A review', *Virology Journal*, 9, pp. 1–8.

Valério, N., Oliveira, C., Jesus, V., Branco, T., Pereira, C., Moreirinha, C. and Almeida, A. (2017) 'Effects of single and combined use of bacteriophages and antibiotics to inactivate *Escherichia coli*', *Virus Research*. Elsevier, 240, pp. 8–17.

Vandenheuvel, D., Lavigne, R. and Brüssow, H. (2015) 'Bacteriophage Therapy: Advances in Formulation Strategies and Human Clinical Trials', *Annual Review of Virology*, 2(1), pp. 599–618.

Verma, V., Harjai, K. and Chhibber, S. (2009) 'Restricting ciprofloxacin-induced resistant variant formation in biofilm of *Klebsiella pneumoniae* B5055 by complementary bacteriophage treatment', *Journal of Antimicrobial Chemotherapy*, 64(6), pp. 1212–1218.

Verstappen, K. M., Tulinski, P., Duim, B., Fluit, A. C., Carney, J., Van Nes, A. and Wagenaar, J. A. (2016) 'The effectiveness of bacteriophages against methicillin-resistant *Staphylococcus aureus* ST398 nasal colonization in pigs', *PLoS ONE*, 11(8), pp. 1–10.

Vieira, A., Silva, Y. J., Cunha, A., Gomes, N. C. M., Ackermann, H.-W. and Almeida, A. (2012) 'Phage therapy to control multidrug-resistant *Pseudomonas aeruginosa* skin infections: in vitro and ex vivo experiments.', *European journal of clinical microbiology & infectious diseases*, 31(11), pp. 3241–9.

Viertel, T. M., Ritter, K. and Horz, H. P. (2014) 'Viruses versus bacteria-novel approaches to phage therapy as a tool against multidrug-resistant pathogens', *Journal of Antimicrobial Chemotherapy*, 69(9), pp. 2326–2336.

Vos, D. D. and Pirnay, J.-P. (2015) 'Phage therapy: could viruses help resolve the worldwide antibiotic crisis?', *Alternatives to Antibiotics*, pp. 110–114.

Vranakis, I., Goniotakis, I., Psaroulaki, A., Sandalakis, V., Tselentis, Y., Gevaert, K. and Tsiotis, G. (2014) 'Proteome studies of bacterial antibiotic resistance mechanisms', *Journal of Proteomics*. Elsevier B.V., 97, pp. 88–99.

Wang, R., Van Dorp, L., Shaw, L. P., Bradley, P., Wang, Q., Wang, X., Jin, L., Zhang, Q., Liu, Y., Rieux, A., Dorai-Schneiders, T., Weinert, L. A., Iqbal, Z., Didelot, X., Wang, H. and Balloux, F. (2018) 'The global distribution and spread of the mobilized colistin resistance gene mcr-1', *Nature Communications*. Springer US, 9(1), pp. 1–9.

Wang, Y., Barton, M., Elliott, L., Li, X., Abraham, S., O'Dea, M. and Munro, J. (2017) 'Bacteriophage therapy for the control of *Vibrio harveyi* in greenlip abalone (*Haliotis laevigata*)', *Aquaculture*. Elsevier B.V., 473, pp. 251–258.

Wang, Z. and Goldenfeld, N. (2010) 'Fixed points and limit cycles in the population

dynamics of lysogenic viruses and their hosts', *Physical Review E - Statistical, Nonlinear, and Soft Matter Physics*, 82(1), pp. 1–18.

Weber-Dabrowska, B., Jończyk-Matysiak, E., Zaczek, M., Łobocka, M., Łusiak-Szelachowska, M. and Górski, A. (2016) 'Bacteriophage procurement for therapeutic purposes', *Frontiers in Microbiology*, 7, pp. 1–14.

Weber-Dabrowska, B., Mulczyk, M. and Górski, A. (2000) 'Bacteriophage therapy of bacterial infections: an update of our institute's experience.', *Archivum immunologiae et therapiae experimentalis*, 48(6), pp. 547–51.

Weinbauer, M. G. (2004) 'Ecology of prokaryotic viruses', *FEMS Microbiology Reviews*, 28(2), pp. 127–181.

Weiss, M., Denou, E., Bruttin, A., Serra-Moreno, R., Dillmann, M.-L. and Brüssow, H. (2009) '*In vivo* replication of T4 and T7 bacteriophages in germ-free mice colonized with *Escherichia coli*', *Virology*, 393(1), pp. 16–23.

WHO (2014a) 'Antimicrobial resistance.', *Bulletin of the World Health Organization*, 61(3), pp. 383–94.

WHO (2014b) Antimicrobial resistance: global report on surveillance. World Health Organization

Williamson, S. J., McLaughlin, M. R. and Paul, J. H. (2001) 'Interaction of the \varphiHSIC virus with its host: lysogeny or pseudolysogeny?', *Applied and environmental microbiology*, 67(4), pp. 1682–1688.

Wishart, D. S., Knox, C., Guo, A. C., Shrivastava, S., Hassanali, M., Stothard, P., Chang, Z., Woolsey, J. and Tg, C. (2006) 'DrugBank : a comprehensive resource for in silico drug discovery and exploration', 34, pp. 668–672.

Withey, S., Cartmell, E., Avery, L. M. and Stephenson, T. (2005) 'Bacteriophages - Potential for application in wastewater treatment processes', *Science of the Total Environment*, 339(1–3), pp. 1–18.

Wittebole, X., De Roock, S. and Opal, S. M. (2014) 'A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens.', *Virulence*, 5(1), pp. 226–235.

Wommack, K. E. and Colwell, R. R. (2000) 'Virioplankton: viruses in aquatic ecosystems.', *Microbiology and molecular biology reviews*. American Society for Microbiology (ASM), 64, pp. 69–114.

Wright, A., Hawkins, C. H., Änggård, E. E. and Harper, D. R. (2009) 'A controlled clinical trial

of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa* ; a preliminary report of efficacy', *Clinical Otolaryngology*, 34(4), pp. 349–357.

Young, R., Wang, I. N. and Roof, W. D. (2000) 'Phages will out: Strategies of host cell lysis', *Trends in Microbiology*, 8(3), pp. 120–128.

Zhang, G., Zhao, Y., Paramasivan, S., Richter, K., Morales, S., Wormald, P.-J. and Vreugde, S. (2017) 'Bacteriophage effectively kills multidrug resistant *Staphylococcus aureus* clinical isolates from chronic rhinosinusitis patients', *International Forum of Allergy & Rhinology*, 8(3), pp. 406–414.

Zhang, Q. G. and Buckling, A. (2012) 'Phages limit the evolution of bacterial antibiotic resistance in experimental microcosms', *Evolutionary Applications*, 5(6), pp. 575–582.

Annex 1

New insights on phage efficacy to control *Aeromonas salmonicida* in aquaculture systems: An in vitro preliminary study

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Abstract

A major source of financial loss for the fish-farming industry is the occurrence of bacterial infections. Phage therapy can be a useful alternative tool to conventional treatments to control bacterial infections in aquaculture. The promising results obtained with phage AS-A to control the agent of furunculosis, *Aeromonas salmonicida*, led us to isolate two new phages and evaluate their dynamics, in cocktails and individually, to control this pathogenic bacterium. Moreover, considering that in outdoor facilities aquaculture water is exposed to the natural variability of physical and chemical parameters, the influence of pH, salinity, temperature, solar radiation and UV radiation on phage AS-D stability was also evaluated in this study, in order to develop an effective phage therapy protocol. Phages were assigned to the family *Myoviridae* and revealed identical morphological characteristics. Phage AS-A presents a higher burst size and a shorter latent period, decreasing the concentration of *A. salmonicida* sooner than phages AS-D and AS-E. However, phage AS-D presented higher rate of bacterial reduction, inducing also less bacterial resistance. Bacterial control with the cocktails was higher, namely when phage AS-A was combined with one of the two new phages or with both, but the main difference in the bacterial control was in the treatment time. Phage cocktails decrease the concentration of *A. salmonicida* sooner than single suspensions. The use of phage cocktails, in general, decreased phage-resistant mutants. The survival of ASD phage was mostly affected by sunlight exposure (decrease of 3 log PFU/mL after 12 h) and high temperatures (decrease of 3 PFU/mL after 21 days and of 7 log PFU/mL after 49 days at 37 °C, but no decrease after 21 and 49 days at 25 °C and decrease of only 1 and 2 log PFU/mL after 21 and 49 days, respectively, at ambient temperature). The high bacterial control and low development of phage-resistant bacterial clones suggest that these phages can be used to control the furunculosis in aquaculture. Nonetheless, the stability of the phages was affected by solar radiation, this can be overcome by the application of phages at the end of the day or at night.

Keywords: Phage treatment, phage cocktails, fish pathogenic bacteria, multidrug resistance bacteria, physico-chemical factors

3.1. Introduction

Fisheries and aquaculture remain important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world (FAO, 2016). World aquaculture production of fish accounted for 44.1% of total production from capture fisheries and aquaculture

in 2014, up from 42.1% in 2012 and 31.1% in 2004 (FAO, 2016). World aquaculture combined production of fish and plants reached 101.1 million tons in live weight in 2014, for an estimated total farm gate value of US$165.8 billion (FAO, 2016). However, aquaculture industries frequently suffer heavy financial losses that threaten their growth and sustainability, due mainly to uncontrolled microbial diseases (Almeida *et al.*, 2009). Aquaculture fish are subjected to greater stress than wild conspecifics, which contributes to a reduction of their natural immune system and often favors bacterial infections, namely during their early life stages (Shao, 2001; Wahli *et al.*, 2002; Silva *et al.*, 2016). This scenario can favor opportunistic infections and cause significant economic losses to producers.

Aeromonas salmonicida, the causative causative agent of furunculosis and fish septicaemia, is a relevant pathogen in aquaculture, responsible for causing significant economic losses worldwide (González *et al.*, 2004; Janda and Abbott, 2010; Noga, 2011; Diamanka *et al.*, 2013; Dallaire-Dufresne *et al.*, 2014; Tewari *et al.*, 2014) due to high mortality and morbidity in a variety of fish species (e.g., salmon (Ringø *et al.*, 2004; Verner–Jeffreys *et al.*, 2007)), trout (Nikoskelainen *et al.*, 2001; Imbeault *et al.*, 2006), turbot (Farto *et al.*, 2011; Lago *et al*., 2012; Coscelli *et al.*, 2014), Atlantic cod (Arnesen *et al.*, 2010), rockfish (Kim *et al.*, 2013), seabream (Zorrilla *et al.*, 2003) and wolffish (Grontvedt and Espelid, 2004)). The consequences of furunculosis are especially severe in farmed fish in the absence of vaccination (Bergh, 2008). Although efficient vaccines are available, they are still expensive and often associated with undesirable side effects (Midtlyng, 1997; Björnsdóttir *et al.*, 2005; Cipriano and Austin, 2011). The regular use of artificial food supplemented with antibiotics in intensive and semi-intensive aquaculture system, to prevent the spread of diseases and their massive use to control infections, has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotic treatments (Martínez and Hipólito-Morales, 2013). The recent emergence of antibiotic resistant *A. salmonicida* strains in aquaculture has been observed (Reith *et al.*, 2008; Kim *et al.*, 2011), making alternative control methods to treat furunculosis urgently needed.

Phage therapy is a proven eco-friendly alternative approach to prevent and control pathogenic bacteria in aquaculture (Nakai *et al.*, 1999; Park *et al.*, 2000; Nakai and Park, 2002; Park and Nakai, 2003; Vinod *et al.*, 2006; Karunasagar *et al.*, 2007; Higuera *et al.*, 2013; Silva *et al.*, 2014b, 2016). The use of phages to prevent infection or to control different fish pathogenic bacteria is well documented (Nakai *et al.*, 1999; Park and Nakai, 2003; Crothers-Stomps *et al.*, 2010; Silva *et al.*, 2014b, 2016). A number of phages infecting Aeromonadaceae isolated from different environments around the world have already been characterized (Beilstein and

Dreiseikelmann, 2008; Kim *et al*., 2012a, 2012 b; Silva *et al.*, 2016), and, in last years, *A. salmonicida* phages have been successfully used to treat furunculosis in brook trout (*Salvelinusn fontinalis*) (Imbeault *et al.*, 2006), Atlantic salmon (*Salmo salar*) (Verner–Jeffreys *et al.*, 2007), rainbow trout (Verner–Jeffreys *et al.*, 2007) and Senegalese sole (*Solea senegalensis*) (Silva *et al.*, 2016). In one recent study of our group, it was showed the application of AS-A phage that inhibited the growth of *A. salmonicida* both in batch cultures and in *Solea senegalensis* juveniles (Silva *et al.*, 2016). After 72 h, fish juveniles treated with AS-A phage after exposure to *A. salmonicida* showed no mortality, contrarily to juveniles that were only exposed to the bacterium, which presented a mortality of 36% (Silva *et al.*, 2016). However, some bacteria after treatment developed resistance to phage AS-A, but the rate of resistance was, however, low (2.24×10^{-4}). The development of phage-resistance can be overcame by the combined use of more than one phage at the same time, that can be achieved by the use of phage cocktails (Crothers-Stomps *et al.*, 2010; Chan *et al*., 2013; Mateus *et al.*, 2014). A cocktail of several phages, each one virulent to the target bacterial strain but binding to different surface receptors, will make it very difficult for the bacteria to develop resistance. To the best of our knowledge, here is only one report about the use of phage cocktails to control the agent of the furunculosis (Verner–Jeffreys *et al.*, 2007). In this study, juvenile fish were initially infected with *A. salmonicida* subsp. *salmonicida* and treated with a cocktail of 3 lytic phages. Fish treated with the phage cocktail lived longer but still died of furunculosis by the end of the study (within 96 h). The time until death was dependent on how long the phage was administered after injection of the bacterium. The fish that were treated with phages cocktail immediately after infection died more slowly than those that were either not treated with phages, or treated 24 h post-infection (Verner–Jeffreys *et al.*, 2007).

For an effective treatment in aquaculture, phages should be stable over time when exposed to the natural variability of physical and chemical parameters. Consequently, the effect of the physico-chemical factors in the stability and survival of phages should be taken into account. It is known that the stability of a virus can be negatively affected by various factors such as pH, salinity, UV-light and temperature (Jończyk *et al.*, 2011; Maura and Debarbieux, 2011; Ly-Chatain, 2014). For phage best activity, pH values should not be lower than 6 or higher than 8, salt must be at low concentrations and temperature should be near the optimal for each phage. UV radiation is considered the most important factor to be controlled, as it modifies genetic material, therefore killing the viruses (Jończyk *et al.*, 2011; Maura and Debarbieux, 2011; Ly-Chatain, 2014). Some authors assume a connection between a phage morphology and its occurrence and infectivity in adverse environments, but evidence supportive of that hypothesis is lacking (Lasobra *et al.*, 1997).

Under unfavorable environmental conditions, phage stability can be affected through damage of its structural elements (head, tail, envelope), lipid loss, and/or DNA structural changes (Ackermann *et al.,* 2004). In general, bacteriophage stability is highly variable and sensitivity of individual phage classes is highly diversified (Jończyk *et al.*, 2011). Pereira *et al.* (2011a) showed that the pattern of survival of phages infecting important aquaculture pathogenic bacteria is different in culture waters. An *A. salmonicida* phage survived in fish-farm water during 90 days. In contrast, the abundance of Vibrio parahaemolyticus phage strongly decreased during the incubation period, showing a survival time of 16 days (Pereira *et al.*, 2011a).

In our previous study, phage AS-A has demonstrated a remarkable efficacy in the control of *A. salmonicida* both in batch cultures and in juveniles of Senegalese sole (Silva *et al.*, 2016), but some phage-resistant mutants emerged. So, in the present study, we extended our research to the isolation of new phages and we studied the efficiency of cocktails of two and three phages to control the emergence of resistant bacteria. Additionally, taking into account that in outdoor facilities, aquaculture water is exposed to natural variation of the environmental factors, and that the survival and persistence of phages are affected by physico-chemical factors, in this study the influence of pH, salinity, temperature, solar radiation and UV radiation on phage AS-D stability was also evaluated in order to develop an effective phage therapy protocol.

3.2. Material and methods

3.2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *A. salmonicida* (CECT 894), *Aeromonas hydrophilla* (ATCC 7966), *Vibrio fischeri* (ATCC 49387), *Salmonella enterica* serovar Typhimurium (ATCC 13311 and ATCC 14028), *Escherichia coli* (ATCC 25922 and 13,706), *Vibrio parahaemolyticus* (DSM 27657), *Vibrio anguillarum* (DSM 21597) and *Photobacterium damselae* subsp. *damselae* (DSM 7482) were purchased from CECT, ATCC and DSM collection, respectively. The bacterial strains, *Aeromonas* sp. K15-2FS, *Aeromonas salmonicida* AH010906–3, *Aeromonas salmonicida* subsp*. achromogenes* AT4175 and *Vibrio ordalii* VI-30, used in this study were previously isolated in another laboratory. All other bacterial strains used in the present study were isolated in previous works from water samples collected in Ria de Aveiro (Louvado *et al.*, 2012; Silva *et al.*, 2016; Pereira *et al.*, 2016a, 2017b). Fresh plate cultures were maintained in Tryptic Soy Agar medium (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was transferred to 10 mL of Tryptic Soy Broth medium (TSB; Liofilchem, Italy) and was grown overnight at 25 °C. An aliquot of this culture (100 µL) was transferred to 10 mL of fresh TSB

medium and grown overnight at 25 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells/mL.

3.2.2. Phage isolation and purification

The phage AS-A used in this study was previously isolated in our laboratory (Silva *et al.*, 2016) and the other two phages (AS-D and ASE) were isolated from sewage network of Aveiro (SIMRIA Multi Sanitation System of Ria de Aveiro - station EEIS9) collected at different times. Sewage water was filtered through 0.45 µm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to double-concentrated TSB medium with 1 mL of fresh culture of the host, *A. salmonicida* CECT 894. The mixtures were incubated at 25 °C for 18 h at 80 rpm, and then filtered through a 0.2 µm membrane (Millipore Bedford, MA, USA). Plates were incubated at 25 °C and observed for the presence of lytic plaques after 12 h. One single plaque was removed from the agar and added to TSB medium with a fresh culture of the host. The sample was centrifuged, being the supernatant used as a phage source for a second isolation procedure. Three successive single-plaque isolation cycles were performed in order to obtain pure phage stocks. All lysates were centrifuged at 10.000 g for 10 min at 4 °C, to remove intact bacteria or bacterial debris. The phage stocks were kept at 4 °C. The phage suspension titers were determined by the double-layer agar method using TSA (TSA soft with 0.6% of agar in tubes and TSA with 1.5% of agar in plates) as culture medium (Adams, 1959). The plates were incubated at 25 °C for 12 h and the number of lysis plaques was counted. The results were expressed as plaque forming units per milliliter (PFU/mL).

3.2.3. Electron microscope examination

Phage particles of a highly concentrated suspension (10^9 PFU/mL) were negatively stained with 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, UK) and electron micrographs were taken using a JEOL 1011 transmission electron microscope (JEDL USA Inc., Peabody, MA, USA) operating at 100 kV and images were acquired with a Gatan CCD-Erlangshen ES100W.

3.2.4. Phage host range and efficiency of plating (EOP) analysis

Firstly, phage host range was determined by spot testing according to Adams (1959). The plates were incubated at 25 °C and examined for plaques after 12 h. Bacterial sensitivity to a phage was established by a lysis cleared zone at the spot. According to the clarity of the spot, bacteria were differentiated into two categories: clear lysis zone (+) and no lysis zone (−). The

efficiency of plating (EOP) was determined for bacteria with positive spot tests (occurrence of a clear lysis zone), using the double-layer agar method (Adams, 1959). The EOP was calculated (average PFU on target bacteria/average PFU on host bacteria) (Kutter, 2009) along with the standard deviation for the three measurements. For each phage, three independent experiments were done.

3.2.5. One step growth assays

Exponential host bacterial cultures of *A. salmonicida* were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). Ten microliters of the phage suspensions (final concentration: 10^6 PFU/mL) were added to 10 mL of the bacterial culture in order to have a MOI of 0.001 and incubated at 25 °C (Mateus *et al.*, 2014). The mixture was centrifuged at 12.000 g for 5 min, the pellet was resuspended in 10 mL of TSB at 25 °C and then it was diluted and titrated. The plates were incubated at 25 °C and examined for plaques after 4–8 h (Mateus *et al.*, 2014). Three independent assays were done.

3.2.6. Bacterial kill curves

Phage therapy was performed using phages AS-D and AS-E individually and with phage cocktails (two or all the three phages mixed together, each phage at the same concentration) using the bacterium *A. salmonicida* as host, at a MOI of 100. In order to obtain a MOI of 100, 2.5 µL of 10^9 CFU/mL of the overnight *A. salmonicida* culture (final concentration of 10^5 CFU/mL) and 200 µL of 10^9 PFU/mL of the phage suspension (final concentration of 10^7 PFU/mL) were inoculated in sterilized glass erlenmeyers with 30 mL of TSB medium and incubated at 25 °C without agitation (B+P). The tested phage cocktails were: ASD/AS-E, AS-A/AS-D, AS-A/AS/E and AS-A/AS-D/AS-E phages. For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). The bacterial control was inoculated with *A. salmonicida* but not with phages and the phage controls were inoculated with phages but not with bacteria. Controls and test samples were incubated in the same conditions. Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. In all assays, the phage titre was determined in duplicate through the double agar layer method after an incubation period of 8–12 h at 25 °C. Bacterial concentration was determined in duplicate in TSA medium after an incubation period of 24 h at 25 °C. Three independent experiments were performed for each condition.

3.2.7. Determination of the rate of emergence of phage-resistant mutants

In order to determinate the frequency of phage-resistant bacteria, ten isolated colonies from a plate with sensitive bacteria were selected and were inoculated into ten tubes with 5 mL of TSB medium, grown at 25 °C for 24 h (concentration around 10^9 CFU/mL). Aliquots of 0.1 mL from the 100 to 10^{-2} dilutions of the bacterial culture and aliquots of 0.1 mL of the phage from a stock solution of 10^9 PFU/mL were inoculated in tubes with TSA soft (MOI 1), plated on TSA and incubated at 25 °C for 3–5 days (because some of the phage-resistant mutants grow very slowly). Simultaneously, 0.1 mL aliquots of 10^{-5} to 10^{-7} dilutions of the bacterial culture were plated on TSA plates without phage and incubated at 25 °C for 24 h (Filippov *et al.*, 2011). The calculation of the frequency of mutants was done by dividing the number of resistant bacteria (obtained from the ten isolated colonies) by the total number of sensitive bacteria (prepared from the culture without phages) (Filippov *et al.*, 2011). Three independent assays were performed.

3.2.8. Isolation of phage-resistant mutants

Sensitive *A. salmonicida* (CECT 894) was grown at 25 °C during 16 h. A soft agar overlay seeded with 300 mL culture was then spotted with 10 mL of each of two lytic phage suspensions. Using high phage titers, clear zones where there was complete absence of visible bacterial growth were created. However, after 24 h of incubation, resistant colonies were observed in these zones. The phage resistant colonies were picked up and purified by successive sub-culturing in TSA agar, in order to remove attached phage particles and were used in further experiments.

3.2.9. Fitness of phage resistant mutants

The growth of the bacterial populations was quantified in the presence and in the absence of phage AS-D. This phage was selected for this experiments because was the most efficient phage to control *A. salmonicida*. Exponential host bacterial cultures of sensitive *A. salmonicida* strain (without phage contact) and resistant mutants of phage AS-D were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). Fitness of each bacterial population was analyzed in two ways: (i) determining bacterial concentration (CFU/mL) after 6 and 12 h of incubation and (ii) determining population growth curve using the optical density (OD).

Sensitive *A. salmonicida* was added to 2 of the 4 samples to obtain a final concentration of 10^5 CFU/mL. One of the inoculated samples with sensitive *A. salmonicida* was inoculated with phage AS-D (sensitive with phage AS-D) to obtain a final concentration 10^7 PFU/mL and the remaining infected sample was not added of phages (sensitive bacteria without phage). Resistant

mutants to phage AS-D were added to 2 of 4 samples to obtain a final concentration of 10^5 CFU/mL. One of these samples was inoculated with phage AS-D (resistant with phage AS-D) to obtain a final concentration 10^7 PFU/mL and the remaining infected samples were not added of phages (resistant bacteria without phage). Samples were incubated at 25 °C and bacterial concentration was determined in duplicate by the spread method in TSA medium, after an incubation period of 6 and 12 h post inoculation. The plates were incubated at 25 °C and bacterial concentration (CFU/mL) was calculated after 24 h incubation. Three independent experiments were performed for each condition.

In parallel with this assay, we determined 12 h growth curves using a spectrophotometer (Halo DB- 20, Dynamica Scientific) using culture turbidity as a proxy for bacterial density. OD 600 nm was taken at 0, 2, 4, 6, 8, 10 and 12 h post inoculation. Three independent experiments were performed for each condition.

3.2.10. Assessment of the effect of physico-chemical factors in the phage survival

The phage survival was determined, having into account the annual variations observed on Ria de Aveiro (Rebelo, 1992; Vargas, Vaz and Dias, 2017) for temperature, salinity, pH and irradiation (sunlight and UV-B light). Phage AS-D was selected for this experiment. The survival of ASD phage was tested in sterile synthetic seawater prepared by mixing Tropic Marin ProReef salt (Tropic Marine, Germany) with freshwater purified by a reverse osmosis unit, according to Silva et al. (2016). Thirty milliliters of water were filtered through 0.45 µm and then by 0.22 µm pore-size membranes (Poretics, USA), followed by the addition of phage suspensions at a final concentration of about 10^7 PFU/mL. In the experiments to evaluate the effect of pH, temperature and salinity, aliquots were collected after 0, 7, 14, 21, 49, 77, 107 days of incubation. To evaluate the effect of UV- B, the aliquots were collected after 0, 2, 4, 6, 8, 12 h of incubation. To assess the effect of solar radiation, the aliquots were collected after 0, 2, 4, 6, 8, 10 and 12 h. Phage titre was determined in duplicate through the double agar layer method and plates were incubated at 25 °C for 18 h. Three independent experiments were performed for each condition.

3.2.10.1. pH experiments

In order to evaluate the effect of water pH, suspensions of phage ASD were added to sterile synthetic seawater with pH values of 5.5, 7 and 8. During these experiments, the temperature of the samples was kept at 25 °C and salinity at 35 g/L.

3.2.10.2. Temperature experiments

To evaluate the effect of the temperature, the samples were kept at constant temperature (25, 37 °C and ambient temperature) in an incubator. The experiments were performed in sterile synthetic seawater at pH 7.0 and salinity at 35 g/L.

3.2.10.3. Salinity experiments

To assess the effect of the salinity, sterile synthetic seawater was prepared with a final salinity of 15, 20 and 35 g/L. During these experiments, the temperature of the samples was kept at 25 °C and the pH at 7.0.

3.2.10.4. UVB experiments

In order to evaluate the effect of the UVB (290–320 nm) it was used an ultra-violet type B lamp TL 20 W/12 RS (Philips, Holland) at a distance of 25 cm. The experiments were performed in sterile synthetic seawater at pH 7.0, salinity at 35 g/L and ambient temperature.

3.2.10.5. Solar radiation experiments

To evaluate the effect of solar radiation (200–1000 Wm2), suspensions of phage AS-D were added to sterile synthetic seawater with pH 7.0 and salinity at 35 g/L and exposed to natural solar radiation.

3.2.11. Statistical analysis

The statistical analysis of data was performed using the IBMSPSS Statistics 22.0 software. Kolmorov-Smirnov test was used to check for normal distributions and Levene test for homogeneity of variances. The significance of bacterial and viral concentrations between the treatments and during the experiments was tested using two-way ANOVA and Bonferrori post-hoc test. For different treatments, the significance of differences was evaluated by comparing the result obtained in the test samples with the results obtained for the correspondent control samples, for the different times (Section 3.4). One way analysis of variance was used to analyze the statistical differences of the rates of phage-resistant mutants between single phage suspensions and phage cocktails (Section 3.7). Student's t-test was used to examine the differences between the concentration of resistant and sensitive bacteria in the presence of the phage after 6 and 12 h of incubation, and the differences between the growth of resistant and sensitive bacteria in absence of the phage after 6 and 12 h incubation (Section 3.3.8). Two-way

ANOVA with repeated measures was used to analyze the statistical differences between the growth curves of the sensitive and resistant bacteria in presence and absence of the phage during the sampling time (Section 3.3.8). The significance of the effect of physico-chemical factors on the AS-D phage and incubation time was assessed by one way analysis of variance (Section 3.3.10). A value of $p < 0.05$ was considered to be statistically significant.

3.3. Results

3.3.1. Phage isolation and enrichment

Phages AS-D and AS-E show similarities to phage AS-A (Silva *et al.*, 2016), forming clear plaques on the host strain with a diameter of 0.5–2mm (Figure 3.1). High titre suspensions (10^9 PFU/mL) were obtained for the three phages.

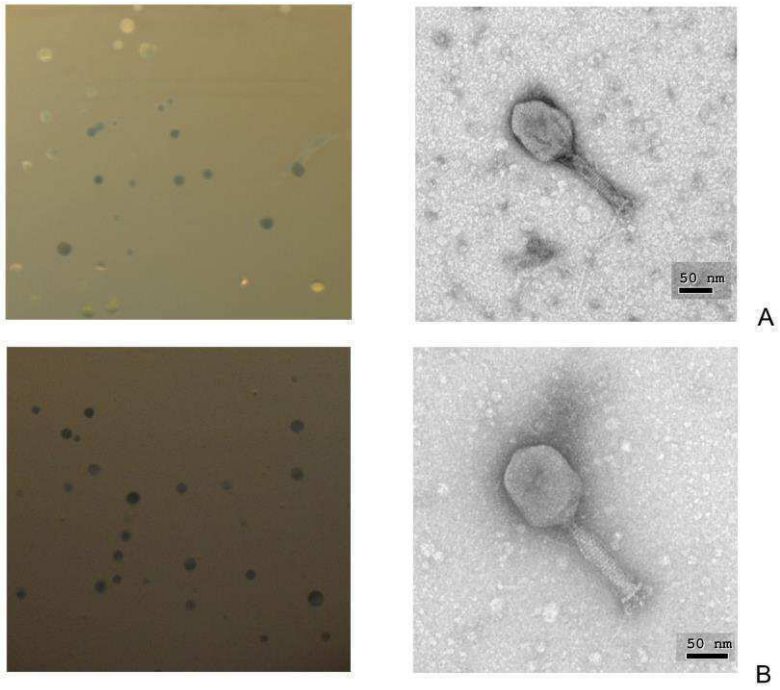

Figure 3.1: Phage plaque morphologies and electron micrographs of *A. salmonicida* phages. (A) Phage AS-D and (B) Phage AS-E.

3.3.2. Virion morphology

Based on the morphological analysis by TEM (Figure 3.1), all three phages were identified as order Caudovirales and family *Myoviridae* of double-stranded DNA phages (Figure 3.1). The phages have an elongated icosahedral head and a rigid tail with approximately 220 ± 5 nm long.

3.3.3. Phage host range determination and efficiency of plating (EOP) analysis

Similarly to phage AS-A (Silva *et al.*, 2016), AS-D and AS-E phages infect only its host without infecting *Aeromonas* sp. K15-2FS, *A. salmonicida* AH010906-3, *A. salmonicida achromogenes*, *V. ordalii* VI-30, *Aeromonas caviae*, *A. hydrophila*, *V. parahaemolyticus*, *V. anguillarum, V. fischeri*, *P. damselae damselae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas gingeri*, *Pseudomonas segetis*, two strains of *S. enterica* and eleven strains of *E. coli* (Table 3.1).

Table 3.1: Host range and efficiency of plating of three *A. salmonicida* (CECT 894) phage isolates determined on 29 bacterial strains included in six genera. Clear lysis zone (+), no lysis zone (−).

Species	**Infectivity of phage**		**Efficacy of plating (%)**	
	AS-D	**AS-E**	**AS-D**	**AS-E**
A. salmonicida CECT 894	+	+	100	100
A. salmonicida AH010906–3	-	-	0	0
A. salmonicida achromogenes AT4175	-	-	0	0
Aeromonas sp. K15-2FS	-	-	0	0
A. caviae	-	-	0	0
A. hydrophilla ATCC 7966	-	-	0	0
V. parahaemolyticus DSM 27657	-	-	0	0
V. anguillarum DSM 21597	-	-	0	0
V. fischeri ATCC 49387	-	-	0	0
Vibrio ordalii VI-30	-	-	0	0
P. damselae damselae DSM 7482	-	-	0	0
P. aeruginosa	-	-	0	0
P. fluorescens	-	-	0	0
P. putida	-	-	0	0
P. segetis	-	-	0	0
P. gingeri	-	-	0	0
S. Typhimurium ATCC 13311	-	-	0	0
S. Typhimurium ATCC 14028	-	-	0	0
E. coli ATCC 25922	-	-	0	0
E. coli ATCC 13706	-	-	0	0
E. coli B30	-	-	0	0

E. coli AE11	-	-	0	0
E. coli AD6	-	-	0	0
E. coli AF15	-	-	0	0
E. coli AN19	-	-	0	0
E. coli AC5	-	-	0	0
E. coli Aj23	-	-	0	0
E. coli BN65	-	-	0	0
E. coli BM62	-	-	0	0

3.3.4. Burst size and explosion time

Growth curves for phages AS-A, AS-D and AS-E were determined in TSB at 25 °C (Figure 3.2). From the triphasic curves obtained, an eclipse period of 20 min, a latent period of 30 min and a burst size of 22 ± 5 PFU/host cell were calculated for AS-A. The AS-D phage is characterized by an eclipse time of 30 min, a latent period of 40 min and each infected bacterium produced 5 ± 1 PFU/host cell. AS-E is characterized by an eclipse period of 30 min, a short latency of 40 min and burst size of 10 ± 1 PFU/host cell.

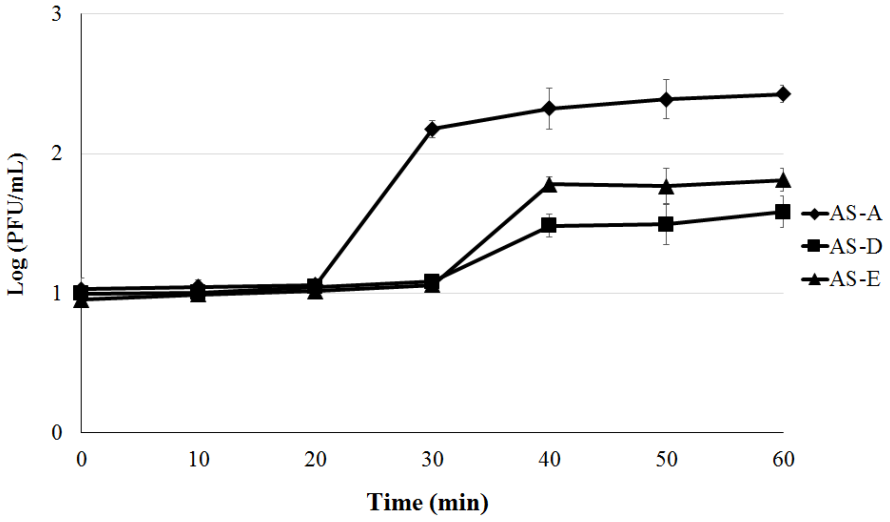

Figure 3.2: One-step growth curves of AS-A, AS-D and AS-E phages in the presence of *A. salmonicida* as host. Values represent the mean of three experiments; error bars represent the standard deviation.**9**

3.3.5. Bacterial killing curves

3.3.5.1. Bacterial killing curves using single-phage suspensions

The maximum of bacterium decrease with the AS-A, AS-D and AS-E phages was 4.0, 6.3 and 6.1 log CFU/mL (ANOVA, $p < 0.05$) achieved after 8, 12 and 10 h, respectively, when compared with those of the bacterial control (BC) (Figure 3.3A). After 8 h of treatment, the rate of bacterial decrease with the phage AS-D was, in general, significantly higher (ANOVA, $p < 0.05$) than the one obtained with the phage AS-A. However, after 4 and 6 h of treatment, the bacterial reduction with phage AS-A was significantly higher (ANOVA, $p < 0.05$) than the obtained with the phages AS-D and AS-E (Figure 3.3A). After 12 h, the bacterial reduction was still considerably high (2.1, 6.3 and 5.3 log CFU/ mL for phages AS-A, AS-D and AS-E ANOVA, $p < 0.05$) for three phages (Figure 3.3A).

Bacterial density in the BC increased by 3.5 log CFU/mL (ANOVA, $p < 0.05$) during the 12 h of incubation (Figure 3.3A). The phage alone (PC) was constant during all of the time (ANOVA, $p > 0.05$) and when the phages were incubated in the presence of its host a significant increase (ANOVA, $p < 0.05$) of 0.8, 1.7 and 1.7 log PFU/mL was observed for AS-A, AS-D and AS-E, respectively, after 4, 12 and 10 h of incubation (Figure 3.3B).

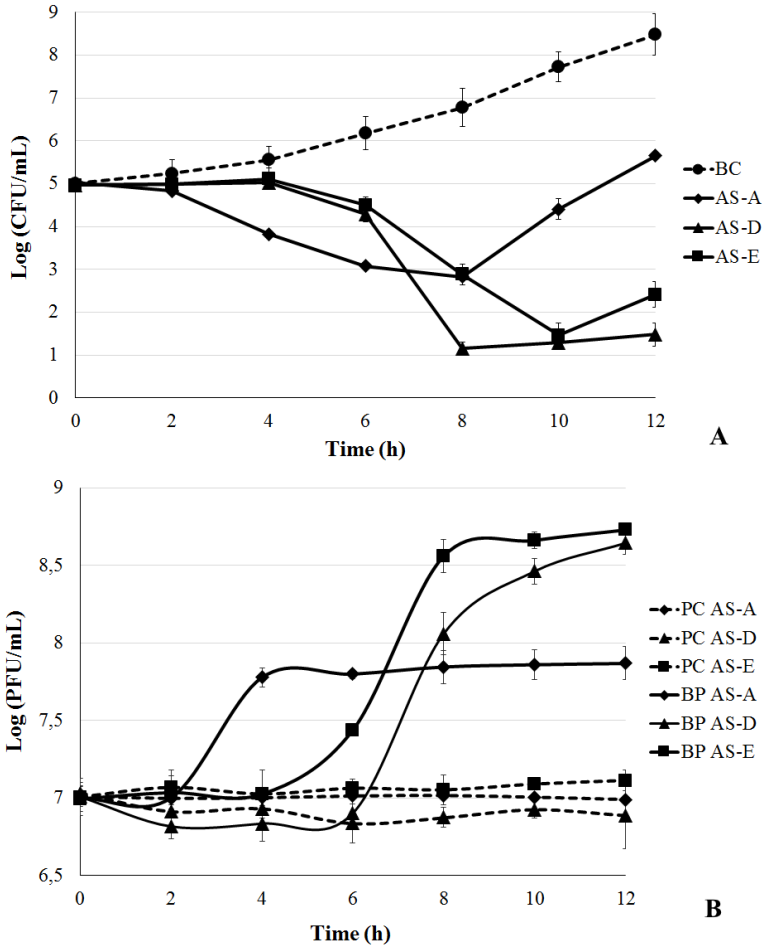

Figure 3.3: Reduction of *A. salmonicida* by the three phages (AS-A, AS-D and AS-E) at a MOI of 100 during 12 h. A. Bacterial concentration: BC—bacteria control; BP—bacteria plus phage. B. Phage concentration:

PC—phage control; BP—bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

3.3.5.2. Bacterial killing curves using phage cocktails

The bacterial reduction was statistically different (ANOVA, $p < 0.05$) for the four phage cocktails (Figure 3.4A).

The maximum of bacterium reduction with the AS-A/AS-D phage cocktail was already 5.4 log CFU/mL (ANOVA, $p < 0.05$) after 10 h of incubation, being statistically different from the values obtained in the phage therapy with the single phage suspensions of AS-A and AS-D (ANOVA, $p < 0.05$) (Figure 3.4A). However, after 4, 6 and 8 h of incubation, the bacterial reduction with AS-A/AS-D phage cocktail was statistically different from that obtained with single phage suspensions of AS-D (ANOVA, $p < 0.05$) and similar to that obtained for the phage AS-A (ANOVA, $p > 0.05$) (Figure 3.4A).

With the phage cocktail AS-A/AS-E, the maximum of bacterial decrease was 5.6 log CFU/mL (ANOVA, $p < 0.05$) after 10 h incubation. With the phage cocktail AS-A/AS-E, the maximum of bacterial reduction was 5.6 log CFU/mL (ANOVA, $p < 0.05$) after 10 h incubation. After 2 and 4 h of incubation, the decrease was significantly higher (2.7 and 3.6 log CFU/mL, respectively) from that obtained with the phage AS-A (0.9 and 2.1 log CFU/mL, respectively) and phage AS-E (0.7 and 0.8 log CFU/mL, respectively) (ANOVA, $p < 0.05$) (0.7 and 0.8 log CFU/mL, respectively). However, after 8 h of incubation, the rate of bacterial decrease with phage AS-E was significantly higher (6.1 log CFU/mL) from that obtained with the phage cocktail AS-A/ASE (5.6 log CFU/mL).

In the phage therapy assays with the phage cocktail AS-D/AS- E, the maximum rate of bacterium reduction was 6.4 log CFU/mL after 12 h of incubation, which was significantly similar from that obtained with the phages AS-D and AS-E. The rates of bacterial reduction during the treatment, were statistically similar (ANOVA, $p > 0.05$) for the phage cocktail AS-D/AS-E and phages AS-D and AS-E (Figure 3.4A).

When the assays of phage therapy were performed with the three phages altogether, AS-A/AS-D/AS-E cocktail, the maximum rate of bacterial decrease was 5.7 log CFU/mL, achieved after 10 h of incubation, being statistically different (ANOVA, $p > 0.05$) from the experiments with the phages AS-A and AS-D (Figure 3.4A). However, for the same period, no significant differences were observed in the therapy assays with the phage AS-E (ANOVA, $p < 0.05$). After 6 h of incubation, the rate of bacterial decrease with the phage cocktail AS-A/AS-D/AS-E was 3.4 log CFU/mL, which was not statistically different from the therapy with the phage AS-A (ANOVA, $p > 0.05$), but was significantly different from the therapy with the other two phages alone (AS-D and

AS-E) (ANOVA, $p > 0.05$). However, after 8, 10 and 12 h of incubation, the bacterial reduction with the phage cocktail AS-A/AS-D/AS-E was similar to the one obtained with the phage AS-E (ANOVA, $p > 0.05$) (Figure 3.4A). Comparing the values of the BC during 12 h of the experiment, none significant differences were observed among the controls (ANOVA, $p > 0.05$) (Figure 3.4A).

Bacterial density in the BC increased by 3.3 log CFU/mL (ANOVA, $p < 0.05$) during the 12 h of incubation (Figure 3.4A). Phage concentration was constant (ANOVA, $p > 0.05$) during the 12 h of experiment for the phage cocktail controls (PC) (Figure 3.4B). The suspensions with the phage cocktails, when incubated in the presence of the host, presented a significant increase (ANOVA, $p < 0.05$). An increase of 0.7, 0.9, 0.9 and 1.6 log PFU/mL was observed after 8 h of incubation (ANOVA, $p < 0.05$) for the phage cocktails AS-A/AS-D, AS-A/AS-E, AS-D/AS-E and AS-A/AS-D/AS-E, respectively (Figure 3.4B).

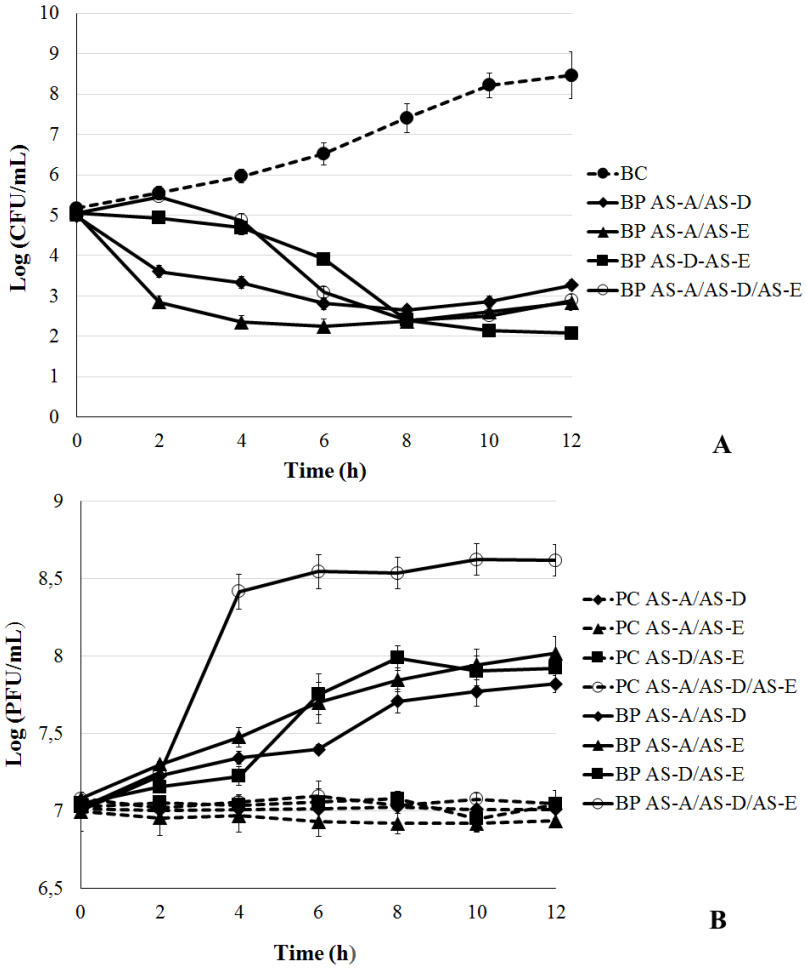

Figure 3.4: Reduction of *A. salmonicida* by phage cocktails at a MOI of 100 during 12 h. (A) Bacterial concentration: BC—bacteria control; BP—bacteria plus phage. (B) Phage concentration: PC—phage control; BP—bacteria plus phage. AS-A - phage AS-A; AS-D - phage AS-D; AS-E – phage AS-E. Values represent the mean of three experiments; error bars represent the standard deviation.

3.3.6. Rate of emergence of phage-resistant mutants

A. salmonicida showed different rates of phage-resistant mutants for single phage suspensions and for phage cocktails (Table 3.2), ANOVA, $p < 0.05$. Phage AS-D presented lower rates of phage - resistant mutants when compared with phage AS-A and AS-E (Table 3.2, ANOVA, $p < 0.05$).

Table 3.2: Frequency of *A. salmonicida* spontaneous phage-resistant mutants for single phage suspensions (AS-A, AS-D, AS-E) and for phage cocktails (AS-A/AS-D, AS-A/AS-E, AS-D/AS-E and AS-A/AS-D/AS-E).

	Control sample (CFU/mL)	**Sample treated with phages**
AS-A	$1.75 \pm 0.15 \times 10^9$	$3.81 \pm 0.18 \times 10^5$
AS-D	$1.68 \pm 0.21 \times 10^9$	$1.53 \pm 0.24 \times 10^5$
AS-E	$1.82 \pm 0.25 \times 10^9$	$2.31 \pm 0.23 \times 10^5$
AS-A/AS-D	$1.71 \pm 0.23 \times 10^9$	$2.80 \pm 0.31 \times 10^5$
AS-A/AS-E	$1.63 \pm 0.27 \times 10^9$	$3.20 \pm 0.29 \times 10^5$
AS-D/AS-E	$1.75 \pm 0.21 \times 10^9$	$1.83 \pm 0.25 \times 10^5$
AS-A/AS-D/AS-E	$1.77 \pm 0.23 \times 10^9$	$3.01 \pm 0.31 \times 10^5$

3.3.7. Fitness of phage resistant mutants

In the presence of AS-D, after 6 h incubation, no differences were found between the growth of resistant bacteria and the growth of sensitive bacteria (Figure 3.5, $t=2.890$, $p=0.1018$). However, after 12 h incubation was observed differences between the growth of resistant bacteria and the growth of sensitive bacteria (Figure 5, $t=10.14$, $p=0.0096$). Resistant bacteria reach a higher concentration at 6 and 12 h when compared to sensitive bacteria (Figure 3.5). In the absence of phage no differences were found between the concentration of resistant bacteria to the phage AS-D and the concentration of sensitive bacteria (Figure 3.5, $t=0.4468$, $p=0.6987$ at 6 h and $t=0.4167$, $p=0.7173$ at 12 h).

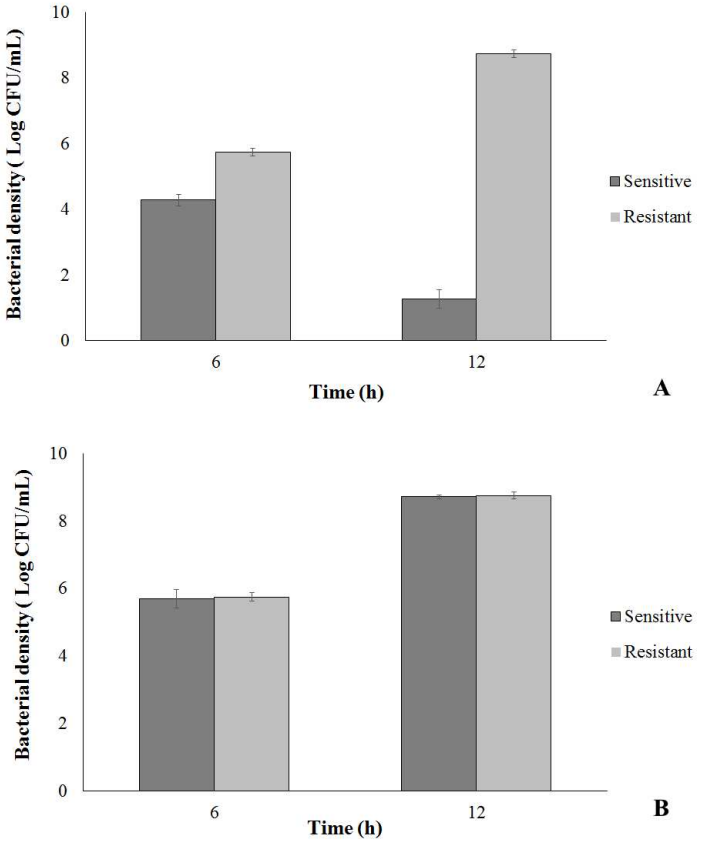

Figure 3.5: Bacterial concentration of resistant mutants versus their sensitive bacteria in the presence (A) or absence (B) of phage AS-D after 6 and 12 h.

In parallel with this assay, we determined the growth curves using the sensitive bacteria and resistant bacteria for the phage AS-D (Figure 3.6). Significant differences (ANOVA, $p < 0.05$) between the growth rate of the sensitive bacteria in presence the phage, and sensitive and resistant bacteria in absence of the phage and resistant bacteria with phage, after 8 h of incubation were detected. No difference in growth curves (ANOVA, $p > 0.05$) between the sensitive and resistant bacteria in the absence of phage and resistant bacteria with phage, during the 12 h of incubation, was observed.

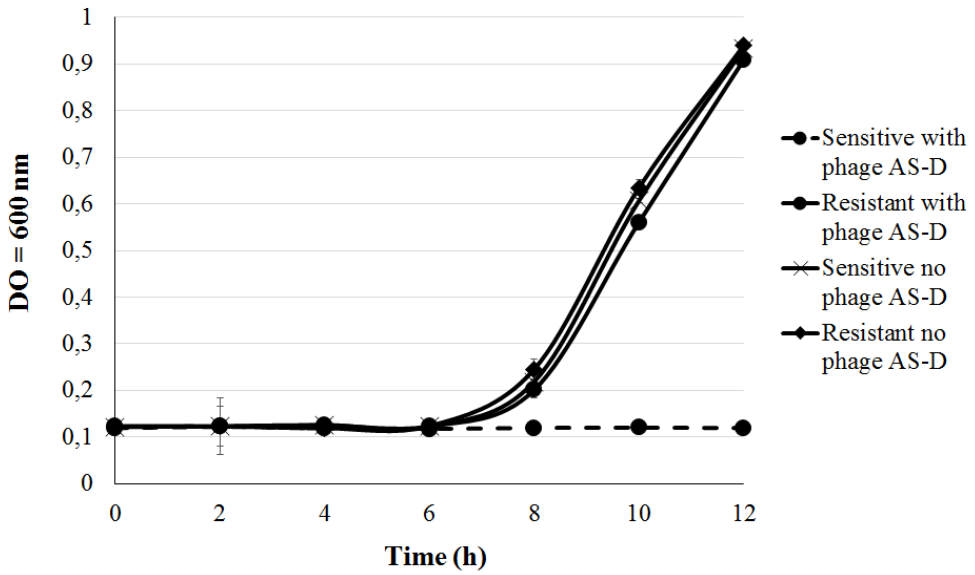

Figure 3.6: Growth curves of sensitive bacteria and resistant mutants in presence or absence of phages during 12 h, with optical density readings at 600 nm.

3.3.8. Assessment of the effect of environmental factors in the phage survival

3.3.8.1. pH experiments

When different pH values (5.5, 7 and 8) were tested, it was observed that the phage concentration slightly decreased with the increase of the pH; however, the differences among the three values of pH were not statistically significant (Figure 3.7, ANOVA, $p > 0.05$). In the three studied pH, phage AS-D persisted viable for at least 107 days at 25 °C (Figure 3.7). The abundance of phage AS-D remained constant during 49 days, then decreased about two orders of magnitude between 49 and 107th day (Figure 3.7, ANOVA, $p < 0.05$).

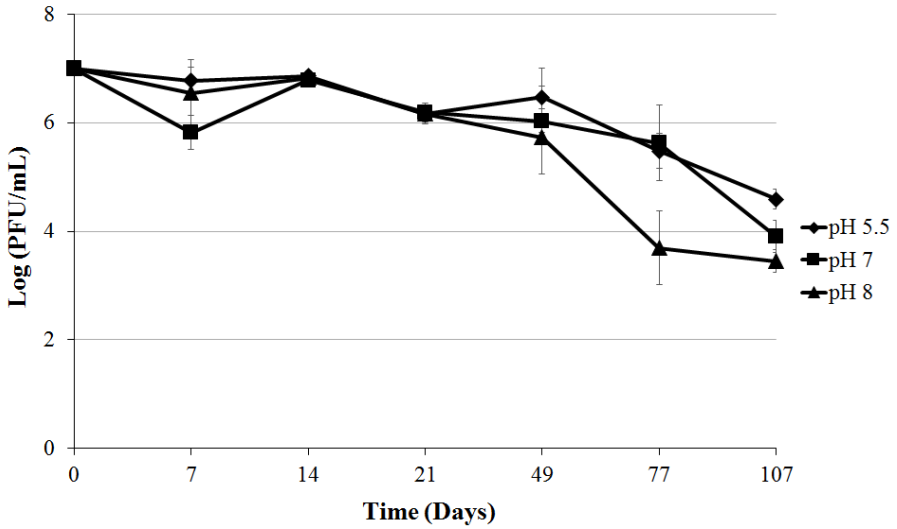

Figure 3.7: Survival of phage AS-D at different pH values. Values represent the mean of three experiments; error bars represent the standard deviation.

3.3.8.2. Temperature experiments

The reduction of the concentration of phage AS-D was higher at 37 °C than at 25 °C and ambient temperature (ANOVA, $p < 0.05$). A maximum decrease of 7 log PFU/mL was observed after 49 days when the samples were kept at a temperature of 37 °C, but after 21 days the decrease was of only 2 log PFU/mL. When the temperature was decreased to 25 °C, the rate of maximum reduction slightly increased to 2.0 log PFU/mL after 107 days of incubation, without any decrease after 21 days. The difference between these two temperatures was statistically significant (ANOVA, $p < 0.05$). At ambient temperature, the abundance of phage AS-D remained constant during 14 days (Figure 3.8, ANOVA, $p > 0.05$), then decreased about two orders of magnitude between day 14 and 107 (Figure 3.8, ANOVA, $p < 0.05$).

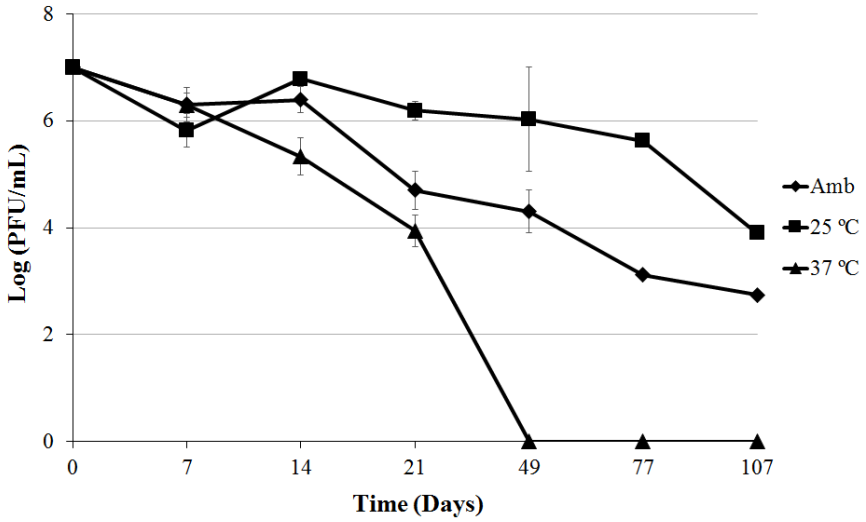

Figure 3.8: Survival of phage AS-D at different temperature values. Values represent the mean of three experiments; error bars represent the standard deviation. Amb: ambient temperature.

3.3.8.3. Salinity experiments

When different salinity concentrations (15, 20 and 30%) were tested, it was observed that the phage concentration decreased along incubation time, however, the differences among the three concentrations of salinity were not statistically significant (Figure 3.9, ANOVA, $p > 0.05$). For the three salinity concentrations, phage AS-D persisted viable for at least 107 days at 25 °C (Figure 3.9). The abundance of phage AS-D decreased about three orders of magnitude after 107 days of incubation (Figure 3.9, ANOVA, $p < 0.05$).

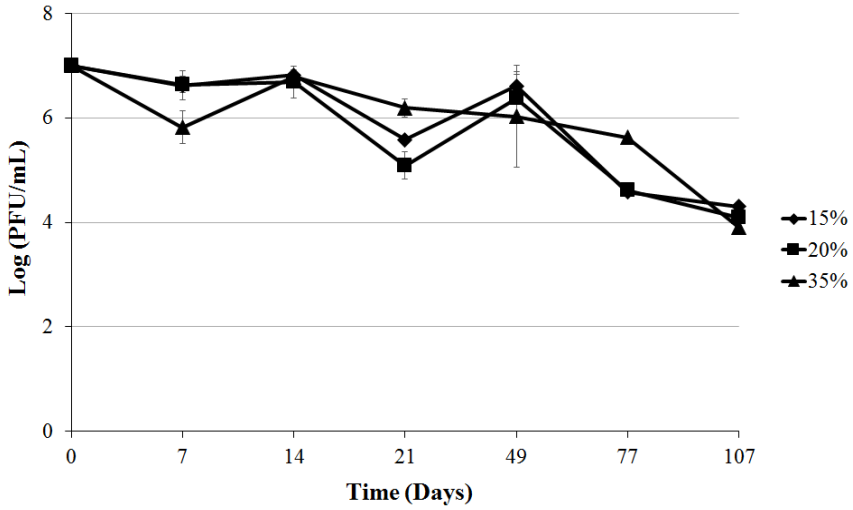

Figure 3.9: Survival of phage AS-D at different salinity concentrations. Values represent the mean of three experiments; error bars represent the standard deviation.

3.3.8.4. UVB experiments

When the phage AS-D was exposed to UV-B irradiation, it was observed that the phage concentration decreased during 12 h of incubation (Figure 3.10). The abundance of phage AS-D exposed to UV-B irradiation was similar to the control during first 8 h of incubation (Figure 3.10, ANOVA, $p > 0.05$), then decreased about 1.3 log PFU/mL between 10 and 12 h (Figure 3.10, ANOVA, $p < 0.05$).

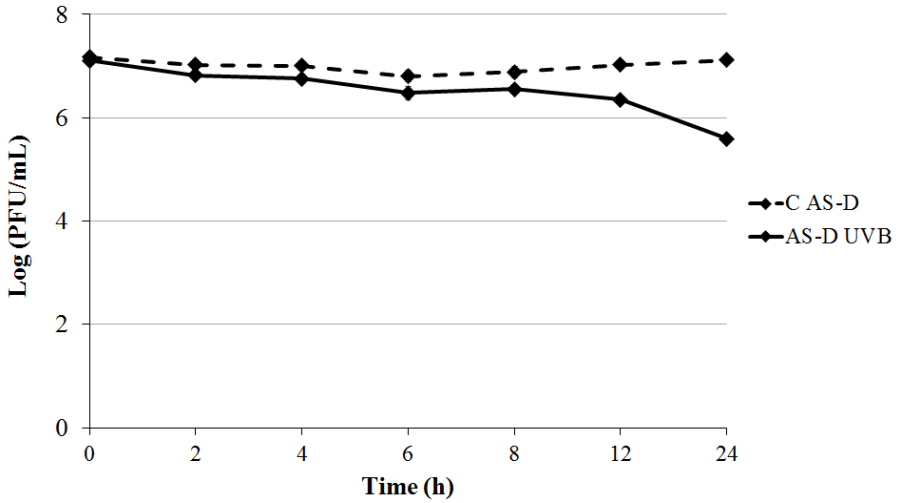

Figure 3.10: Effect of UV-B irradiation on the phage AS-D during 12 h. Values represent the mean of three experiments; error bars represent the standard deviation. UVB: UV-B irradiation.

3.3.8.5. Solar radiation experiments

When the phage AS-D was exposed to solar radiation, it was observed that the phage concentration decreased during 12 h of incubation (Figure 3.11, ANOVA, $p < 0.05$). After 12 h of incubation, the abundance of phage AS-D decreased 2.7 log PFU/mL when compared with control (C AS-D) (Figure 3.11, ANOVA, $p < 0.05$).

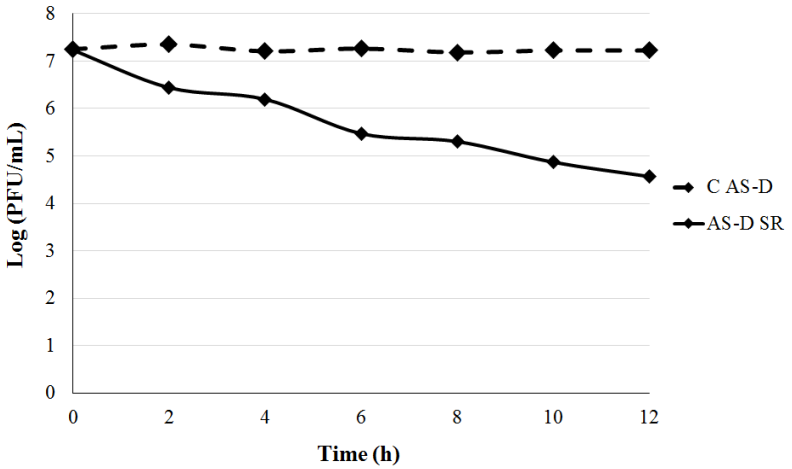

Figure 3.11: Effect of solar radiation on the phage AS-D during 12 h. Values represent the mean of three experiments; error bars represent the standard deviation. SR: solar radiation.

3.4. Discussion

There are few studies about the use of phages to control furunculosis in aquaculture. In a previous work of our group, phage AS-A successfully controlled *A. salmonicida* in juvenile fish (Silva *et al.*, 2016), however, some bacteria developed resistance. As it has been reported that the development of phage-resistance can be circumvented by the use of phage cocktails (Crothers-Stomps *et al.*, 2010; Filippov *et al.*, 2011; Chan, Abedon and Loc-Carrillo, 2013), we isolated two more phages that were used together with phage AS-A as cocktail in order to control phage-resistance emergence. In fact, the results of this study showed that, in general, phage cocktails were more effective to control the bacterium. The bacterial reduction with the cocktails was higher, namely when phage AS-A was combined with one of the two new phages or with both, but the main difference in the bacterial decrease was found in the treatment time. When the phage cocktails were used, the maximum of bacterial reduction was observed after 4–6 h, but with the single suspensions the maximum decrease was observed after 8–10 h of treatment. The two new phages, although needing a long period to control *A. salmonicida* than the phage AS-A, reduced the bacterial concentration more effectively. This can explain the fact that cocktail including phage AS-A and one of the new ones was more effective to control the agent of the furunculosis than the other phage combinations. Additionally, when the cocktails were used, in general, the number of bacteria that regrowth was lower than that observed when the phages were tested as single phage suspensions. These results are in accordance with other studies (Wagner and Waldor, 2002; O'Flynn *et al.*, 2006; Mateus *et al.*, 2014) that achieved a faster and higher bacterial decrease by using phage cocktails, than that obtained with single phage

suspensions. However, the use of phage cocktails, as well as the use of single-phage suspensions, did not prevent the occurrence of phage-resistant mutants. Nonetheless the frequency of phage-resistant mutations was low, 10^{-4}–10^{-5} CFU/mL, and was slightly lower for phage cocktails than for single phage suspensions, as it was observed in other studies (Pereira *et al.*, 2016a, 2017b). With such a small mutation frequency, phage resistance should not hinder the use of phages as biocontrol agents against pathogenic bacteria, as stated before by other authors (Flynn *et al.*, 2004; Tanji *et al.*, 2004). It has been suggested by other authors that phage exposure could be fitness cost for bacteria (Bohannan *et al.*, 1999; Brockhurst *et al.*, 2005), which can contribute to their elimination from the environment faster than their wild-type parents. However, this hypothesis can vary across environment and degree of competition for resources (Lennon *et al.*, 2007; Quance and Travisano, 2009). In this study, the experiments of fitness of phage resistant mutants, showed no differences between the concentration of sensitive bacteria and resistant mutants when grown in the absence of phage AS-D. However, these experiments were done in nutrient rich medium (culture medium) without the presence of competition. Similar results have been observed for *Pseudomonas syringae* (Meaden *et al*., 2015) and *Escherichia coli* (Pereira *et al.*, 2017b). Further studies are necessary to evaluates the cost of resistance in natural aquaculture systems.

As phage resistance may result mainly from mutations that alter cell surface receptors, restriction modification, or abortive infection associated with the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) in the bacterial genome (Allison and Klaenhammer, 1998; Barrangou *et al.*, 2007) therefore, when combining phages it is important to select different ones. The three phages presented similar morphology, however, evident differences between them, namely in their burst size, explosion time and bacterial kill curves, were observed. The growth characteristics of phage AS-A were clearly different from those of phages AS-D and AS-E. Phage AS-A presented the highest burst size (22 ± 5 PFU/host cell) around 4 and 2 times higher than that of phage AS-D (5 ± 1 PFU/host cell) and AS-E (10 ± 1 PFU/host cell), respectively, and the shortest latent period, 30 min (40 min for the phages AS-D and AS-E). Consequently, the bacterial reduction by phage AS-A starts sooner than that when the other two phages were tested. However, phages AS-D and AS-E caused a higher reduction of *A. salmonicida* (maximum reduction of around 6 log CFU/mL) than phage AS-A (maximum reduction of around 4 log CFU/ mL). This can explain the more effective bacterial control when phage AS-A was combined with phage AS-D or phage AS-E. However, no significant difference in the rate of emergence of phage-resistant mutants was observed when these two phage combinations were

tested. Further evaluation trials must still be performed (e.g., whole genome sequencing) to identify the presence of any genes encoding toxins and/or antibiotic resistance.

Considering that in outdoor facilities aquaculture water is exposed to natural variation in physical and chemical parameters, thorough investigations addressing different field conditions are required for the design and implementation of effective phage therapy protocols. This becomes particularly important when phage therapy is applied to semi-intensive marine earth ponds in temperate climates, a culture technique widely employed throughout the world, because in these systems, environmental parameters can shift significantly through the year.

One important factor that influences phage stability is the acidity of the environment (Jończyk *et al.*, 2011). The pH has been shown to be an important control factor, influencing attachment, infectivity, intracellular replication and multiplication of phages (Pirisi, 2000; Leverentz *et al.*, 2001, 2004). Generally, pH values < 5 and over 10 have shown to be less efficient in studies on the lytic activity of phages, being the optimum conditions around a neutral pH of 6–8 [31, 48, 79]. Unfavorable pH can interfere with the lysozyme enzyme and/or with other capsids proteins, thus preventing phage attachment to receptor sites on the host cell (Leverentz *et al.*, 2001, 2004). In this study, pH values ranging from 5.5 to 8 were tested, which are included within the optimum neutral range of pH values, and survival of the phages was not significantly affected. This means that the seasonal variation of pH in aquaculture water would not affect the efficacy of phage therapy.

Temperature is also a crucial factor for bacteriophage survivability (Nasser and Oman, 1999; Olson *et al.*, 2004). It plays a fundamental role in attachment, penetration and multiplication (Jończyk *et al.*, 2011). At low temperatures, fewer phage genetic material enter into bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. High temperatures can extend the length of the latent stage (Tey *et al.*, 2009). In this study, the decrease in concentration of phage AS-D was significantly higher at 37° than at 25 °C and ambient temperature. However, as in aquaculture water this high temperature is not reached, temperature would not be a problem to apply phage therapy in the aquatic environment.

Phages require salts at low concentrations to be successful during the infection process and growth (Baross *et al.*, 1978). At low concentrations, salt ions interact with proteins and stabilize protein structure by neutralizing protein charges. However, at higher concentrations, NaCl increases the thermal denaturation of the proteins and can also adversely affect the structural stability of the phage nucleic acid (Fennema, 1996). In this study, salinity concentrations ranging from 15 to 35% were tested and phage AS-D survival was not significantly affected. Similar results

were also obtained for other phages (Wu and Chao, 1987). This means that phage AS-D can be used to control furunculosis in marine and freshwater fish (the phage AS-D as well as the other two phages are stable in buffer for more than one year).

Solar radiation or, more specifically, UV radiation (UV) has been recognized as the most important factor in the loss of phage infectivity in surface coastal water. UV radiation destroys viruses by chemically modifying their genetic material, DNA and RNA. In fact, the abundance of phage AS-D was decreased when exposed to solar radiation (decreased 2.7 log PFU/mL) and UV-B radiation (decreased 1.3 PFU/mL). Suttle and its coworkers observed that sunlight is the dominant factor implicated in the loss of infectivity in seawater viruses (Suttle and Chen, 1992; Suttle and Chan, 1994). A way to overcome this problem would be to apply phage therapy at the end of the day or during night period, in which UV radiation is limited.

Overall, phage AS-D was affected by the variation of the physicochemical factors, namely by solar radiation and temperature, this can be overcome by the application of phage at the end of the day or at night. Although water temperature in outdoor aquaculture facilities does not reach 37 °C, applying the phages at the end of the day or during night period, the temperature can also be more easily controlled, allowing phage therapy to achieve better results. Taking into account the results of this study and the fact that the variation of pH and salinity in aquaculture waters is moderate (Pereira *et al.*, 2011b), these parameters should not affect phage survival in field conditions.

In future near, it will be essential to understand the efficiency of these single phage suspensions and cocktails in the field, in aquaculture systems. In fact, one of the current challenges faced when performing phage biocontrol studies is to demonstrate their feasibility *in vivo*. According to several authors, the *in vitro* experiments are not sufficient to understand phage–bacteria interactions *in vivo* (Silva *et al.*, 2014a; Pereira *et al.*, 2016c, 2017b; Silva *et al.*, 2016). Numerous other factors, beside the physical and chemical characteristics tested in this study, can affect the survival and lytic properties of the phages in aquaculture systems, e.g., delivery mechanism, fish immune response, presence of zooplankton, phytoplankton and bacterioplankton (Jończyk *et al.*, 2011; Maura and Debarbieux, 2011; Madsen *et al.*, 2013; Ly-Chatain, 2014; Sabouri *et al.*, 2017). In future, it will be essential understand how the interaction of these factors influences the efficiency of phage therapy in aquaculture tanks. Application of phages to control fish pathogens in aquaculture requires also detailed insight to the efficiency of dispersal and persistence of infective phages in the target organs, as well as the factors that regulates their infectivity in fish.

3.5 Conclusions

The results of the present work suggest that phage therapy using phages combined in cocktails or even single phage suspensions can be an effective alternative to control the agent of the furunculosis. In outdoor aquaculture facilities, phages should be applied at the end of the day or during night period in order to have a higher bacterial control. Nonetheless, further studies are needed, namely those performed in aquaculture systems, to understand the true potential of these three phages to control *A. salmonicida*.

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References

Ackermann, H.-W., Tremblay, D. and Moineau, S. (2004) 'Long-term bacteriophage preservation', *WFCC Newsletter*, 38(8), pp. 35–40.

Adams, M. H. (1959) *Bacteriophages*. John Wiley and Sons Inc, New York.

Allison, G. E. and Klaenhammer, T. R. (1998) 'Phage resistance mechanisms in lactic acid bacteria', *International Dairy Journal*, 8(3), pp. 207–226.

Almeida, A., Cunha, Â., Gomes, N. C. M., Alves, E., Costa, L. and Faustino, M. A. F. (2009) 'Phage therapy and photodynamic therapy : Low environmental impact approaches to inactivate microorganisms in fish farming plants', *Marine Drugs*, 7, pp. 268–313.

Arnesen, K. R., Mikkelsen, H., Schrøder, M. B. and Lund, V. (2010) 'Impact of reattaching various *Aeromonas salmonicida* A-layer proteins on vaccine efficacy in Atlantic cod (Gadus morhua)', *Vaccine*, 28(30), pp. 4703–4708.

Baross, J. A., Liston, J. and Morita, R. Y. (1978) 'Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples', *Applied and Environmental Microbiology*, 36(3), pp. 492–499.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Patrick Boyaval, Moineau, S., Romero, D. and Horvath, P. (2007) 'CRISPR provides acquired resistance against viruses in prokaryotes', *Science*, 315(5819), pp. 1709–1712.

Beilstein, F. and Dreiseikelmann, B. (2008) 'Temperate bacteriophage O18P from an *Aeromonas* media isolate: Characterization and complete genome sequence', *Virology*, 373(1), pp. 25–29.

Bergh, O. (2008) 'Bacterial diseases of fish', in Eiras, J., Segner, H., Whali, T., and Kapoor, B. (eds) *Fish diseases*. Enfield, NH, USA: Science Publishers, pp. 239–277.

Björnsdóttir, B., Gudmundsdóttir, S., Bambir, S. H. and Gudmundsdóttir, B. K. (2005) 'Experimental infection of turbot, *Scophthalmus maximus* (L.), by *Aeromonas salmonicida* subsp. *achromogenes* and evaluation of cross protection induced by a furunculosis vaccine', *Journal of Fish Diseases*, 28(3), pp. 181–188.

Bohannan, B. J. M., Travisano, M. and Lenski, R. E. (1999) 'Epistatic interactions can lower the cost of resistance to multiple consumers', *Evolution*. Wiley, 53(1), pp. 292–295.

Brockhurst, M. A., Buckling, A. and Rainey, P. B. (2005) 'The effect of a bacteriophage on diversification of the opportunistic bacterial pathogen, *Pseudomonas aeruginosa*', *Proceedings Biological Sciences*, 272(1570), pp. 1385–1391.

Chan, B. K., Abedon, S. T. and Loc-Carrillo, C. (2013) 'Phage cocktails and the future of phage therapy', *Future Microbiology*, 8(6), pp. 769–783.

Cipriano, R. and Austin, B. (2011) 'Furunculosis and other Aeromonad diseases', in Woo, P. and Br

Coscelli, G. A., Bermúdez, R., Losada, A. P., Faílde, L. D., Santos, Y. and Quiroga, M. I. (2014) 'Acute *Aeromonas salmonicida* infection in turbot (*Scophthalmus maximus* L.). Histopathological and immunohistochemical studies', *Aquaculture*. Elsevier B.V., 430, pp. 79–85.

Crothers-Stomps, C., Høj, L., Bourne, D. G., Hall, M. R. and Owens, L. (2010) 'Isolation of lytic bacteriophage against Vibrio harveyi', *Journal of Applied Microbiology*, 108, pp. 1744–1750.

Dallaire-Dufresne, S., Tanaka, K. H., Trudel, M. V., Lafaille, A. and Charette, S. J. (2014)

'Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis', *Veterinary Microbiology*, 169(1–2), pp. 1–7.

Diamanka, A., Loch, T. P., Cipriano, R. C. and Faisal, M. (2013) 'Polyphasic characterization of *Aeromonas salmonicida* isolates recovered from salmonid and non-salmonid fish', *Journal of Fish Diseases*, 36(11), pp. 949–963.

FAO (2016) *The State of World Fisheries and Aquaculture 2014*. Rome: FAO.

Farto, R., Milton, D. L., Bermúdez, M. B. and Nieto, T. P. (2011) 'Colonization of turbot tissues by virulent and avirulent *Aeromonas salmonicida* subsp. *salmonicida* strains during infection', *Diseases of Aquatic Organisms*, 95(2), pp. 167–173.

Fennema, O. (1996) *Food Chemistry*. 3rd edn. Marcel Dekker, Inc, New York, USA

Filippov, A., Sergueev, K. V., He, Y., Huang, X. Z., Gnade, B. T., Mueller, A. J., Fernandez-Prada, C. and Nikolich, M. P. (2011) 'Bacteriophage-resistant mutants in *Yersinia pestis*: Identification of phage receptors and attenuation for mice', *PLoS ONE*, 6(9), pp. 1–11.

Flynn, G. O., Ross, R. P., Fitzgerald, G. F. and Coffey, A. (2004) 'Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7.', *Appl. Environ. Microbiol.*, 70(6), pp. 3417–3424.

González, S. F., Krug, M. J., Nielsen, M. E., Santos, Y. and Call, D. R. (2004) 'Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray', *Journal of Clinical Microbiology*, 42(4), pp. 1414–1419.

Grontvedt, R. N. and Espelid, S. (2004) 'Vaccination and immune responses against atypical *Aeromonas salmonicida* in spotted wolffish (*Anarhichas minor* Olafsen) juveniles', *Fish and Shellfish Immunology*, 16(3), pp. 271–285.

Higuera, G., Bastías, R., Tsertsvadze, G., Romero, J. and Espejo, R. T. (2013) 'Recently discovered *Vibrio anguillarum* phages can protect against experimentally induced vibriosis in Atlantic salmon, Salmo salar', *Aquaculture*, 392–395, pp. 128–133.

Imbeault, S., Parent, S., Lagacé, M., Uhland, C. F. and Blais, J.-F. (2006) 'Using bacteriophages to prevent furunculosis caused by *Aeromonas salmonicida* in farmed brook rout', *Journal of Aquatic Animal Health*, 18(3), pp. 203–214.

Janda, J. M. and Abbott, S. L. (2010) 'The genus Aeromonas: Taxonomy, pathogenicity, and infection', *Clinical Microbiology Reviews*, 23(1), pp. 35–73.

Jończyk, E., Kłak, M., Międzybrodzki, R. and Górski, A. (2011) 'The influence of external factors on bacteriophages—review', *Folia Microbiologica*, 56(3), pp. 191–200.

Karunasagar, I., Shivu, M. M., Girisha, S. K., Krohne, G. and Karunasagar, I. (2007)

'Biocontrol of pathogens in shrimp hatcheries using bacteriophages', 268, pp. 288–292.

Kim, D. H., Choi, S. Y., Kim, C. S., Oh, M. J. and Jeong, H. D. (2013) 'Low-value fish used as feed in aquaculture were a source of furunculosis caused by atypical *Aeromonas salmonicida'*, *Aquaculture*, 408–409, pp. 113–117.

Kim, J. H., Hwang, S. Y., Son, J. S., Han, J. E., Jun, J. W., Shin, S. P., Choresca, C., Choi, Y. J., Park, Y. H. and Park, S. C. (2011) 'Molecular characterization of tetracycline- and quinolone-resistant *Aeromonas salmonicida* isolated in Korea', *Journal of Veterinary Science*, 12(1), pp. 41–47.

Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S. P., Han, J. E., Jun, J. W., Kang, D. H., Oh, C., Heo, S. J. and Park, S. C. (2012a) 'Isolation and characterization of a lytic Myoviridae bacteriophage PAS-1 with broad infectivity in *Aeromonas salmonicid*a', *Current Microbiology*, 64(5), pp. 418–426.

Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S. P., Han, J. E., Jun, J. W. and Park, S. C. (2012b) 'Complete genomic sequence of a T4-like bacteriophage, phiAS4, infecting *Aeromonas salmonicida* subsp. *salmonicida*', *Archives of Virology*, 157(2), pp. 391–395.

Kutter, E. (2009) 'Phage host range and efficiency of plating', in Clokie, M. R. J. and Kropinski, A. M. (eds) *Bacteriophages: Methods and protocols*. Humana Press, pp. 141–149.

Lago, E. P., Nieto, T. P. and Farto, R. (2012) 'Virulence factors of *Aeromonas salmonicida* subsp. *salmonicida* strains associated with infections in turbot Psetta maxima', *Diseases of Aquatic Organisms*, 99(2), pp. 145–151.

Lasobra, J., Muniesa, M., Frías, J., Lucena, F. and Jofre, J. (1997) 'Relationship between the morphology of bacteriophages and their persistence in the environment', *Water Science and Technology*, 35(11–12), pp. 129–132.

Lennon, J. T., Khatana, S. A. M., Marston, M. F. and Martiny, J. B. H. (2007) 'Is there a cost of virus resistance in marine cyanobacteria?', *The ISME journal*, 1(4), pp. 300–312.

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., Chighladze, E. and Sulakvelidze, A. (2001) 'Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study', *J Food Prot.*, 64(1116–1121), pp. 1116–1121.

Leverentz, B., Conway, W. S., Janisiewicz, W. and Camp, M. J. (2004) 'Optimizing concentration and timing of a phage spray application to reduce *Listeria monocytogenes* on honeydew melon tissue', *Journal of Food Protection*, 67(8), pp. 1682–1686.

Ly-Chatain, M. H. (2014) 'The factors affecting effectiveness of treatment in phages therapy', *Frontiers in Microbiology*, 5, pp. 1–7.

Madsen, L., Bertelsen, S. K., Dalsgaard, I. and Middelboe, M. (2013) 'Dispersal and survival of *Flavobacterium psychrophilum* phages in vivo in rainbow trout and *in vitro* under laboratory conditions: Implications for their use in phage therapy', *Applied and Environmental Microbiology*, 79(16), pp. 4853–4861.

Martínez, S. F. and Hipólito-Morales, A. (2013) 'Efficacy of phage therapy to prevent mortality during the vibriosis of brine shrimp', *Aquaculture*. Elsevier B.V., 400–401, pp. 120–124.

Mateus, L., Costa, L., Silva, Y. J., Pereira, C., Cunha, A. and Almeida, A. (2014) 'Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture', *Aquaculture*. Elsevier B.V., 424–425, pp. 167–173.

Maura, D. and Debarbieux, L. (2011) 'Bacteriophages as twenty-first century antibacterial tools for food and medicine', *Applied Microbiology and Biotechnology*, 90(3), pp. 851–859.

Meaden, S., Paszkiewicz, K. and Koskella, B. (2015) 'The cost of phage resistance in a plant pathogenic bacterium is context-dependent', *Evolution*, 69(5), pp. 1321–1328.

Midtlyng, P. (1997) 'Vaccination against furunculosis', in Gudding, R., Lillehaug, A., and Evensen, Ø. (eds) *Furunculosis: Multidisciplinary Fish Disease Research*. London, UK: Academic Press, pp. 382–404.

Nakai, T. and Park, S. C. (2002) 'Bacteriophage therapy of infectious diseases in aquaculture', *Research in Microbiology*, 153(1), pp. 13–18.

Nakai, T., Sugimoto, R., Park, K. H., Matsuoka, S., Mori, K., Nishioka, T. and Maruyama, K. (1999) 'Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail', *Diseases of Aquatic Organisms*, 37(1), pp. 33–41.

Nasser, A. M. and Oman, S. D. (1999) 'Quantitative assessment of the inactivation of pathogenic and indicator viruses in natural water sources', *Water Research*, 33(7), pp. 1748–1752.

Nikoskelainen, S., Ouwehand, A., Salminen, S. and Bylund, G. (2001) 'Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*', *Aquaculture*, 198(3–4), pp. 229–236.

Noga, E. (2011) *Fish disease: diagnosis and treatment*. 2nd edn. John Wiley & Sons, Iowa, USA

O'Flynn, G., Coffey, A., Fitzgerald, G. and Ross, R. (2006) 'The newly isolated lytic bacteriophages st104a and st104b are highly virulent against *Salmonella enterica*.', *J Appl Microbiol*, 101(1), pp. 251–259.

Olson, M. R., Axler, R. P. and Hicks, R. E. (2004) 'Effects of freezing and storage temperature on MS2 viability', *Journal of Virological Methods*, 122(2), pp. 147–152.

Park, S. E. C., Shimamura, I., Fukunaga, M., Mori, K. and Nakai, T. (2000) 'Isolation of bacteriophages specific to a fish pathogen , *Pseudomonas plecoglossicida* , as a candidate for disease control', *Applied and Environmental Microbiology*, 66(4), pp. 1416–1422.

Park, S. and Nakai, T. (2003) 'Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu', *Dis Aquat Organ*, 53(1), pp. 33–39.

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Cunha, Â., Delgadillo, I., Romalde, J. L., Nunes, M. L. and Almeida, A. (2016a) 'Bacteriophages with potential to inactivate *Salmonella* Typhimurium: Use of single phage suspensions and phage cocktails', *Virus Research*. Elsevier B.V., 220, pp. 179–192.

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Romalde, J. L., Nunes, M. L. and Almeida, A. (2017b) 'Characterization and in vitro evaluation of new bacteriophages for the biocontrol of *Escherichia coli*', *Virus Research*, 227.

Pereira, C., Moreirinha, C., Rocha, R. J. M., Calado, R., Romalde, J. L., Nunes, M. L. and Almeida, A. (2016c) 'Application of bacteriophages during depuration reduces the load of *Salmonella* Typhimurium in cockles', *Food Research International*, 90, pp. 73–84.

Pereira, C., Salvador, S., Arrojado, C., Silva, Y., Santos, A. L., Cunha, A., Gomes, N. C. M., Gomes, N. and Almeida, A. (2011b) 'Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy.', *Journal of environmental monitoring*, 13(4), pp. 1053–8.

Pereira, C., Silva, Y. J., Santos, A. L., Cunha, Â., Gomes, N. C. M. and Almeida, A. (2011a) 'Bacteriophages with potential for inactivation of fish pathogenic bacteria : Survival , host specificity and effect on bacterial community structure', *Marine Drugs*, 9, pp. 2236–2255.

Pirisi, A. (2000) 'Phage therapy—advantages over antibiotics?', *Lancet*, 356(9239).

Quance, M. A. and Travisano, M. (2009) 'Effects of temperature on the fitness cost of resistance to bacteriophage T4 in *Escherichia coli*.', *Evolution*, 63(6), pp. 1406–1416.

Rebelo, J. (1992) 'The ichthyofauna and abiotic hydrological environment of the Ria de Aveiro, Portugal', *Estuarines and Coasts*, 15(3), pp. 403–413.

Ringø, E., Jutfelt, F., Kanapathippillai, P., Bakken, Y., Sundell, K., Glette, J., Mayhew, T. M., Myklebust, R. and Olsen, R. E. (2004) 'Damaging effect of the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* on intestinal enterocytes of Atlantic salmon (*Salmo salar* L.)', *Cell and Tissue Research*, 318(2), pp. 305–312.

Sabouri, S., Sepehrizadeh, Z., Amirpour-Rostami, S. and Skurnik, M. (2017) 'A minireview on the *in vitro* and *in vivo* experiments with anti-*Escherichia coli* O157:H7 phages as potential

biocontrol and phage therapy agents', *International Journal of Food Microbiology*. Elsevier B.V., 243, pp. 52–57.

Shao, Z. J. (2001) 'Aquaculture pharmaceuticals and biologicals: Current perspectives and future possibilities', *Advanced Drug Delivery Reviews*, 50(3), pp. 229–243.

Silva, Y. J., Costa, L., Pereira, C., Cunha, A., Calado, R., Gomes, N. C. M. and Almeida, A. (2014) 'Influence of environmental variables in the efficiency of phage therapy in aquaculture', *Microbial Biotechnology*, 7(5), pp. 401–413.

Silva, Y. J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., Gomes, N. C. M., Pardo, M. A., Hernandez, I. and Almeida, A. (2014) 'Phage Therapy as an Approach to Prevent *Vibrio anguillarum* Infections in Fish Larvae Production', *PLoS ONE*, 9(12).

Silva, Y. J., Moreirinha, C., Pereira, C., Costa, L., Rocha, R. J. M. R. J. M., Cunha, Â., Gomes, N. C. M. N. C. M., Calado, R. and Almeida, A. (2016) 'Biological control of *Aeromonas salmonicida* infection in juvenile *Senegalese sole* (*Solea senegalensis*) with Phage AS-A', *Aquaculture*, 450, pp. 225–233.

Suttle, C. A. and Chan, A. M. (1994) 'Dynamics and distribution of cyanophages and their effect on Synechococcus spp', *Applied and Environmental Microbiology*, 60(9), pp. 3167–3174.

Suttle, C. A. and Chen, F. (1992) 'Mechanisms and rates of decay of marine viruses in seawater mechanisms and rates of decay of marine viruses in seawatert', *Applied and Environmental Microbiology*, 58(11), pp. 3721–3729.

Tewari, R., Dudeja, M., Nandy, S. and Kumar Das, A. (2014) 'Isolation of *Aeromonas salmonicida* from Human Blood Sample: A case report', *Journal of Clinical and Diagnostic Research*, 8(2), pp. 139–140.

Tey, B. T., Ooi, S. T., Yong, K. C., Yeen, M., Ng, T., Ling, T. C. and Siang Tan, W. (2009) 'Production of fusion m13 phage bearing the di-sulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen', *African Journal of Biotechnology*, 8(2), pp. 268–273.

Vargas, C. I. C., Vaz, N. and Dias, J. M. (2017) 'An evaluation of climate change effects in estuarine salinity patterns: Application to Ria de Aveiro shallow water system', *Estuarine, Coastal and Shelf Science*, 189, pp. 33–45.

Verner–Jeffreys, D. W., Algoet, M., Pond, M. J., Virdee, H. K., Bagwell, N. J. and Roberts, E. G. (2007) 'Furunculosis in Atlantic salmon (*Salmo salar* L.) is not readily controllable by bacteriophage therapy', *Aquaculture*, 270(1–4), pp. 475–484.

Vinod, M. G., Shivu, M. M., Umesha, K. R., Rajeeva, B. C. and Krohne, G. (2006) 'Isolation of

Vibrio harveyi bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments', 255, pp. 117–124.

Wagner, P. L. and Waldor, M. K. (2002) 'Bacteriophage control of bacterial virulence', *Infection and Immunity*, 70(8), pp. 3985–3993.

Wahli, T., Knuesel, R., Bernet, D., Segner, H., Pugovkin, D., Burkhardt-Holm, P., Escher, M. and Schmidt-Posthaus, H. (2002) 'Proliferative kidney disease in Switzerland: Current state of knowledge', *Journal of Fish Diseases*, 25(8), pp. 491–500.

Wu, J. and Chao, W. (1987) 'The epizootic of milkfish vibriosis and its biological control by bacteriophage AS10', *The memoir of Virology and Pharmacology in fish disease*. Taipei: The WorldFish Center and SEAFDEC, p. 3.

Zorrilla, I., Chabrillón, M., Arijo, S., Díaz-Rosales, P., Martínez-Manzanares, E., Balebona, M. . and Moriñigo, M. (2003) 'Bacteria recovered from diseased cultured gilthead sea bream (*Sparus aurata* L.) in southwestern Spain', *Aquaculture*, 218(1–4), pp. 11–20.