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In vitro evaluation of an antibiofilm product: a combination between bacteriophages and Portuguese honey

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que cai sobre nós..."

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RESUMO

As feridas crónicas representam um grande encargo tanto a nível económico como social. A *Pseudomonas aeruginosa* e a *Escherichia coli* fazem parte dos organismos colonizadores mais comuns nas feridas crónicas, sendo formadoras prolíficas de biofilmes. Estes tornaram-se um dos grandes problemas em infeções devido à crescente dificuldade no seu controlo e erradicação, e tolerância a vários fármacos prescritos. Assim, são necessários métodos alternativos. Os bacteriófagos (fagos) e o mel são vistos como estratégias promissoras para o controlo de biofilmes associados a infeções. Os fagos apresentam especificidade relativa a um género, espécie ou mesmo estirpe, auto replicam-se e não promovem a disbiose. O mel tem ganho reconhecimento devido às suas propriedades antibacterianas, antioxidantes, anti-inflamatórias, assim como em tratamento de feridas. Este trabalho aborda os efeitos antibacterianos dos fagos, mel e a sua combinação em biofilmes simples e mistos de *P. aeruginosa* e *E. coli*. Esta avaliação foi conseguida através de ensaios de atividade antimicrobiana padrão e análise de citometria de fluxo. O fago selecionado para *P. aeruginosa* (PAO1-D), um fago *Podoviridae*, apresentou placas rodeadas por um halo, um indicativo de atividade de depolimerase, lisou 41.6% das estirpes usadas, libertou 40 fagos por cada célula infetada e manteve atividade antibacteriana mesmo 24 h após tratamento. O fago de *E. coli* (EC3a), com 530 fagos libertados por célula infetada, também apresentou atividade de depolimerase e pertence à família *Siphoviridae.* Dois méis portugueses (U3 e C1) com diferente origem floral revelaram a mesma concentração mínima inibitória (25% (w/v)), no entanto diferente atividade antibacteriana *in vitro.* Nas abordagens individuais, os fagos revelaram uma eficácia maior em tratamentos de menor duração, contrastando com os méis que foram mais eficazes em períodos mais longos. A combinação com mel 4 vezes diluído (fago–$U3_{25\%}$) resultou num sinergismo 24 h após aplicação em biofilmes de *P. aeruginosa*, um resultado suportado pela contagem de células viáveis e análise de citometria de fluxo, que revelou um aumento de restos celulares quando comparado com a aplicação de mel individualmente. Esta mesma abordagem resultou num efeito aditivo às 12 h em biofilmes de *E. coli.* As células danificadas não tiveram capacidade de crescimento, confirmação obtida pelos ensaios antibiofilme. Finalmente, a presença de uma segunda espécie num consórcio não afetou a eficácia do tratamento. O trabalho desenvolvido revela uma estratégia promissora para o tratamento de biofilmes de *P. aeruginosa* e *E. coli* associados a infeções em feridas.

PALAVRAS-CHAVE: FAGO, MEL, BIOFILMES, SINERGIA

ABSTRACT

Chronic skin wounds represent a major burn both economically and socially. *Pseudomonas aeruginosa* and *Escherichia coli* are among the most common colonizers of infected wounds and are prolific biofilm formers. Biofilms are a major problem in infections due to their increasingly difficult control and eradication, and tolerance to multiple prescribed drugs. As so, alternative methods are necessary. Bacteriophages (phages) and honey are both seen as a promising approach for biofilm related infections. Phages have specificity towards a bacterial genus, species or even strain, self-replicating nature, and avoid dysbiosis. Honey has gained acknowledgment due to its antibacterial, antioxidant and anti-inflammatory and wound healing properties. This work presents insights into the antibacterial effects of phage, honey and their combination on *P. aeruginosa* and *E. coli* mono and dual species biofilms. This evaluation was achieved through standard antimicrobial activity assays and flow cytometry studies. The selected *P. aeruginosa* phage (PAO1-D), a *Podoviridae*, presented plaques surrounded by halo which is an indicative of depolymerase activity, was able to lyse 41.6% of the used strains, had a burst size of 40 released phages per infected cell, and possessed antibacterial activity even after 24 h of treatment. The *E. coli* specific phage (EC3a), with a burst size of 530 phages per infected cell, possessed also depolymerase activity and belongs to the *Siphoviridae* family. Two Portuguese honeys (U3 and C1) with different botanical source revealed the same minimum inhibitory concentration value (25% (w/v)) however different *in vitro* antibacterial activities. In single approaches, phages revealed better efficiency for treatments of short duration, oppositely to honeys that were more effective at longer periods. The combination with 4-fold diluted honey (phage–U3$_{25\%}$) in *P. aeruginosa* biofilms resulted in synergism after 24 h of application (2.84 log viable cell reduction), a result supported by viable cell counts and flow cytometry analysis that revealed an increase of cellular debris when compared to honey treatment alone. Additive effect was perceived at 12 h for *E. coli* biofilms (3.27 log viable cell reduction) using the combinatorial approach. Compromised cells were not able to regrow, as confirmed by antibiofilm assays. Finally, the presence of a second microorganism in a consortium of species did not affect the effectiveness of the treatment. The work developed reveals a promising approach for the treatment of *P. aeruginosa* and *E. coli* biofilm related wound infections.

KEYWORDS: PHAGE, HONEY, BIOFILMS, SYNERGY

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

×g	Gravitational force
°C	Celsius degrees
µg/mL	Microgram *per* milliliter
µL	Microliter
µm	Micrometer
C1	Chestnut 1
CFU	Colony-forming unit
CFU/mL	Colony-forming unit *per* milliliter
cm	Centimeter
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ECM	Extracellular matrix
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FAS	Ferrous ammonium sulfate
FDA	Food and Drug Administration
FnBPA	Proteinaceous fibronectin-binding protein A
g/L	Grams *per* liter
h	Hour(s)
IL-1β	Interleukin-1 beta
Kb	Kilobase
kDa	Kilodalton
LPS	Lipopolysaccharide
M	Molar
MBEC	Minimum biofilm eliminating concentration
mg/kg	Milligram *per* kilogram
MGO	Methylglyoxal
MIC	Minimum inhibitory concentration

min	Minute(s)
mL	Milliliter
mL/L	Milliliter *per* liter
mM	Millimolar
MOI	Multiplicity of infection
MRJP1	Major royal jelly protein 1
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*
nm	Nanometer
nM	Nanomolar
OD	Optical density
OprF	Outer membrane porin F
PDGF	Platelet-derived growth factor
PEG 8000	Poly(ethylene glycol) 8000
PF2	Polyfloral 2
PFU	Plaque-forming unit
PFU/mL	Plaque-forming unit *per* milliliter
PIA	Polysaccharide intercellular adhesion
PNA-FISH	Peptide nucleic acid-based fluorescence in situ hybridization
PUSH	Pressure Ulcer Scale for Healing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations *per* minute
ssRNA	Single stranded RNA
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor alpha
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U3	Urze 3
v/v	Volume *per* volume
w/v	Weight *per* volume

Motivation

and aim of the thesis

The decades of extensive and misused application of antibiotics, allied to the high mutation frequencies and exchange of genetic information seen in bacteria, has led to a serious global health crisis due to antibiotic resistance. The current dissemination of antibiotic resistant bacteria calls for alternative approaches. The use of phages, the natural predators of bacteria, is listed by the US National Institute of Allergy and Infectious Diseases as a research priority for addressing this antibiotic crisis. Another approach is to use honey that is a natural product, used since ancient times to treat several infectious conditions. Honey has several attractive properties and the most interesting is the fact that no resistant microorganisms to honey have yet been found. Both these treatments can be used in chronic wounds, infected laceration or burn injuries colonized by diverse pathogenic bacteria, including multi-resistant to antibiotics.

The work developed in this thesis aimed to evaluate the use of these two natural antimicrobial agents, honey and phage, towards bacteria frequently present in chronic wounds - *P. aeruginosa* and *E. coli* - and assess if using co-therapy would provide synergistic effect. Isolation and characterization of phages was performed, as well as analysis of honeys antiviral effect. The study relied in the use of *in vitro* models of established mature biofilms of the individual species and, then, their consortia.

1. Introduction

1.1 Chronic wounds

Wound healing is a complex process divided in several phases. When one of them becomes compromised, a chronic wound may develop. Chronic wounds are commonly defined as wounds which have failed the sequential reparative process that could repair the anatomic and functional integrity of the damaged tissue in a period of four to eight weeks[1,2].

Although difficult to establish exact numbers on the prevalence and incidence of chronic wounds[3,4] it is well acknowledged that these wounds lead to considerable morbidity and costs associated with treatment, which represents an increasing burden on public and health systems worldwide. In fact, in 2009, there was an annual incidence of 4 million individuals with wounds in Europe and about 20% of hospital inpatients suffered with a pressure ulcer[5]. In the same year, only in United States of America, about 6.5 million patients were affected by chronic wounds leading to a cost of 25 billion dollars spent annually[6]. There is no general data in literature regarding wound management costs in Portugal. Besides the socio-economic impact, it is important to highlight the problem as a concern for the patient, affecting one's mobility, psychological state and eventually reduced quality of life[6].

The majority of chronic wounds is associated with one of four primary diagnosis, namely ischemia, diabetes mellitus, venous stasis, and pressure[7]. For example, in diabetic patients, the presence of diabetic neuropathy or damage to the foot's sensory nerves leads frequently to foot deformities and ulcerations. In fact, it is estimated that up to 25% of all diabetics will develop a diabetic foot ulcer during lifetime[8]. Numbers of chronic ulcers incidence and wound management costs are likely to increase due to the ageing of population, the fraction of society most affected by chronic wounds[9], but also due to the growth of the incidence of associated diseases as obesity, diabetes and cardiovascular diseases[10–12]. Based on the underlying condition, they are divided in three major categories: venous ulcers, pressure ulcers and diabetic ulcers, but they all share common physiological processes of development[13].

1.1.1 Development of chronic wounds

The normal response to a wound comprises four sequential phases: haemostasis following a structural damage to the skin, inflammation, proliferation, and tissue remodeling. This process involves interactions among a variety of cell types, structural proteins, growth factors, and proteinases[14].

Several factors can influence the phases of the process, leading to a delayed response, which can result in a chronic wound. The development of an inflammatory reaction in a chronic wound is, contrasting to the normal self-limiting process seen in acute wounds, a continuous process that acts as

a positive feedback, promoting the constant recruitment of neutrophils[15]. The former lead to an excessive non balanced amount of degradative matrix metallic proteinases, when compared to the amount of their correspondent tissue inhibitors, resulting in the destruction of extracellular matrix (ECM)[16,17]. Associated with this environment, there are pro-inflammatory cytokines, as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), present in high levels and decreased growth factors – such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF) - activity when compared to acute wounds[18]. Reactive oxygen species (ROS) production is also increased due to this inflammatory environment, contributing as well to ECM damage[19]. Wound healing is also impaired by tissue ischemia[20]. It is acknowledged that fibroblasts are essential to the production of new extracellular matrix[21]. The fluids present in chronic ulcers seem to inhibit the proliferation of human dermal fibroblasts and development of vascular endothelial cells[22]. Additionally, fibroblasts with premature senescence and, therefore, reduced activity have been reported in this context[23].

Bacteria have a significant role in promoting the healing of wounds, as it has been stated during years. Several microorganisms from endogenous (patient's own flora) and exogenous (treatment facilities) environments have the ability to access and to replicate within the wound without tissue damage. When critical colonization happens and a host's immune system fails to eliminate the pathogenic bacteria, they lead to an infective state[24]. Hence, inflammation is prolonged once both bacteria and endotoxins promote an extended increase of the pro-inflammatory cytokines stated before. Microbiology of chronic wound is diverse and complex. In recent years, strong scientific evidence has supported the fact that the microbiome of chronic wounds is mostly associated with the biofilm phenotype[25–31] which is responsible for a delayed healing answer. Given the importance of biofilm structures on the wound healing process, the understanding of biofilm development and behavior is essential for an appropriate response strategy.

1.2 Biofilms

1.2.1 Biofilm characterization and development

Bacterial growth can be characterized by at least two different phenotypes: single cells (planktonic) or sessile communities, which are referred as biofilms. The concept "biofilm" was introduced in 1981[32], but bacterial aggregation has been reported much earlier, when Anthony van Leeuwenhoek, in 1684, observed clusters of bacteria in the 'scurf of the teeth'[33]. Biofilms are defined by Costerton[34] as a three-dimensional microbial structure, consisting of a multicellular community composed of prokaryotic and/or eukaryotic cells embedded in a self-produced matrix containing extracellular polymeric substances

(EPS) such as polysaccharides, and other substances, namely proteins, extracellular DNA (eDNA), membrane vesicles, and other polymers. In spite of beneficial in several contexts, they are associated with the majority of human infections, being critical in clinical settings as they are extremely recalcitrant to elimination by antimicrobial agents and the host's immune system[35].

The development of a biofilm involves sequential stages, represented in Figure 1. Generally, the process is initiated with the adhesion of planktonic bacteria to a surface[36]. This is facilitated by adhesion molecules that bind to receptors or other molecules at the site of attachment[37,38]. Appendages found on the surface of certain bacteria such as *Pseudomonas* and *Klebsiella* spp., namely fimbriae and pili, may also play a role[39,40]. Following an initial reversible attachment, microbial cells attach irreversibly and begin to proliferate into small clusters or microcolonies and to produce a polymer matrix around them. Microcolonies gradually develop into mature biofilm. At this stage, cells form a three-dimensional bulbous, holding water channels within it that act as a transport system for the movement of nutrients and waste products[41]. Dispersal of bacterial cells finally occurs, through the release of matrix polymer-degrading enzymes, resulting in cell detachment[36].

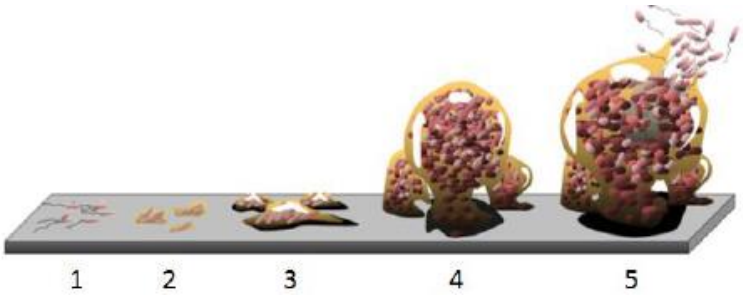


Figure 1. Developmental stages of a *Pseudomonas aeruginosa* biofilm. **1)** Initial reversible attachment of planktonic cells to a surface. **2)** Irreversible attachment to the surface, characterized by the loss of flagella, and development of microcolonies. **3)** Biofilm maturation due to increased proliferation and EPS secretion to form the matrix. **4)** As the mature biofilm develops, colony growth slows and biofilm dynamics are established. **5)** Dissemination of biofilm cells by environmental shear forces or the release of planktonic cells. Adapted from Monroe[42].

1.2.2 Biofilm resistance traits

Biofilms are considered a protected mode of growth that allows bacteria to survive in hostile environments, being their physiology and behavior significantly different from their planktonic equivalents. As example, they present an altered growth rate[43] and gene expression[44], which translates in a variety of phenotypic characteristics[45]. There is strong evidence indicating that the biofilm phenotype promotes increased resistance and tolerance to antimicrobial products. In fact, when aggregated, bacteria have the capacity to be 10 to 1000-fold less susceptible to various antimicrobial agents than the same bacterium growing as a free floating culture[46,47].

Resistance traits are found in the biofilm mode of growth, and increased mutation rates in biofilms which enhance resistance development have been reported[48,49]. The existence of efflux pumps allows the active export of antimicrobials without reaching lethal concentrations within the bacterium. These systems have been characterized in several biofilm forming pathogens, such as *Escherichia coli*[50], *Pseudomonas aeruginosa*[51] and *Staphylococcus aureus*[52]. The production of antibiotic degrading-enzymes, such as β-lactamase enzyme, by biofilm forming strains can also decrease the action of antibiotics[53].

Within the chronic wound, the biofilm tolerance to several antibiotics and host defenses is promoted by several factors, as seen in Figure 2. Firstly, the biofilm matrix - composed of complex polysaccharide polymers, peptidoglycan, lipoproteins, eDNA - offers structural stability and increased tolerance to antimicrobials and immune cells[54], acting as diffusional barrier both to antibiotics[55] and to host defenses[56]. Some biofilms impair Immunoglobulin G and complement deposition, resulting in reduced phagocyte-mediated killing and cytotoxins released by bacteria attack host immune cells[57]. Also, eDNA can be exchanged among bacteria and has been demonstrated to function as a protective shield against aminoglycosides[58]. Persister cells are cells with slow growth and severely limited metabolic activity that may be present in biofilms and are highly tolerant to antibiotics[59]. They can reactivate after such stress, leading to the regrowth of the biofilm after treatment[60]. Furthermore, in general, multispecies biofilms give fitness advantages (ability to form biofilm and persist under a certain environment or environmental stress) to bacterial species compared to their single-species biofilms[61].

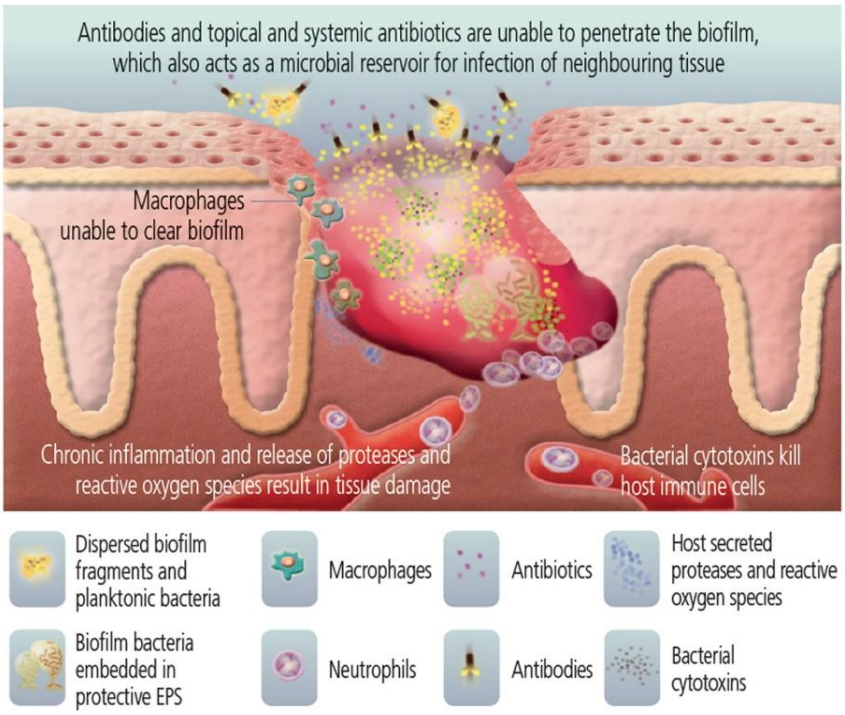


Figure 2. Biofilm characteristics that allow bacteria to proliferate and survive. Adapted from Phillips *et al.*[62].

1.2.3 Clinical evidence and relevance of biofilms in chronic wounds

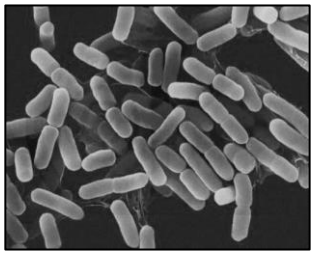
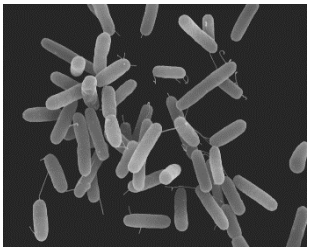
The first publication regarding the presence of a biofilm in a wound was in 1985[63] when Gristina and colleagues analyzed sutures and staples removed from surgical wounds using scanning electron microscopy. In their analysis, consistent colonization by bacteria placed in a fibrous extracellular matrix – a structure that resembled a biofilm, was observed. Since then, the role of biofilms in wound healing has been debated for years (extensively reviewed in[35,64]).

The implementation of molecular and microscopy techniques has allowed a stronger characterization of the microbial flora in wound. For instance, in 2008, James and colleagues[25] observed chronic and acute wounds specimens to search for biofilms. Through the use of light and scanning electron microscopy techniques, they identified that biofilm phenotype predominates in 60% of chronic wounds studied, whereas only 6% of acute wound specimens was characterized as containing biofilm. A study of 22 specimens of chronic wounds using peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH) method revealed that large bacterial aggregates of bacteria were present in approximately 60% of the samples[28]. Also, *P. aeruginosa*, present in 70% of chronic wounds, was found aggregated in the deeper regions of the wounds as microcolonies imbedded in an alginate matrix[29]. These results were corroborated in a study regarding distribution of *P. aeruginosa* and *S. aureus* in chronic wounds[30]. Diabetic foot ulcers also revealed the presence of dense aggregated colonies of bacteria often surrounded by EPS and host-cell debris. In these ulcers, single cells but also large aggregates of grape-like clusters, characteristic of biofilms were observed[31].

Wound microbiota identification and characterization has revealed a diverse community of bacteria. Dowd and colleagues[65] have conducted a broad survey of wounds using molecular methods to reveal the major populations of bacteria that occur in the pathogenic biofilms of chronic wounds. Different types of chronic wounds had marked differences in bacterial populations, but the main prevalent populations evident in all chronic wound types included *Staphylococcus, Pseudomonas, Peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia*, and *Serratia* spp.. All three types of evaluated chronic wounds (diabetic foot ulcers, venous leg ulcers, and pressure ulcers) revealed the presence of a large portion of anaerobic bacteria (approximately 80%, considering both strict and facultative anaerobes)[65]. Coaggregation of bacteria is suggested to lead to a synergistic effect that provides the biofilm community with the necessary factors to maintain chronic biofilm infections[65,66]. In spite of this claim, some authors defend that although multiple species can be present in the same wound, they are normally aggregated as single species[28,29].

E. coli and *P. aeruginosa* are two frequently identified organisms in a chronic wound. It is important to mention that both species can be found in the human body, such as in the gastrointestinal tract[67], the skin, throat, and stool[68]. Both bacteria are highly difficult to eradicate[69] and the factors that may promote biofilm development[70,71] are presented in **Table 1.**

Table 1. Organisms of study and factors promoting their biofilm development.

Organism	Characteristics	Factors for biofilm development
E. coli		
	• Family: *Enterobacteriaceae;* • Gram-negative bacillus; • Facultative anaerobic; • Dimensions: 2-3 μm in length × 0.5 μm in width.	**Adhesion and development** Flagella, adhesion factors, type 1 pili, curli fimbriae, conjugative pili **Maturation of the biofilm structure** Production of EPS, PGA, cellulose, colanic acid, LPS O-antigen, *E. coli* capsules **Quorum sensing**
P. aeruginosa		
	• Family: *Pseudomonadaceae;* • Gram-negative bacillus; • Nonfermentative aerobic; • Dimensions: 1.5-3.0 μm in length × 0.5-0.8 μm in width • High nutritional and survival temperature versatility	**Adhesion** Pili, lipopolysaccharides **Maturation** Alginate, rhamnolipids **Disruption of host cell structures** Elastase, toxin A and exoenzyme S, pyocyanin **Quorum sensing** **Mobile genetic elements** **Transference of resistance genes**

.

Fighting biofilm progress aiming a faster and more effective treatment of chronic wounds becomes evident. Several strategies have been developed during recent years with limited success[72] leading to the search of other strategies such as bacteriophages (phages) and honey.

1.3 Bacteriophages

1.3.1 Brief historical context

Phages are viruses that infect and replicate within bacteria. They are considered the most abundant entities in the world, exceeding bacteria in number by tenfold[73] and can be found wherever bacterial development is observed. Their discovery is attributed to the bacteriologist Frederick Twort in 1915[74] and, independently, to the microbiologist Felix d'Herelle in 1917[75]. D'Herelle noticed clear spots on cultures of bacteria, suggesting elimination of the organisms. Later, when investigating dysentery, he acknowledged that the bacterial culture was destroyed by an unknown agent in the filtrate where the clear areas were present. He suggested that these entities were viruses and they were named bacteriophage, from the words "bacteria" and "phagein", meaning "eaters of bacteria". During his career, he isolated phages for bacteria responsible for such diseases as cholera, bubonic plague or anthrax[76].

In the early 1930's, a few studies[77–79] presented fairly promising results and the model of 'phage therapy', aiming cellular destruction of pathogenic bacteria while remaining completely innocuous to host cells due to phage selectivity[80], was considered as a possible 'magic bullet' in public health. At this time, several companies, as d'Herelle's commercial laboratory, in Paris, and Eli Lilly Company, in Indianapolis, among others, began large-scale production of phages against various bacterial pathogens[81].

However, about 20 years after the discovery of phages, Alexander Fleming revealed the first antibiotic – penicillin – and its success along with some early phage clinical failures as well as scientific controversies and ethical concerns, dictated the end of phage therapy in the US and in most Western European Countries. Phages continued to be used therapeutically, combined with antibiotics or alone, in Poland and Georgia, and in the former Soviet Union. Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences, in Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, in Poland, were the main centers of activities regarding therapeutic phage research and production[81].

Recently, public health concerns regarding bacterial resistance to antibiotics have driven research groups to develop novel or adopt old strategies to overcome the issue giving a new opportunity to phages.

1.3.2 Characterization and classification of phages

More than 5500 different phages have been identified under electronic microscopy which allowed a deeper knowledge of phage morphology and nature of nucleic acid and, ultimately, the possibility of a complete taxonomic classification[82].

Phage is an entity that consists of a nucleic acid (genetic material) and proteins responsible for its structure and enzymatic activity. Genetic material may be DNA or RNA, and the molecule can be single or double stranded. Majority of phages genetic material is double stranded DNA - dsDNA. Genome sizes are highly variable; they can range from 3.5 kb single stranded RNA (ssRNA) in phage MS2[83] (*Leviviridae* family) to 497 kb dsDNA genome in *Bacillus magaterium* phage G[84] (*Myoviridae* family). Phage enormous diversity is also seen in morphologic characteristics. They can be tailed, polyhedral, filamentous or pleomorphic. Some have a lipid-containing envelope or contain lipids as part of their particle wall. Phages belonging to the *Caudovirales* order account for approximately 96% of all reported phages[85]. They are divided in 3 families: *Myoviridae*, *Siphoviridae* and *Podoviridae*. All of them contain dsDNA in a linear segment, and are morphologically formed by an icosahedral head with tail.

The International Committee on Taxonomy of Viruses is the international organ responsible for the development of a universal taxonomic scheme for all viruses. Phage classification is mainly based on the nature of nucleic acid and morphology. Other properties such as content in lipids, proteins and carbohydrates, physicochemical and physical, and biological properties are considered as well. According to last report, published in August of 2016[86], phages are currently divided in ten families: three from *Caudovirales* order (*Myoviridae*, *Siphoviridae* and *Podoviridae) and* seven without assigned order (*Corticoviridae*, *Plasmaviridae*, *Tectiviridae*, *Inoviridae*, *Microviridae*, *Cystoviridae* and *Leviviridae*).

1.3.3 Life cycles

Phages are viruses. As so, they do not possess a metabolism factory and are considered obligate parasites of a bacterial cell. Phages are capable of thriving in several life cycles: lytic, lysogenic, pseudolysogenic and chronic infections[87]. As the main life cycles observed in environments, the first two will be characterized next. A scheme of these cycles is presented in Figure 3.

The lytic life cycle of a prokaryotic virus can be distinguished in several characteristic steps: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release and transmission[88]. The first step, **adsorption**, is highly specific. Phage attachment to a host cell is facilitated by viral tail filaments to specific complementary receptors on the surface of a susceptible bacterial cell. The specificity of the receptors determines the range of phage host organisms[88]. The receptor sites are surface components of a bacterial cell, including lipopolysaccharide (LPS), peptidoglycan, teichoic acids, outer membrane proteins, oligosaccharides[89]. In some cases the attachment sites might be present on the cell capsule, flagella or conjugative pili (male-specific phages)[90].

A review on the specific host cell structures within each group of recognition sites and the mechanism of phage adsorption and penetration into microbial cell was published by Rakhuba and colleagues in 2010[91].

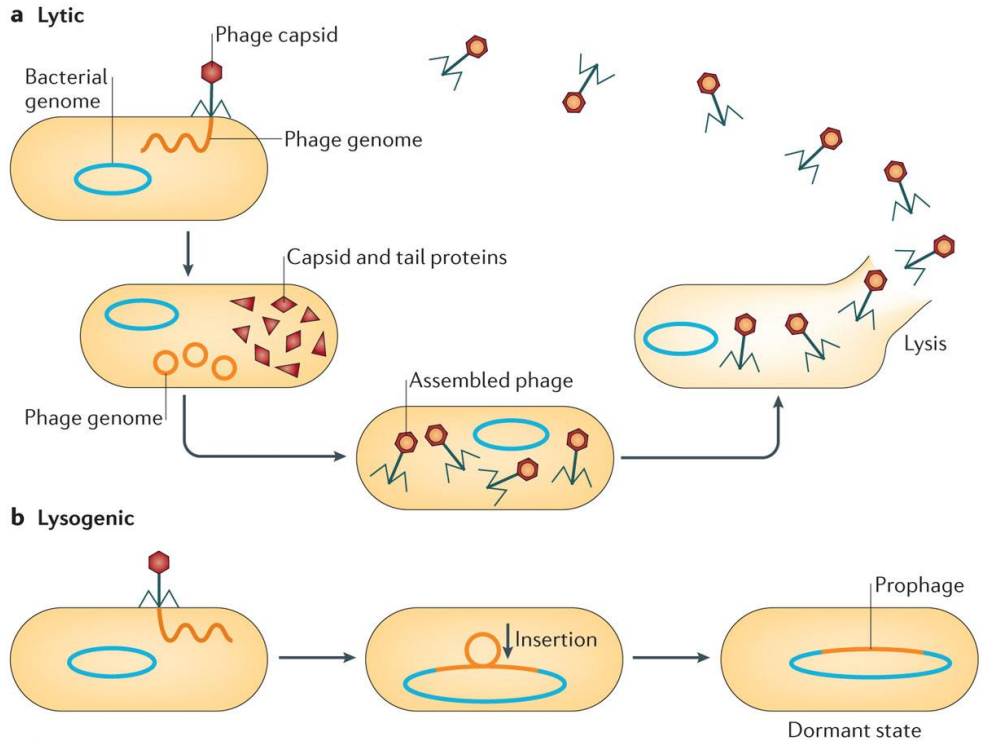


Figure 3. Schematic of lytic and lysogenic phage replication cycles. **a)** Lytic cycle; **b)** Lysogenic cycle. Adapted and modified from Feiner *et al.*[73].

This adsorption is initially reversible[92] by diluting the reaction mixture or killing the bacteria and the phage may be dissociated retaining their infectious capacity. After the initial period, the association becomes irreversible, leading to the penetration of the viral genetic material into the bacteria cell. In the second step, **separation of nucleic acids from protein coat**, peptidoglycan is degraded and pores are formed in the bacterial cell wall so the genetic material can be transferred into the cell. Third step, **expression and replication of the nucleic acids**, is involved in directing the host metabolism to viral nucleic acid replication and phage protein synthesis. When the constituents of phages are formed, **virion assembly** and packing of phage particles can be done. The final step is characterized by bacterial cell lysis allowing **phage progeny release and transmission** to other bacteria. Phages express enzymes, generically termed muralytic enzymes or endolysins, that attack the bacterial peptidoglycan, which can be lysozymes that target sugar bonds, endopeptidases that target peptide linkages or amidases that act on amide bonds[93]. Another enzyme, referred as holin, is necessary to create a lesion in the cytoplasmic membrane through which endolysins access the peptidoglycan[94]. Together, they are responsible for host cell lysis and virion burst to the environment.

In lysogenic cycle, after penetration, phage genetic material is integrated into the host cell genome and become a prophage[88]. It does not promote cell death or the production of phage particles and it can persist in a latent state for thousands of bacteria generations. Adverse environmental conditions for the host bacterium may activate the prophage, so the lytic cycle can proceed[73]. At the end, the newly formed phage particles are ready to lyse the host cell. Worthy noticing, the presence of a prophage confers some kind of immunity to infection by other phages due to the synthesis of a repressor protein that blocks the transcription of phage own genes and also those of closely related phages[90].

1.3.4 Advantages and concerns of phage therapy

Bacterial infections are a major cause of concern worldwide today, as resistance to conventional antibiotics is increasing in an alarming rhythm after years of abusive use. Due to this current problem, the use of phages as an antimicrobial therapy is a promising alternative to conventional antibiotics, bringing several advantages over them.

Phages can only target bacteria through attachment to host cell surface receptors. Moreover, each phage will only attack one species or in some cases a single strain of bacterium[90]. This can be seen as a dual effect. On one hand, use of phage therapy has to be carefully studied; it is essential to know the etiological factor causing the infection, as the phage-bacteria interaction is specific and therefore limited. On the other hand, it warrants no influence on normal flora because phages only eradicate the targeted strain. Phages replicate only at the site of infection, lowering the probability of allergies and secondary infections, as observed after antibiotic treatment. In fact, no significant adverse reactions have been observed in several experiments[95–97]. Their lytic cycle characteristics allow an exponential growth as the virus multiplies within the susceptible bacterial host and is subsequently released, leading to a lower administration when compared to antibiotics dosage[98]. Phage production costs are low, when compared to the process of development of a new antibiotics. Bacterial resistance to phages, although observed[99], is considerably lower than that to antibiotics. Also, some strategies to deal with this problem have been studied and include the use of a different phage, especially ones with fast adsorption rate and large burst size so the bacterial population is rapidly lowered and resistance becomes unlikely[100]. Release of endo- and exo-toxins after bacterial lysis by lytic phages is a concern; however bactericidal antibiotics can produce the same effect[101]. Phages may be recognized by the human immune system as foreign bodies and therefore neutralized[102]. However, this effect was only reported when administration was intravenous; not associated with oral and local administration[103].

1.3.5 Phage therapy in chronic wounds

Phage therapy has been evaluated almost since their discovery. It has been studied and reported, mainly in Poland and former Soviet Union, as a potential solution for diverse human infections[103]. Regarding infected wounds, phage therapy was successfully used during World War II by the Russian army[103]. Its effectiveness (orally and locally administered) in chronic suppurative infections of the skin caused by *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Proteus* and *Escherichia* was already confirmed 30 years ago with approximately 50% of the studied cases resulting in an "outstanding" improvement[104]. In 2002, a phage impregnated polymer – PhagoBioDerm – used to treat infected venous stasis skin ulcers achieved complete healing in 70% of the patients[105].

Research using animal models has also given strong evidence, both in safety and efficacy, that phage therapy can be used in the treatment of chronic wounds. Mendes *et al.*[106], in 2013, used rat and pig models with diabetic cutaneous wounds to evaluate the effects of topical phage cocktails on bacterial counts and wound healing. Results revealed that phage treatment effectively decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps, in *S. aureus* and *P. aeruginosa* infections, although it was not effective against *Acinetobacter baumannii*. The potential of bacteriophage AB 38 specific for *A. baumannii* wound infection in uncontrolled diabetic rats was investigated in an *in vivo* assay that resulted in a significant reduction in infection, period of epithelization, and wound contraction in phage-challenged group when compared to the other conditions in study[107].

Phage therapy for use in other skin conditions such as burns or other chronic infections is showing encouraging therapeutic results[108,109]. In 2009, the first controlled randomized, double-blind, placebo-controlled Phase I/II clinical trial was conducted to access the efficacy and safety of a therapeutic phage cocktail preparation (Biophage-PA) targeting antibiotic-resistant *P. aeruginosa* in chronic otitis of 24 patients. Results revealed a significant reduction both in bacterial presence, as well as a decrease of disease symptoms and increase in phage numbers *in situ*[95]. PhagoBurn clinical trial is a Phase I/II ongoing project involving three European countries with the aim of evaluating the safety and efficacy of phages for the treatment of burn wounds infected with *E. coli* and *P. aeruginosa* (www.phagoburn.eu). Regarding chronic wounds, in 2008, a phage phase I safety trial approved by Food and Drug Administration (FDA) against venous leg skin ulcerations and other wounds was completed in 42 patients. A cocktail of two phages active against *S. aureus*, five against *P. aeruginosa* and one against *E. coli*, was added during 12 weeks to chronic infections without observation of significant side effects[97]. Furthermore, FDA approved a phase I trial of a phage cocktail for the treatment of infected chronic ulcers occurring in diabetic foot by the company Technophage, in Portugal (www.technophage.pt/)[110].

1.3.6 Synergistic effects of combinatory therapy

The traditional phage therapy methodology for biofilm control can be improved by combining one or more lytic phages with other antimicrobials agents. Several reports reveal an additive or synergistic effect on biofilm reduction resulting from a combinatory strategy, mostly with antibiotics[111–113].

In one of the first reports, Hagens *et al.* noted an increased sensitivity of two *P. aeruginosa* strains to several antibiotics in the presence of filamentous phages. A reduction of more than 99.9% in viable bacteria was observed with combinatory treatment with gentamicin in one of the strains, while both treatments alone had no reduction, revealing a high synergistic effect[111]. This effect was also obtained with ceftriaxone in subinhibitory concentrations and bacteriophage σ-1 against *P. aeruginosa* planktonic cells. Additionally, visualization of cell morphology confirmed significant elongation of *P. aeruginosa* cells after the administration of subinhibitory concentrations of ceftriaxone and ciprofloxacin[112]. Another study demonstrated a synergistic effect combining phages and antibiotics against *P. aeruginosa* cells; carbenicillin had a particular strong effect, with 3 times higher reduction of bacterial density than the sum of the two individual treatments[113].

Synergic efficacy maybe influenced by the order of addition; in fact, combined treatment of phage and streptomycin against *P. aeruginosa* PAO1 was particularly marked when antibiotic was added 12 h after the addition of phage[114]. More recently, a study assessed the effect of co-therapy of two isolated lytic phages and several antibiotics of different classes in *P. aeruginosa* mature biofilms. Addition of gentamycin and tobramycin 24 h after phage significantly reduced cell density compared to treatments administered simultaneously. Furthermore, simultaneous combined treatment of biofilms revealed a synergistic effect for ceftazidime and a facilitated action for ciprofloxacin and tobramycin[115].

Phage-cefotaxime combination against *E. coli* biofilms was evaluated, revealing an increase of plaque along with increase of concentration of cefotaxime, as well as burst size and phage concentration compared to no addition of antibiotic. Reduction in cefotaxime Minimum Biofilm Eradication Concentration (MBEC) with the addition of phage titers, that alone produced no significant effect in in biofilm reduction, was observed[116]. *E. coli* biofilms were also reduced synergistically by the combined treatment of phage and tobramycin[117]. Although not synergic, a greater destruction of biofilms of *Klebsiella pneumoniae* was observed when phages and amoxicillin[118] and ciprofloxacin[119] were used together. *Burkholderia cepacia complex* cells were also the target of a study that revealed synergism of both agents for six antibiotics through assessment of phage plaque diameters. Meropenem promoted the greatest increase in plaque size and bacterial load was considerably reduced with the combinatory addition of

phage and either ciprofloxacin, meropenem, or tetracycline, tendency also observed with phage alone, even though less expressive[120].

S. aureus is one of the most studied bacteria due to its impact in several infectious conditions. Combination of phage with rifampicin had a biofilm removal activity considered synergic, with approximately 65% of the biofilm cells eliminated[121]. The development of an experimental system to evaluate the pharmacodynamics of a dual therapy of phage and gentamicin - in a continuous culture system of *S. aureus* revealed, after 72 h, that viable cell density was lower than the drug therapy alone and phage density revealed an increase 24 h after inoculation[122]. A synergic effect for the combined action of phage and cefoxitin, ciprofloxacin and polymyxin B was also achieved against an antibiotic resistant *S. aureus* strain[123]. Chhibber and colleagues reported that the bacteriophage MR-10 when combined to the antibiotic linezolid was found to be more effective in controlling the entire process of hindpaw infection by Methicillin-resistant *S. aureus* (MRSA) and Methicillin-susceptible *S. aureus* (MSSA) strains in diabetic mice as compared to antibiotic or phage given alone. Other criteria, such as edema and lesion score were also slightly smaller than other treatments and significantly reduced compared to the control[124].

Novel antimicrobials are also been tested. The effect of limiting iron availability in the formation of *K. pneumoniae* biofilms was accessed by the combination of an iron antagonizing molecule – Co [II] – and a phage. Combinatory treatment completely reduced young biofilms, which was significantly higher that each antimicrobial alone. Authors suggest that degradation of exopolysaccharide matrix of biofilm by depolymerase enzyme in the phage enabled the diffusion of cobalt ions and consequent action of both agents. Nevertheless, no substantial inhibitory effects were seen on the older biofilms regarding individual as well as combinatory treatment[125]. The elimination of a mixed species biofilm of *K. pneumoniae* and *P. aeruginosa* by the individual treatment with specific phages or their combination with the sugar alcohol xylitol resulted in a synergic effect, as there was complete eradication of *Klebsiella* and a superior significant reduction in *Pseudomonas* bacterial count. These results suggest that the use of two phages allowed the *P. aeruginosa* phage to reach its host through the disruption of matrix caused by the *K. pneumoniae* phage, and the addition of xylitol caused a synergic effect[126]. The efficacy of phages against two types of biofilm extracellular matrices, namely polysaccharide intercellular adhesion (PIA) and proteinaceous fibronectin-binding protein A (FnBPA), in MSSA and MRSA strains, when combined with PIA-disturbing or protein denaturing chemical adjuvants in order to increase the biofilm matrix permeability and the exposure of phage binding receptors on MSSA and MRSA cell walls was evaluated. Benzethonium chloride and ethanol, both in a sublethal dose, were considered as the best disintegrators

of PIA type biofilms and the best protein denaturing chemical for FnBPA type biofilms, respectively, both when combined with phages[127].

Some conclusions taken from several studies associate biofilm matrix disintegration by phage-associated depolymerases to consequent exposure of the deep bacterial cells to both lytic phages and antibiotics, increasing the antimicrobial therapy[119,126]. However, success of combinatory treatment was shown to be dose dependent[111,113] and influenced by the order of treatment application[114,115]. Pharmacological characteristics, namely the mechanism of action, might also have an impact on phage-antibiotic synergy[112,113,128]. Cells injured or with irregular morphology[121,123] and cell elongation/filamentation[111,112] are phenotypes characteristic of synergic combination. Several reports also emphasize the fact that emergence of resistant variants is reduced in combinatory treatment, when compared to the single antibiotic treatment[113,114,117,123,129,130]. For example, resistance assays revealed a >99.99% decrease in tobramycin resistant cells and a 39% decrease in T4 phage resistant cells when combinatory treatment of these agents was administered. In the same way, tobramycin resistant cells and bacteriophage PB-1 resistant cells had a decrease of 60% and 99%, respectively, for the combined treatment, which are important results to validate the use of combinatory therapy for the decrease of emergent resistant cells[117].

1.4 Honey

1.4.1 Characterization and properties of the honey

Honey is a viscous solution derived from nectar gathered and modified by the species *Apis mellifera*, the traditional honeybee. It is composed of approximately 31.3% glucose, 38.2% fructose, 1% sucrose and 17% water, as well as other minor components highly variable, depending on source and geographical location, such as acids, proteins, amino acids, vitamins, minerals and enzymes[131]. The use of honey in the management of wounds was first documented by the ancient Egyptians 4000 years ago and it has been used since ancient times by Romans, Greeks and Chinese in the treatment of wounds and gastrointestinal diseases[132]. With antibiotics discovery, honey research and use as antimicrobial was abandoned or left as a last resource. However, with the rise of antibiotic resistant bacteria, this treatment was considered again and its healing properties object of study. In 1999, the first commercial available and sterile topical medical preparation based on honey was licensed in Australia: Manuka honey, which is native to New Zealand and parts of Australia and derives from *Leptospermum scoparium*[133].

The difference in antimicrobial capacity among the different honeys can be more than 100-fold, depending on their geographical, seasonal and botanical source as well as harvesting, processing and storage conditions[134]. **Antimicrobial properties** of honey are associated to (i) the high osmolarity, namely, the sugar concentration that allows osmosis to occur. Bacterial reproduction is impaired due to the low availability of water[134]; (ii) the production of hydrogen peroxide due to the combination of the glucose oxidase in honey with glucose and water helps to suppress antimicrobial growth[135,136]. The release of hydrogen peroxide is slow and concentrations accumulated are reported to be approximately 1000 times lower than that associated with the 3% hydrogen peroxide solutions, considered potential harmful, being non-toxic and non-damaging to the surrounding tissue[137,138]; (iii) honey has normally acidic pH levels which inhibits the growth of most microorganisms[139]; (iv) the direct action of antimicrobial chemicals present within it, namely methylglyoxal (MGO) – discovered in 2008 as the dominant antimicrobial agent of Manuka honey[140]. MGO can react non-specifically with macromolecules such as DNA, RNA and proteins[140,141] and is found in Manuka honey at concentrations of 828 mg/kg compared with 24 mg/kg in non-Manuka honeys. Bee defensin-1, an antimicrobial bee-derived peptide is responsible for activity in Revamil honey, an active honey produced from an undisclosed source[142].

Honey presents a **wound debridement action** due to the high osmolarity that allows the drawing of exudate and lymph from the deeper wound bed and the tissues. This maintains a constant supply of proteinases with autolytic action at the interface of the wound bed and the overlying sloughy and necrotic tissue[143], also favoring a moist wound environment. Hydrogen peroxide activates the neutrophils leading to the production of cytokines which strengthen the inflammatory response by recruiting and activating leucocytes[144]. An **anti-inflammatory effect** of honey has been supported histologically by biopsies taken from superficial burn wounds[145], observed clinically as a reduction in edema and pain. The dominant protein in honey, 55 kDa glycoprotein major royal jelly protein 1 (MRJP1)[146], may also contribute to this effect, as well as natural endotoxins that are present in honeys[147]. **Antioxidant properties** have been attributed to some of the constituents present in honey, such as phenolic acids and flavonoids[148,149], which scavenge the free radicals present in the wound. Honey presents **Deodorizing properties** due to the metabolism of honey's glucose by anaerobic bacterial species to non-malodorous compounds, over to the amino acids in the decomposed serum and tissue proteins that they would normally utilize[150,151]. Finally, honey stimulates **tissue growth** through the synthesis of collagen and development of angiogenesis in the bed of wounds[145,152] and fibroblasts and epithelial cells growth[153]. Acidic pH can indirectly promote granulation and tissue growth[137].

1.4.2 Evidence of honey antibacterial action

Manuka honey has been considered one of the most effective honeys due to its large spectrum of antimicrobial activity. However, other honeys have also revealed good antibacterial properties. For example, 58 strains of *S. aureus* isolated from swabs of infected wounds were all sensitive to both Manuka and pasture polyfloral honey in low concentrations. Pasture polyfloral honey revealed no antibacterial activity when tested in the presence of catalase, rising again in absence of the former, thus bacterial inhibition was mainly due to hydrogen peroxide generation. On the other hand, catalase revealed no influence in antibacterial activity of Manuka honey so its bacterial inhibition was attributable primarily to nonperoxide components[154]. In another study with MRSA and vancomycin-sensitive enterococci isolated from infected wounds and vancomycin-resistant enterococci isolated from hospital environmental surfaces strains, *in vitro* inhibition was accomplished for different honeys, raw honey and artificial honey (honey prepared with similar proportions of the four predominant sugars in natural honey samples) but with concentrations of artificial honey at least three times higher, which confirms antibacterial activity not exclusively due to osmolarity[155]. Similar results were accomplished with the comparison of natural and artificial honey in *Streptococcus mutans* growth and biofilm formation[156].

Polyethylene membranes composed of Manuka honey and pectin (1:1 v/v) were effective in inhibiting the growth of gram-negative bacteria within 3 h, whereas those composed of Honeydew honey needed 24 h to neutralize bacterial growth[157]. These honeys were also effective in decreasing cell viability of wound pathogens (*S. aureus*, *Streptococcus agalactiae* and *P. aeruginosa*) in a mature polymicrobial biofilm[158]. Manuka honey has also shown to impair *in vitro* biofilm formation by *Clostridium difficile*[159] and inhibition of bacterial attachment, as well as reduction of biofilm development of *E. coli* and *Proteus mirabilis* to vinyl substrates[160]. In other study, Manuka honey effectively disrupted and caused extensive cell death in biofilms of *S. aureus*, *P. aeruginosa* and *S. pyogenes*, commonly wound bacteria by the prevention of their adhesion to the fibronectin, fibrinogen and collagen in human keratinocytes[161]. Testing of Sidr and Manuka honeys against MSSA, MRSA and *P. aeruginosa* planktonic cultures and biofilms revealed a 100% killing efficacy of the isolates in the planktonic form. The bactericidal rates for biofilms were 63, 73 and 91% for Sidr honey and 82, 63 and 91 for Manuka honey, respectively, being higher than those observed with single antibiotics commonly used against *S. aureus*[162]. Indigenous black seed honey (*Nigella sativa*)[163], Norwegian forest Honey[164] and Chestnut honey[165] also show effect on impaired biofilm formation, revealing their potential against wound bacteria.

1.4.3 Insights on antibacterial mechanisms of honey

Cellular mechanisms of honeys are not completely known and it is suggested that they may differ accordingly to the organism in study. In fact, three honeys (Manuka, Kanuka and clover honey) showed different effects in growth dynamics and cellular morphology for *Bacillus subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*[166]. Sublethal doses of honey lead to extended lag phases and bacterial cells were also significantly shorter in length than usual for *B. subtilis, S. aureus* and *E. coli*. Concentrations exceeding 8–16% inhibited their growth. For both *B. subtilis* and *S. aureus*, the chromosome looped conformation seen during normal chromosome replication was not observable. Oppositely, there was no extended lag phase and much higher concentrations of honey were required to completely inhibit growth of *P. aeruginosa*, whose cell size appeared longer than usual.

Studies with Manuka honey may give a useful insight of mechanisms behind antibacterial action of different honeys. For MSSA and MRSA, its action is associated with interruption of the cell cycle. Loss of autolysin activity, responsible for digestion of peptidoglycan[167], accumulation of cells with formed septum[168] and downregulation of the universal stress protein, in MRSA, reducing the ability of bacteria to survive cellular and metabolic stress, are reported[167]. Manuka honey was reported to inhibit the development of *S. pyogenes* biofilms, likely due to the differential expression of two major surface adhesins that are known to have a role in biofilm development as they facilitate streptococcal binding to fibronectin. A reduced expression would impede bacterial binding to host proteins in the wound bed, thus preventing initial colonization[169]. Regarding *E. coli* O157:H7, low concentrations of honey were able to reduce biofilm formation, quorum sensing, and virulence[170].

The mode of action of Manuka honey against *P. aeruginosa* is quite different, as explained in Figure 4. It causes structural damage leading to cell lysis and death, being particularly notable the damage to the cell envelope. Genomic analysis support the findings by revealing a reduction in the expression of outer membrane porin F (OprF), an integral membrane protein required for the structural stability of the cell envelope in gram-negative microorganisms[171]. Other factors such as reduced production of virulence factors[172,173] and inhibition of regulatory cascade of flagellum production[174] have been linked to honey effect on *P. aeruginosa* cells.

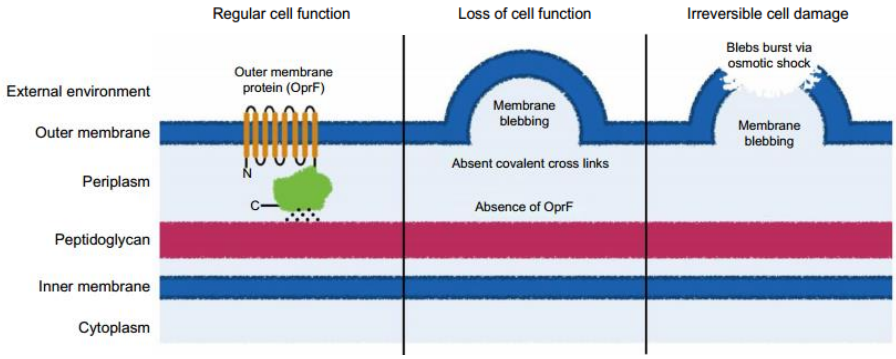


Figure 4. Mechanism of cell envelope disruption by Manuka honey to *P. aeruginosa*, causing membrane blebbing and ultimately cell lysis. Adapted from Jenkins *et al.*[175].

To the date only one study reported an increased resistance of *P. aeruginosa* isolates recovered from biofilms treated with Manuka honey when cultured as planktonic and as a biofilms. These isolates also exhibited increased resistance to antibiotic treatment and biofilm forming capacity[176]. The different antibacterial honey components possibly act together, targeting several cellular processes, which affects the capacity of bacteria to resist and adapt to the new environment[177].

1.4.4 Honey therapy in chronic wounds

A number of recent clinical reports evaluated honey, mainly in topic dressings, for the treatment of chronic wounds to assess its efficacy in comparison with other common antimicrobials. However, some variance in the design of the experiment hampers their outcomes and consequent comparative analysis of results. No doubt it offers a good alternative in certain cases and the results obtained are of considerable value and respected by scientific and medical community, as several dressings using honey are found in the market[137].

A Medihoney dressing used in non-healing venous leg ulcers during 12 weeks revealed a decrease in ulcer pain, size and odor, resulting in a high acceptance by the patients to this alternative treatment[178]. Bacteriological changes in sloughy venous leg ulcers treated with Manuka honey or hydrogel were evaluated in 108 patients[179]. After four weeks, Manuka honey was able to eradicate MRSA in 70% of treated wounds, while only 16% of the hydrogel treated wounds had eradicated MRSA. *P. aeruginosa* was reported in 14% of all wounds at baseline; and here hydrogel produced a better outcome, with 50% eliminated. Another study evaluated the safety and effectiveness of Manuka honey as a dressing for 368 venous ulcers compared to usual care but no significant differences between the groups were observed[180]. In 2009, Medihoney was compared with standard therapy in wound care and the results, although not statistically significant, suggest that healing times after treatment with honey were reduced compared

with conventional treatment, being of clinical significance (median of 100 compared with 140 days, respectively)[181].

Two trials recruited people with diabetes and foot ulcers of Wagner grade (standard classification system of diabetic foot ulcers and lesions) I or II and compared the effects of honey with either saline soaks[182] or povidone-iodine gauze[183]. In both studies participants also received initial debridement and antibiotics as necessary. There was no difference in frequency of healing between honey and standard treatments, however, a significant reduction in the time of healing and rapid disinfection of ulcers was achieved with honey, as well as a more effective decreasing in wound edema and odor. Another clinical trial studied the effect of Beri-honey-impregnated dressings on diabetic foot ulcers and compared it with normal saline dressings, revealing a significant superior healing rate and a lesser healing time for patients treated with honey[184].

Comparison of a honey versus an ethoxy-diaminoacridine plus nitrofurazone dressing in patients with pressure ulcers showed that healing among subjects using a honey dressing was approximately 4 times the rate of healing in the comparison group, accordingly to PUSH (Pressure Ulcer Scale for Healing) scores[185]. Bee honey dressing was evaluated for the treatment of diabetic foot ulcers, revealing clover honey as a clinical and cost-effective dressing for diabetic wound, once complete healing was significantly achieved in 43.3% of ulcers and another 43.3% reported decrease in size and healthy granulation. Failure of treatment was only observed in 6.7% of ulcers[186]. In the same way, Medihoney also proved to eliminate bacterial growth and 90% of patients showed complete wound healing after a period of 4 weeks in chronic pressure ulcers in patients with spinal cord injury[187].

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Two strains of *E. coli* were used in this study: the clinical isolate EC3a that was kindly provided by the Hospital Escala Braga in Portugal and used as phage propagation strain, and the *E. coli* reference strain CECT 434 that was purchased from the Spanish Type Culture Collection and used in all biofilm experiments. *P. aeruginosa* reference strain PAO1 (DSM22644) was purchased from the German Collection of Microorganisms and Cell Cultures. This strain was used for isolation and propagation of phages as well as in all biofilm experiments. In order to evaluate the lytic spectra of the isolated *P. aeruginosa* phages, 36 strains of *P. aeruginosa* were used in this work: 3 reference strains (ATCC 10145, CECT 111, PAO1) and 33 clinical isolates provided by the Hospital de Braga (Braga, Portugal)[188,189].

All strains were grown at 37 °C in Tryptic Soy Broth (TSB, VWR) or in solid Tryptic Soy Agar medium (TSA; TSB containing 1.2% (w/v) of NZYTech agar). Bacterial lawns were done in Tryptic Soy Agar medium (TSA; TSB containing 0.6% (w/v) of agar). For antibiofilm assays, *E. coli* counts were plated in MacConckey Agar (Merck®) and *P. aeruginosa* counts were plated in Pseudomonas agar base (VWR) supplemented with glycerol 5% (w/v) (Fisher BioReagents™), being both incubated at 37 °C for approximately 18 h. All media and reagents were prepared accordingly to manufacturer's instructions and autoclaved at 121 °C for 15 minutes.

2.2 Honey samples

This study involved the analysis of three Portuguese honeys: Urze 3 (U3), Chestnut 1 (C1) and a polyfloral honey (PF2). Honeys U3 and C1 were evaluated regarding antibiofilm activity against *P. aeruginosa* biofilms at 3 different concentrations, 25% (w/v), 50% (w/v) and 75% (w/v), hereafter $U3_{25\%}$, $U3_{50\%}$, $U3_{75\%}$ and $C1_{25\%}$, $C1_{50\%}$, $C1_{75\%}$, respectively. Honey U3 was selected to continue the experiments in *P. aeruginosa*, in *E. coli* biofilms and for flow cytometry analysis of both species. Portuguese honey PF2 25% (w/v), hereafter $PF2_{25\%}$, was evaluated against *E. coli* biofilms through flow cytometry based on previous results[190].

Samples were collected raw and unprocessed from regional beekeepers in the harvesting period of 2015-2016 and maintained at room temperature in the dark until analysis and used in antimicrobial experiments. Commercial 100% medical Manuka honey (Medihoney®, Derma Sciences) was also acquired and analyzed in this study.

2.3 Phage samples

The phage used for infection of the *E. coli*, referenced vB_EcoS_CEB_EC3a and mentioned bellow as EC3a, was previously isolated from raw sewage using the clinical isolate EC3a as host[189]. Phages for infection of *P. aeruginosa* were isolated from two commercial cocktails acquired in Russia – Sextaphage and Intestiphage (Microgen, ImBio Nizhny Novgorod, Russia) within the tasks of this work.

2.3.1 Phage Isolation

The isolation of phages from two commercial cocktails was done as described by Azeredo *et al.*[191]. In order to distinguish phage plaques, 10 µL of each cocktail were placed over an agar plate with a PAO1 bacterial lawn. Sterile paper strips were used to streak the phages across 3 different petri dishes so individual plaques could be identified and plates were incubated overnight at 37 °C. Plaque morphologies were checked for differences in size, presence of halo, and turbidity. Each different isolated phage plaque was picked with a toothpick that was streaked several times (in a line) in an agar plate with a bacterial lawn. Sterile paper strips were used to streak the phages as explained before and plates were incubated at 37 °C overnight. This procedure was repeated 3 consecutive times for each phage in order to obtain uniform phage plaques.

For plaque diameter analysis, two sets of plates were incubated at 37 °C overnight: one plate was afterwards left at room temperature (22 °C) for 48 h; and the other put at 4 °C. The diameter of several individual phage plaques and of their surrounding halos were registered at these two different temperatures.

2.3.2 Determination of phage lytic spectra

One of the criteria to select the best *P. aeruginosa* phage was based on the results of the lytic spectra, performed by the spot test[191]. All isolated phages (PAO1-A, PAO1-C and PAO1-E from Instestiphage, and PAO1-B, vB_PaeP_PAO1-D (abbrev. PAO1-D), PAO1-F, PAO1-G from Sextaphage) were tested against the 36 strains of *P. aeruginosa*. One drop (10 µL) of each phage suspension, with titers of 10^9 PFU/mL, was placed on the different bacterial lawns and incubated overnight at 37 °C. In the following day, the susceptibility of each host to the different phages was evaluated.

2.4 Production of phages

Propagation of phages used in the treatment of *P. aeruginosa* and *E. coli* biofilms, PAO1-D and EC3a, respectively, was done using the plate lysis and elution method[192]. Briefly, 5 µL of phage suspension were spread evenly on host bacterial lawns of several Petri dishes using a paper strip and incubated overnight at 37 °C. Afterwards, approximately 3 mL of SM Buffer (5.8 g/L NaCl, PanReac AppliChem; 2 g/L $MgSO_4 \cdot 7H_2O$, PanReac AppliChem; 50 mL/L 1M Tris-HCl pH 7.5, VWR) were added to each plate and incubated overnight at 4 °C with gentle stirring (50 rpm on a PSU-10i Orbital Shaker (BIOSAN)) to recover the amplified phages. Subsequently, the liquid was collected, centrifuged (10 minutes, 9 000 ×g, 4 °C) and the supernatant collected and filtered (PES, GE Healthcare, 0.22 µm). The concentration of phage suspension was performed according to Adams *et al.* (1959)[193] by PEG 8000/1M NaCl. Briefly, 58.4 g/L of NaCl was added to the phage lysate and the suspension was incubated 1 h at 4 °C under slow agitation (50 rpm). Subsequently, the suspension was centrifuged (10 minutes, 9 000 ×g, 4 °C) and the supernatant collected. Then, 100 g/L of PEG 8000 (ThermoFisher Scientific) were added and the resultant was incubated overnight at 4 °C with agitation (70 rpm). The suspension was then centrifuged as before, the supernatant discarded and the pellet resuspended in SM Buffer. Purification was performed with chloroform, which was added in 1:4 (v/v) proportion, vortexing for 30 seconds. After centrifugation (5 minutes, 3 500 ×g, 4 °C), aqueous phase was filtered-sterilized (0.22 µm) and stored at 4 °C for further use.

2.5 Titration of phages

Phage titer was performed by the double agar technique, as described by Adams[193]. Briefly, successive 10-fold dilutions of the collected samples were done in SM buffer. Then, 100 µL of diluted solution, 100 µL of host bacteria culture, and 3 mL of TSA top agar 0.6% (w/v) were mixed and spread onto a Petri plate containing a thin layer of TSA. After overnight incubation at 37 °C, the plaque forming units (PFU) number were determined as follows:

$$Phage\ concentration\ (\frac{PFU}{mL}) = \frac{\text{Number of plaques } \times \text{Dilution } factor}{Volume\ of\ plaqued\ phage}$$

2.6 Determination of honey minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the honey was determined using the broth microdilution method described in the guidelines of the Clinical and Laboratory Standards Institute [CLSI, Wayne, NJ, USA) (M27-A2)][194]. Briefly, fresh bacterial colonies were selected from a TSA plate, transferred to 10 mL of TSB and incubated at 37 °C, 120 rpm for 16 h. The turbidity of the bacterial culture at 620 nm was adjusted to 0.13 (approximately 3 $\times 10^8$ CFU/mL, Synergy HT – BioTek) and diluted 30-fold in TSB. MICs were determined in a 96-well plate flat bottom plates (Orange Scientific) using a final volume of 100 µL using a honey concentration range from 50% (w/v) to 0% (w/v). Plates were incubated for 20 h at 37 °C and after growth inhibition was confirmed visually and by turbidimetry at 620 nm (Synergy HT – BioTek). Three independent experiments were performed in triplicate.

2.7 Assessment of phage viability in honey

The viability of phages PAO1-D and EC3a in 25% (w/v) and 50% (w/v) U3 and C1 honeys was tested. Briefly, 2×10^9 PFU/mL was tested in 50% (w/v) honey, where honey was directly diluted in the phage suspension, and in 25% (w/v), where honey was diluted to 50% (w/v) in sterile water and the phage suspension in SM buffer was added in a proportion of 1:1 (v/v). Controls were performed in sterile deionized water instead of honey. The solutions were incubated at 37 °C and samples were taken after 1 h, 2 h, 6 h and 12 h. Phages titration was performed according to the double agar overlay technique. Two independent experiments were performed in duplicate.

2.8 Phage growth characterization

The growth characterization of phage EC3a and phage PAO1-D was determined according to the method of described by Pajunen *et al.*[195] with some modifications. Briefly, 10 mL of the host culture was grown until reaching mid-exponential phase (OD_{600}=0.500), harvested by centrifugation (7000 ×g, 5 minutes, 4°C) and resuspended in 5 mL fresh TSB medium in order to obtain an OD_{600}=1. Then, 5 mL of phage with a MOI of 0.001 were added to this suspension and allowed to adsorb for 5 minutes at 37 °C under agitation. The mixture was then centrifuged as before and pellet containing the infected cells was resuspended in 10 mL of TSB followed by incubation at 37 °C under agitation. Samples were taken periodically each 5 minutes during the first 30 minutes and each 10 minutes until 60 minutes of assay, being immediately diluted and titrated by the double-layer technique. Two independent experiments were performed in duplicate.

2.9 Transmission electron microscopy analysis

Analysis of virion particles dimensions and morphology was performed through transmission electron microscopy (TEM). Phage EC3a and Phage PAO1-D particles were sedimented by centrifugation (25 000 ×g, 60 minutes, 4 °C) and washed twice in tap water by repeating the centrifugation step. Subsequently, the suspension was deposited on copper grids with carbon-coated Formvar films, stained with 2% (w/v) uranyl acetate (pH 4.0) (Agar Scientific), and examined using a Jeol JEM 1400 (Tokyo, Japan) transmission electron microscope. Images were digitally recorded using a CCD digital camera Orious 1100W, Tokyo, Japan.

2.10 Biofilm formation

For biofilm formation assays, the turbidimetry of a 16 h *E. coli* or *P. aeruginosa* inoculum grown in TSB was adjusted to 0.13 (~3×10^8 CFU/mL in McFarland scale), diluted 10-fold in TSB, and 200 µL were added to the wells of a 96-well plate. For the formation of dual species biofilms, after adjustment to 0.13, each inoculum was diluted 5-fold in TSB and then mixed together, achieving a final dilution of 10-fold, similarly to the monospecies biofilm condition. Plates were incubated for 24 h or 48 h, at 37 °C and 120 rpm (orbital shaker ES-20/60 (BIOSAN)). Medium replacement in 48 h old biofilms was done after 24 h.

2.11 Biofilm treatment

All the conditions performed in the analysis of *E. coli* and *P. aeruginosa* biofilms are described in the table below (Table 2). Three different biofilm treatments were evaluated: phage, honey and the combination of both agents. Phage treatments were performed with an established concentration of 1×10^9 PFU/mL (~MOI 10) due to the clinical context of the work and the need of a high titer. For dual species biofilms and treatments with phage, each phage suspension was prepared with twice the concentration described and in half of the volume due to the addition of two different phages on the final suspension that will be used in the experiments.

Table 2. Description of each treatment applied to *E. coli* and *P. aeruginosa* biofilms.

Condition	Composition
Control	SM buffer, 4× TSB, deionized water, 2:1:1 (v/v).
Phage	Phage 2 × 10^9, 4× TSB, deionized water, 2:1:1 (v/v).
Honey 25% (w/v)	Honey, 4× TSB, SM buffer, 1:1:2 (w/v).
Honey 50% (w/v)	Honey, 4× TSB, SM buffer, 2:1:1 (w/v).
Phage + Honey 25% (w/v)	Phage 2 × 10^9, Honey, 4× TSB, 2:1:1 (w/v)
Phage + Honey 50% (w/v)	Phage 1.2 × 10^{10}, Honey, 4× TSB, SM buffer 1: 5.5 : 2.5 :2 (w/v)

Biofilms formed on the 96-well plates for 24 h or 48 h were washed twice with fresh TSB medium to remove non-adhered cells. A volume of 200 µL of the desired treatment was added to each well and the plates incubated at 37 °C, 120 rpm (orbital shaker ES-20/60 (BIOSAN)). The control wells were performed in a separate 96-well plate. Samples were taken at 0 h, 6 h, 12 h and 24 h for biofilm viable cells quantification. Three independent experiments were performed in triplicate.

2.12 Quantification of biofilm viable cells

The number of viable cells present in the biofilms at each time point were performed according to Pires *et al.*[188], with some modifications. Briefly, in all conditions, the phage and/or honey was removed and biofilms in the wells were washed thrice with saline solution [0.9% (w/v) NaCl] to remove non-adhered cells. Then, 200 µL of the saline solution were added to each well and the biomass removed from the bottom and walls of the well with the aid of a pipette tip. Samples were collected and serial dilutions were performed in saline solution containing 1 mM ferrous ammonium sulfate (FAS, Applichem Panreac) to assure the destruction of non-infecting phages and further bias in CFUs counts[196]. Samples were plated on MacConkey or Pseudomonas agar base plates, for *E. coli* or *P. aeruginosa* cell counts, respectively, using the microdrop technique[197]. Briefly, a drop of 10 µL of each dilution was placed over the agar and let dry for 15 minutes. After the drops on the agar absorbed, the plates were incubated for 16 h at 37 °C.

2.13 Flow cytometry analysis

Cell viability before and after single and mixed treatments was assessed by flow cytometry as previously optimized[198] with some modifications. Briefly, biofilms were washed as described above, and resuspended in 200 µL saline. Then, in the dark, 20 µL of suspension was added to 180 µL of a saline solution containing 250 nM of SYTO® BC Green Fluorescent Nucleic Acid Stain (ThermoFisher Scientific) and 20 µg/mL of propidium iodide (PI) (ThermoFisher Scientific). The fluorescence of bacteria was measured using an EC800 (SONY, San Jose, CA, USA) flow cytometer. SYTO® BC was detected on the FL1 channel and PI on the FL4 channel. For all detected parameters, amplification was carried out using logarithmic scales. Data were acquired and analyzed using Sony EC800 Flow Cytometry Analyser software. Two independent experiments were performed in duplicate.

2.14 Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 6. Mean and standard deviations (SD) were determined for the independent experiments and the results were presented as mean ± SD. Results were compared using Two-way ANOVA, with Turkey's multiple comparison statistical test. Differences were considered statistically different if $p \leq 0.05$ (95% confidence interval).

3. RESULTS

3.1 The selection and characterization of a phage against *P. aeruginosa*

3.1.1 Phage plaque morphology

The procedure to isolate *P. aeruginosa* phages from the cocktails resulted in the visualization of seven different phage plaques - four phages isolated from Sextaphage and three from Intestiphage. Phages were nominated accordingly to the bacteria from which they were isolated (PAO1) followed by a letter of the roman alphabet (A, B, C, etc.). To characterize these phage plaques, their morphology and dimensions were observed and registered (Table 3 and Figure 5).

Table 3. Determination of phage plaque and halos dimensions for the seven isolated *P. aeruginosa* phages. Data are shown as mean ± SD.

Temperature (°C)		4	≈ 22	
Size (cm)		Plaque ± SD	Plaque ± SD	Plaque_halo ± SD
Phages	PAO1-A	0.36 ± 0.08	0.77 ± 0.09	1.46 ± 0.13
	PAO1-B	0.38 ± 0.07	0.55 ± 0.07	1.25 ± 0.19
	PAO1-C	0.35 ± 0.06	0.60 ± 0.08	1.36 ± 0.31
	PAO1-D	0.39 ± 0.08	0.36 ± 0.10	1.23 ± 0.24
	PAO1-E	0.39 ± 0.06	0.54 ± 0.08	1.02 ± 0.14
	PAO1-F	0.42 ± 0.09	0.70 ± 0.01	1.32 ± 0.31
	PAO1-G	0.29 ± 0.08	0.53	1.44

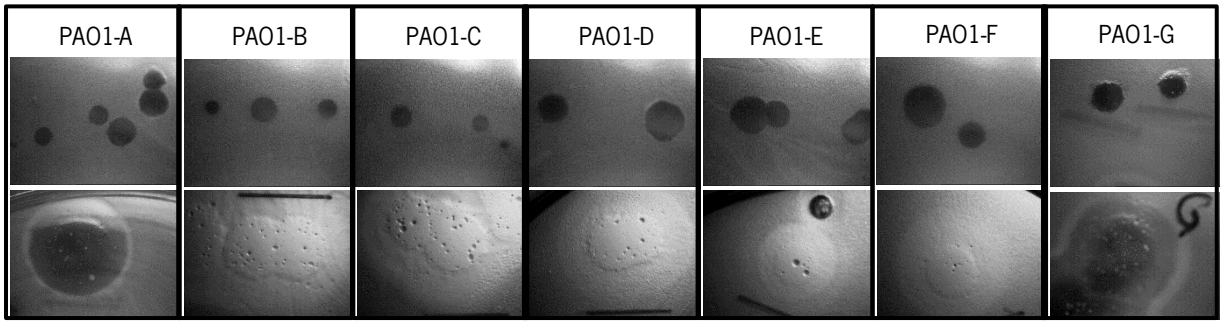


Figure 5. Microscopic observation of plaques and halos of the *P. aeruginosa* isolated phages, after incubation at 37 °C, overnight. Top line displays phages stored at 4 °C and bottom line shows phages and their halos at room temperature. The black bar represents 1 cm.

All phages left at room temperature (22 °C) for 48 h, possess a halo around the phage plaque, indicating the presence of enzymes able to degrade the biofilm matrix (Figure 5). This was not verified when phages were stored at 4 °C, as no halos were observed. At 22 °C, the largest plaques belongs to phage PAO1-A (0.77 ± 0.09 cm) and the smallest to phage PAO1-D (0.36± 0.10 cm). When considering the sum of plaque + halo, the biggest measurement is attributed to phage PAO1-A (1.46 ± 0.13 cm) and the smallest to phage PAO1-E (1.02 ± 0.14 cm). Although this could lead to the interpretation of phage A as the one with largest size and, for that, highest potential of action, when considering the difference between plaque + halo and halo alone, that is, to obtain the dimension of the halo itself, the biggest value belongs to phage PAO1-D (a difference of 0.87 cm), which may suggest the presence of enzymes with bigger efficiency to degrade the polymeric matrix of the biofilms.

3.1.2 Lytic spectra of the isolated phages

The lytic spectra of each of the isolated phages was determined (Table 4). For that, a spot test was performed on every *P. aeruginosa* strain available at the laboratory with the aim to select the ones with higher lytic spectra to posterior evaluation on biofilm control.

It was verified that none of the phages was able to infect 11 strains: the clinical isolates 10, 11, 16, 21, 22, 24, 25, 26, 27, 28 and 29, respectively. On the contrary, all phages were able to completely lyse the 4 strains (clinical isolates 9, 15 and 19 and reference strain PAO1). Furthermore, the lytic spectra presented by each phage was not very distinct. In fact, for example, the profiles of phages PAO1-A and PAO1-B are very similar; the difference is only seen in two clinical isolates strains, where PAO1-B seems to be more effective. The phage with lowest spectra of action is phage PAO1-C that infects only seven strains (~19.5%) and the phage with wider infectivity is phage PAO1-E (17 strains, ~47.2%). As so, this result was taken into account for the selection of the phage.

Table 4. Host-range of isolated phages against *P. aeruginosa* clinical isolates and reference strains.

	Susceptibility to phage *						
	PAO1-A	PAO1-B	PAO1-C	PAO1-D	PAO1-E	PAO1-F	PAO1-G
Pa 1	-	-	-	-	+-	-	-
Pa 2	-	-	-	-	+-	+	-
Pa 3	+-	+-	+-	+-	+	+-	+-
Pa 4	+-	+	+	+	+	+	+
Pa 5	+-	+-	+-	+-	+	+	+-
Pa 6	+-	+-	+-	+	+	+	+-
Pa 7	+-	+	+	+	+	+	+
Pa 8	+-	+-	+-	+	+	+-	+
Pa 9	+	+	+	+	+	+	+
Pa 10	-	-	-	-	-	-	-
Pa 11	-	-	-	-	-	-	-
Pa 12	+-	+-	+	+	+	+-	+
Pa 14	+-	+-	+-	+-	+-	+-	+-
Pa 15	+	+	+	+	+	+	+
Pa 16	-	-	-	-	-	-	-
Pa 17	-	-	-	+-	+-	-	+-
Pa 18	+	+	+-	+-	-	+-	+-
Pa 19	+	+	+	+	+	+	+
Pa 20	+-	+-	+-	+-	+-	+-	+-
Pa 21	-	-	-	-	-	-	-
Pa 22	-	-	-	-	-	-	-
Pa 23	+-	+-	+-	+	+	+	+
Pa 24	-	-	-	-	-	-	-
Pa 25	-	-	-	-	-	-	-
Pa 26	-	-	-	-	-	-	-
Pa 27	-	-	-	-	-	-	-
Pa 28	-	-	-	-	-	-	-
Pa 29	-	-	-	-	-	-	-
Pa S20	+	+	+-	+	+	+	+
Pa S21	+	+	+-	+	+	+	+
Pa S22	+	+	+-	+	+	+	+
Pa S23	-	-	-	+-	+-	+-	-
Pa S24	-	-	-	-	-	-	+-
Ref CECT III	+	+	+-	+	+	+	+
Ref PAO 1	+	+	+	+	+	+	+
Ref ATCC 10145	+	+	+-	+	+	+	+-
Sum of "only +"	10	12	7	15	17	15	13

* "+" completely lysed – no phage resistance colonies were observed; "+-" partially lysed – phage resistant colonies observed; "-" no lysis.

3.1.3 Evaluation of the antibiofilm effect of isolated phages on *P. aeruginosa* biofilms

The seven isolated phages were tested to evaluate their effect on 24 h old *P. aeruginosa* biofilms, as represented in Figure 6.

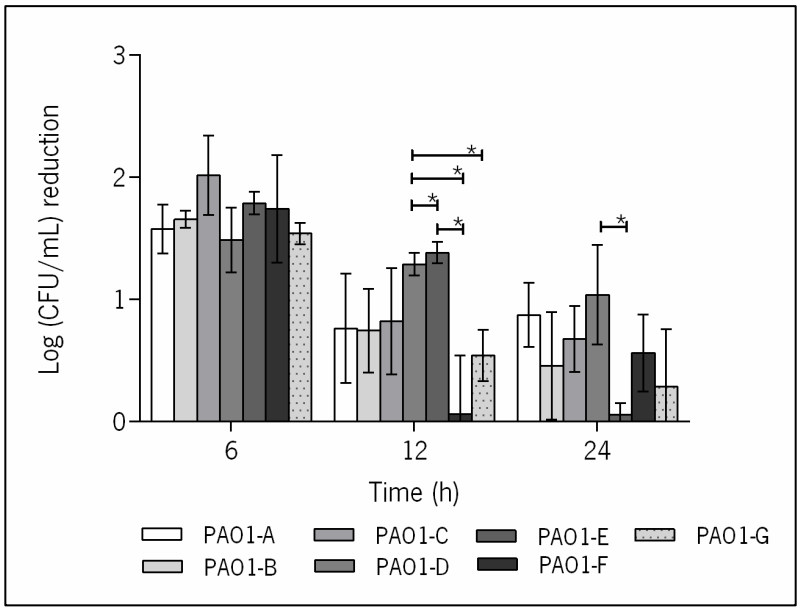


Figure 6. Antibiofilm effect of the seven isolated phages on 24 h old *P. aeruginosa* biofilms. Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

All phages presented similar antibiofilm activity ($p > 0.05$) 6 h after treatment, ranging from 1.49 log to 2.02 log viable cell reductions. This pattern changed at 12 h post treatment where phages PAO1-D and PAO1-E presented an overall better efficacy (in the range of 1.30 log reduction), that was statistically different from the action of phages PAO1-F and PAO1-G ($p < 0.05$). Although some reduction in effect was seen for the remaining phages PAO1-A, PAO1-B and PAO1-C, the values were not significant ($p > 0.05$). After 24 h, the two phages that led to higher reductions in viable cells were phage PAO1-A (0.87 log) and PAO1-D (1.04 log). After the evaluation of these three criteria (phage plaque and halo dimensions, lytic spectra, antibiofilm activity), phage PAO1-D was the selected for the combinatory treatment assays.

3.1.4 Evaluation of phage PAO1-D growth characterization and morphology

In order to characterize the growth parameters of the selected phage, one step growth curve assays were performed (Figure 7). TEM was performed so phage virion particles morphology could be analyzed.

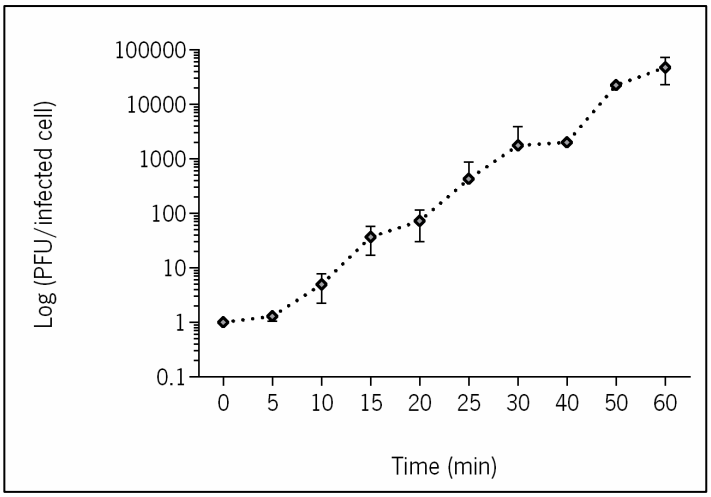


Figure 7. One-step growth curve of phage PAO1-D in *P. aeruginosa* at 37 °C. Error bars represent standard deviations from 2 independent experiments performed in duplicate.

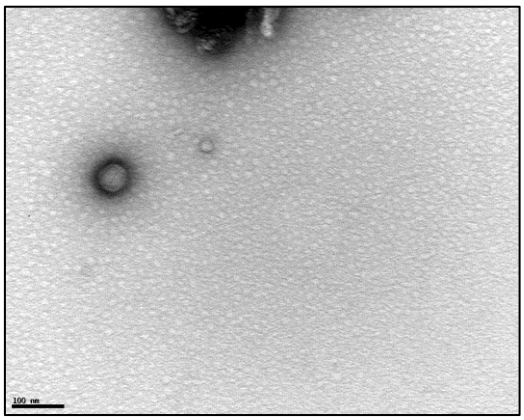


Figure 8. Characteristics of PAO1-D virion particle (scale bar 100 nm).

As observed, it is possible to conclude that this phage presents a very short latent period of approximately 5 minutes and a burst size of ~40 released phages per infected cell after a rise period of 10 minutes. The estimate burst might be lower than the real burst size however, because a new cycle of host infection did not allow its correct calculation. Furthermore, within the 60 minutes assayed, three cycles of infection were observed. TEM images of the phage particles (Figure 8) revealed a 56 × 64 nm icosahedral capsid with a 12 nm short non-contractile tail. Based on phage morphology, this phage belongs to the *Podoviridae* family.

3.1.5 Evaluation of phage PAO1-D viability in contact with honey

Given the final purpose of combinatory therapy of phages and honey, it was important to verify the viability of the selected phage PAO1-D in U3 and C1 honeys (Figure 9) at two concentrations - 25% (w/v) and 50% (w/v).

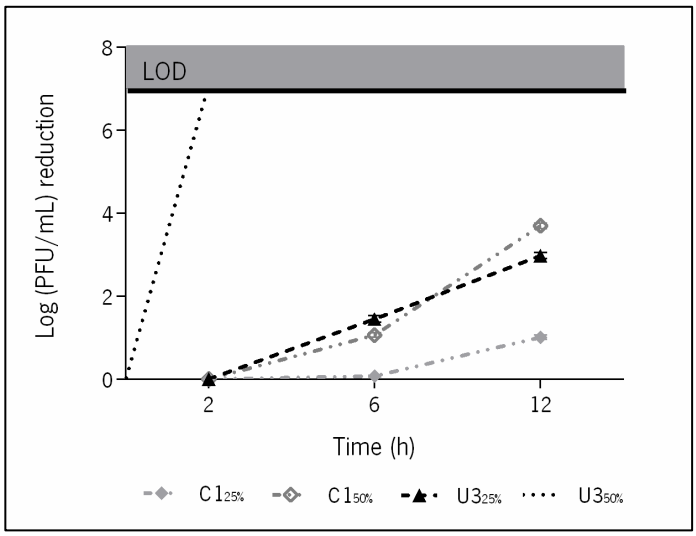


Figure 9. Phage PAO1-D viability. PFU counts after PAO1-D exposure to C1 and U3 honeys at 25% (w/v) and 50% (w/v) concentrations. Error bars represent standard deviations from 2 independent experiments performed in duplicate.

During the 12 h of experiment, only $U3_{50\%}$ influenced drastically the viability of phage with only less than 100 phage plaques being counted (LOD=6.9 log reduction) at 2 h. $C1_{50\%}$ caused a reduction of approximately 4 log after 12 h. Both honeys at 25% (w/v) were less aggressive to the phage. The viability of PAO1-D in $U3_{25\%}$ progressively decreased, nevertheless $\sim 8 \times 10^5$ PFU/mL still remained at 12 h. $C1_{25\%}$ led only to minor changes in phage viability along the assay ($\sim$1 log PFU/mL reduction).

3.2 Evaluation of honey antibiofilm effect on *P. aeruginosa* biofilms

MIC for the Portuguese honeys C1 and U3 were determined and the results presented in Table 5. Both honeys revealed a MIC of 25% (w/v). The value of MIC for Manuka was previously accessed[190], being 25% as well. To select the most efficient honey in reducing/eliminating *P. aeruginosa* biofilms, the antibiofilm activity of C1 and U3 was evaluated at 3 concentrations: 25% (w/v), 50% (w/v), and 75% (w/v) (Figure 10).

Table 5. MIC values for U3 and C1 against *P. aeruginosa.*

	Honey	
	U3	**C1**
MIC [%(w/v)]	25	25

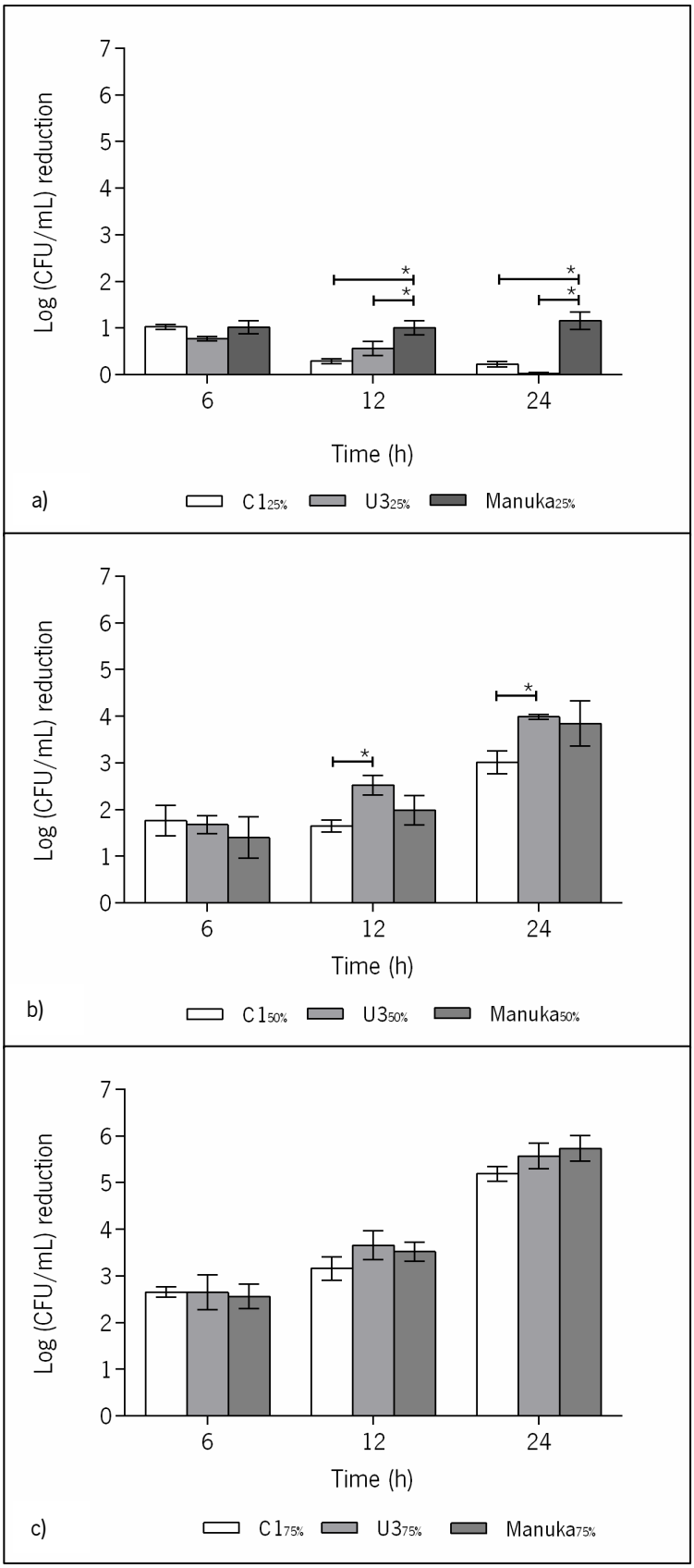


Figure 10. Antibiofilm effect of honey on 24 h old *P. aeruginosa* biofilms. a) 25% (w/v); b) 50% (w/v); c) 75% (w/v). Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

Overall, both Portuguese honeys produced similar reductions in all time points regardless of the honey concentration used. Antibacterial action of both honeys at 25% (w/v) tend to reduce over the course of treatment, reaching maximum reductions at 6 h (~1 log viable cell reduction) and diminishing until no effect is seen after 24 h of treatment. Oppositely, the efficiency of honeys at 75% (w/v) increased significantly ($p < 0.05$) along the treatment period, reaching maximum reductions of biofilm cells 24 h after treatment (5.19 and 5.57 viable cell reduction for $C1_{75\%}$ and $U3_{75\%}$, respectively). Manuka's antibacterial activity was assessed for comparison purposes only, and revealed that this honey, specifically at 25% (w/v), was able to retain its efficacy over time (~1 log viable cell reduction), and was significantly more efficent ($p < 0.05$) after 24 h than the two Portuguese honeys. The effect of Manuka at 50% (w/v) and 75% (w/v) were similar ($p > 0.05$) to those observed for the two Portuguese honeys in all time points evaluated.

The selection of the Portuguese honey to be used in further experiments was based on the intermediary concentration - 50% (w/v) - where reductions were more accentuated in U3. In fact, after 12 h of treatment, $U3_{50\%}$ reduced the viable cells in biofilms by 2.52 log while only a reduction of 1.64 was accomplished with $C1_{50\%}$, being this value significantly inferior. This difference was also notory ($p < 0.05$) at 24 h post treatment where $U3_{50\%}$ reduced by 4 log the viable cells of the biofilm. Bearing in mind these results, the chosen honey to carry on the experiments in the combinatory approach was U3.

3.3 Evaluation of antibiofilm effect of the combinatory approach on *P. aeruginosa* biofilms

This study evaluated the effect of U3, a Portuguese honey, using two different concentrations 25% (w/v) and 50% (w/v), and phage PAO1-D, individually, and the combination of both agents in 24 h old (Figure 11) and 48 h old (Figure 12) *P. aeruginosa* biofilms. The determination of viable cells present in the biofilm after the combinatory approach was done after 6 h, 12 h and 24 h.

Single approach results were already reported when evaluating the several phages and honeys antibacterial activity for the selection of the best agent for the combinatory approach. Phage PAO1-D and honey U3 were selected. This phage revealed to have significantly more efficacy ($p < 0.05$) in reducing the viable cells of biofilms at every time point when compared to lower dilutions of honey - 25% (w/v). Honey $U3_{25\%}$ revealed no effect after 24 h of treatment. Oppositely, a more concentrated honey led to a higher antibiofilm activity than phage, especially after application for longer periods, leading to significant differences at 12 h and 24 h ($p < 0.05$).

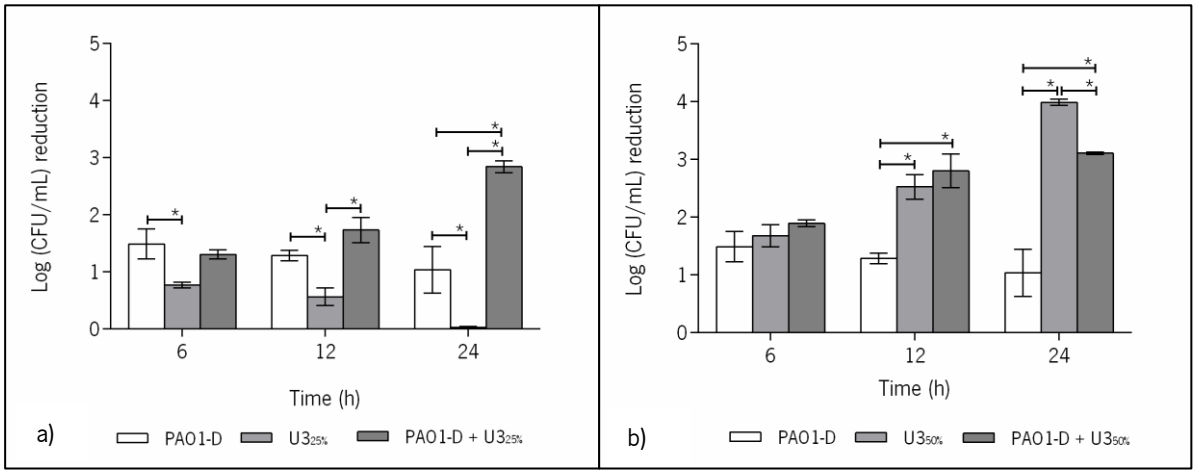


Figure 11. Antibiofilm effect of phage PAO1-D, honey U3 and phage-honey combination on 24 h old *P. aeruginosa* biofilms. a) 25% (w/v); b) 50% (w/v). Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

The combination phage–U3$_{25\%}$ produced similar reductions ($p > 0.05$) to those observed by the phage alone at 6 h and 12 h of treatment. However, after 24 h, reductions were more pronounced in the combinatory approach ($p < 0.05$) compared to the sum of each of the single agents (0.02 U3$_{25\%}$ + 1.04 phage < 2.84 combination), indicating a synergistic effect. Considering the combination phage–U3$_{50\%}$, this was overall more efficient than the previous combination, especially after 6 h and 12 h ($p < 0.05$), where 1.89 log and 2.80 log reduction in viable cell, respectively, were achieved. This combination was not, however, synergistic in any of the time points, being actually less effective than honey after 24 h of treatment.

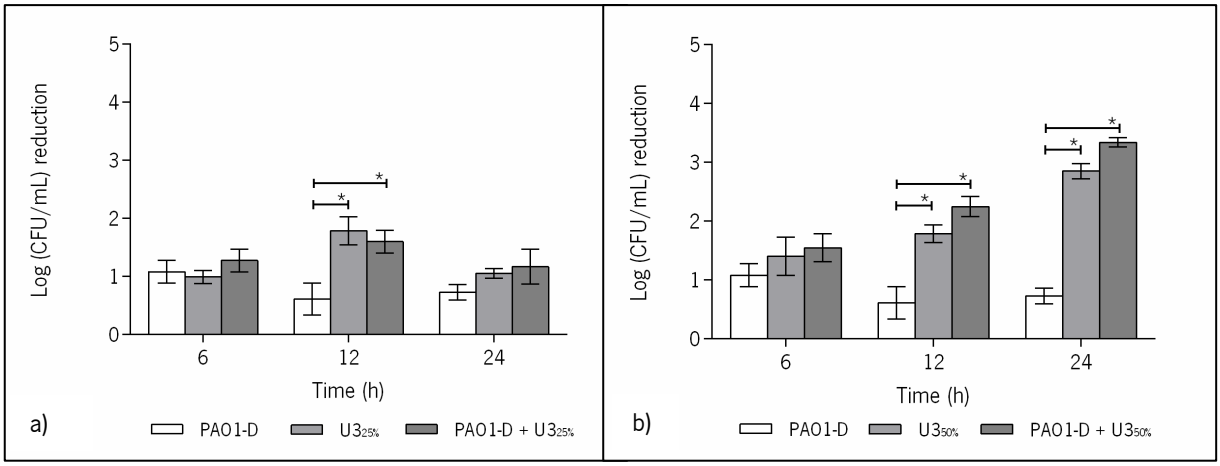


Figure 12. Antibiofilm effect of phage PAO1-D, honey U3 and phage-honey combination on 48 h old *P. aeruginosa biofilms*. a) 25% (w/v); b) 50% (w/v). Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$)

The effect of phage in older biofilms (48 h old) proved to be statistically similar ($p > 0.05$) between the time points, during the 24 h of action, resulting in viable cell reductions in the range of 1.08 log at 6

h and 0.6 log after 12 h of treatment (Figure 12). The efficacy of phage was inferior in 48 h old biofilms than younger 24 h old biofilms. Oppositely, U3$_{25\%}$ was slightly more effective in 48 h old biofilms comparatively to 24 h, presenting a reduction of approximately 1 log at 6 h and 24 h and almost twice the effect at 12 h (1.78 log reduction in viable cells), a value statistically different from the two previous time points. The higher honey concentration – U3$_{50\%}$ - revealed the same tendency as observed in 24 h old biofilms, achieving the maximum reduction 24 h after treatment (2.85 log viable cell reduction). Treatments of longer duration have better results using honey than phage.

Considering the combined approach phage-U3$_{25\%}$, this resulted in no better effect ($p > 0.05$) than honey alone in every time points. The maximum reduction was obtained after 12 h of treatment (1.60 log viable cell reduction), similar to the occurred with honey U3$_{25\%}$. Still in this time point, the combination was proven to be more effective than single phage approach. Biofilms (24 h and 48 h old) were equally affected at the initial phases of treatment (6 h and 12 h time points), but more easily reduced ($p < 0.05$) after 24 h of treatment when they were younger (24 h).

The combination phage–U3$_{50\%}$ revealed a greater antibiofilm reduction along the duration of treatment compared to individual treatments. At 6 h, a reduction of 1.55 log was accomplished which increased until 3.34 log viable cell reduction after 24 h. The combination proved to be significantly better than phage at both 12 h and 24 h post treatment ($p < 0.05$). Nevertheless, a synergistic effect was not accomplished since the combinatory approach was equally effective as honey U3$_{50\%}$. It can be, however, concluded that the use of this concentration in a combinatory approach is substantially more effective ($p < 0.05$) than the use of honey 4 times diluted, in treatments of longer duration, namely 12 h and 24 h after challenge.

3.4 Flow cytometry analysis of *P. aeruginosa* biofilms

Flow cytometry using LIVE/DEAD staining was performed to assess the effect of all treatments on the viability of 24 h old *P. aeruginosa* biofilm cells and to look for alterations of population dynamics. Results are represented in Figure 13. The majority of the population in *P. aeruginosa* control biofilms (Figure 13a) were viable cells as evidenced by the SYTO® BC (SYTO) uptake (SYTO+/PI-); there was also a small fraction of cells on the third quadrant, evidencing compromised/injured cells (SYTO+/PI+) as well as in second quadrant (SYTO-/PI-), accounting for cellular debris. U3$_{25\%}$ (Figure 13b) led to a deviation of the population for the third quadrant, indicating that the cells became compromised, however, no cellular death was verified (SYTO-/PI+). When honey U3$_{50\%}$ (Figure 13c) was used, a greater reduction was

accomplished in live cells (SYTO+/PI-). In fact, the major fraction of the population was constituted of cellular debris (SYTO-/PI-) and there was only a minority of compromised cells. When phage was administered (Figure 13d), the majority of the population remained alive, however compromised cells and cellular debris were observed as well.

In the combinatory therapy, it is possible to observe a shift of the population with cells in different stages. The phage – U3$_{25\%}$ cytogram (Figure 13e) clearly reveals an increase of cellular debris when compared to the honey treatment alone, resulting from the deviation of both live and compromised cells into that quadrant. With the phage - U3$_{50\%}$ combination (Figure 13f), the biofilm population presented a core population of cellular debris, with a small equal proportion of live and compromised cells. The addition of honey to the phage resulted in a higher proportion of compromised cells and cellular debris, as well as a reduction of live cells, compared to phage alone. Nevertheless, use of honey 50% individually caused more damaged than the combination phage-U3$_{50\%}$, once more cellular debris and less live cells were observed.

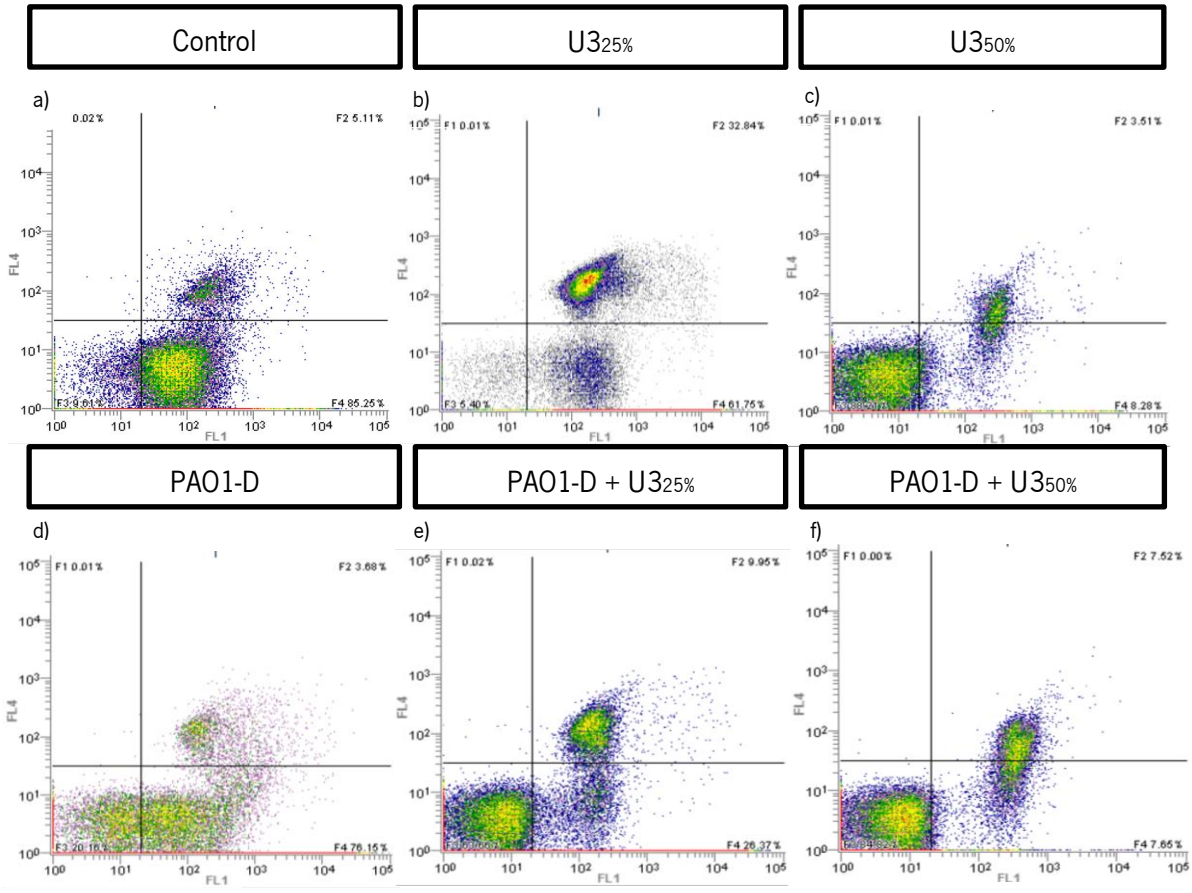


Figure 13. Flow cytometric analysis after 24 h application of single and combined treatments to 24 h old *P. aeruginosa* biofilms. Representative dot plot FL1 (xx axis) vs FL4 channel (yy axis) showing *P. aeruginosa* cells stained with SYTO BC (250 nM) and PI (20 µg/mL). **a)** *P. aeruginosa* control; **b)** U3$_{25\%}$; **c)** U3$_{50\%}$; **d)** PAO1-D phage; **e)** phage-U3$_{25\%}$; **g)** phage-U3$_{50\%}$. Results are a representative example of 2 independent experiments performed in duplicate.

3.5 The characterization of a phage against *E. coli*

3.5.1 Phage morphology

The phage selected to develop this work, phage vB_EcoS_CEB-EC3a here referenced as EC3a, was previously isolated from raw sewage by a member of the group and infected 12 of 31 multidrug resistant *E. coli* clinical isolates[189]. This phage was chosen for further characterization and for evaluation of the antibiofilm effect. The observation of six individual phage plaques (plaque and halo) revealed the formation of clear plaques (plaque = 0.263 ± 0.035 mm) surrounded by a halo (plaque_halo = 1.155 ± 0.161 cm). The morphological characterization of EC3a, performed by TEM, led to the observation of an icosahedral head with 57 nm of diameter and a tapered, non-contractile tail of 192 nm × 11 nm with conspicuous striations. Accordingly to these results, it was possible to conclude that phage EC3a belongs to *Siphoviridae* family, which is characterized by phages with a short non-contractile tail, resembling morphological values observed in T1-like phages.

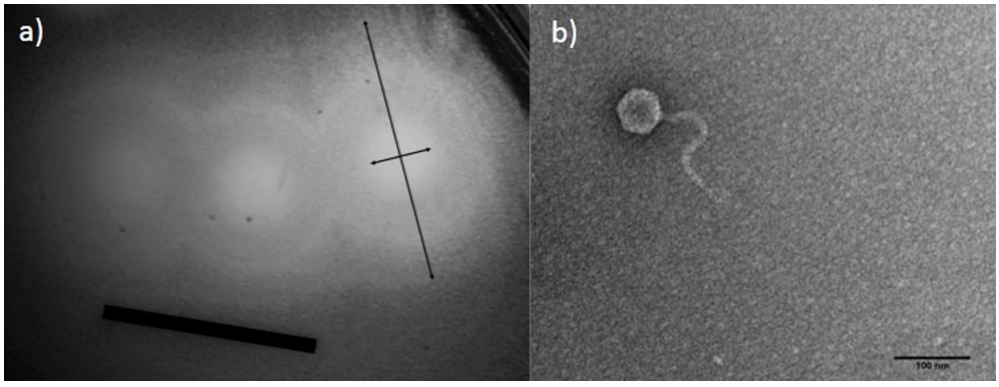


Figure 14. Characteristics of EC3a a) plaque morphology (black arrows indicate diameter of EC3a plaque and diameter of EC3a plaque and the surrounding halo; scale bar 1 cm), b) virion particle (scale bar 100 nm).

3.5.2 Evaluation of phage EC3a growth characterization

The growth parameters of phage EC3a, namely the latent period, rise period and burst size, were determined by one-step growth studies, represented in Figure 15. Two independent experiments showed that EC3a has a short latent period of approximately 15 minutes and a burst size of ~530 released phages per infected cell after a rise period of 10 minutes. Within the 60 minutes of duration of the experiment, a second cycle of replication was evident for this phage.

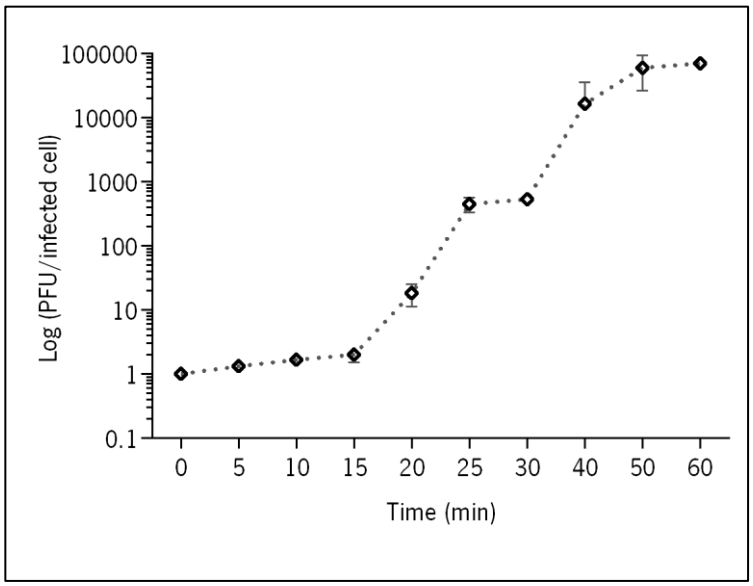


Figure 15. One-step growth curve of phage EC3a in *E. coli* at 37 °C. Error bars represent standard deviations from 2 independent experiments performed in duplicate

3.5.3 Evaluation of phage EC3a viability in contact with honey

The viability of phage EC3a was inspected in U3 honey at two concentrations - 25% (w/v) and 50% (w/v) (Figure 16). As observable, after 12 h of contact with $U3_{25\%}$, there are still phages remaining, with an average concentration of 3.45 log. On the other hand, $U3_{50\%}$ completely inactivated EC3a within 1 h upon contact.

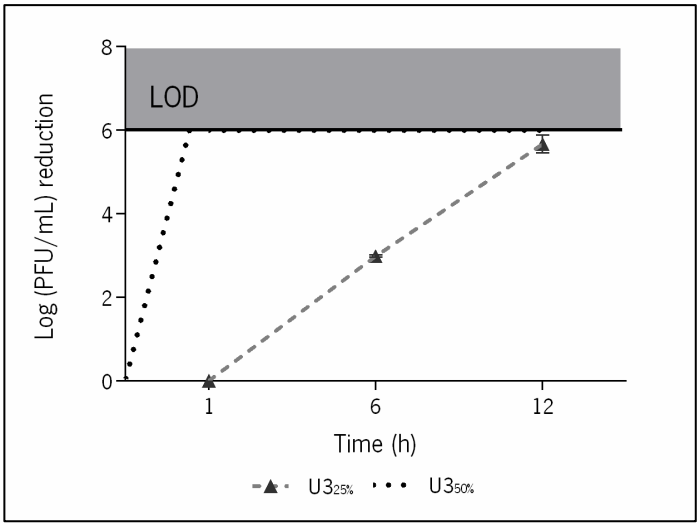


Figure 16. Phage EC3a viability. PFU counts after EC3a exposure to U3 honey at 25% (w/v) and 50% (w/v) concentrations. Error bars represent standard deviations from 2 independent experiments performed in duplicate.

3.6 Evaluation of antibiofilm effect of single and combinatory approaches on *E. coli* biofilms

After characterizing the phage and using U3, the same honey as used in the study with *P. aeruginosa* biofilms, both agents, individually or combined were evaluated for their antibiofilm action in 24 h old (Figure 17) and 48 h old (Figure 18) *E. coli* biofilms.

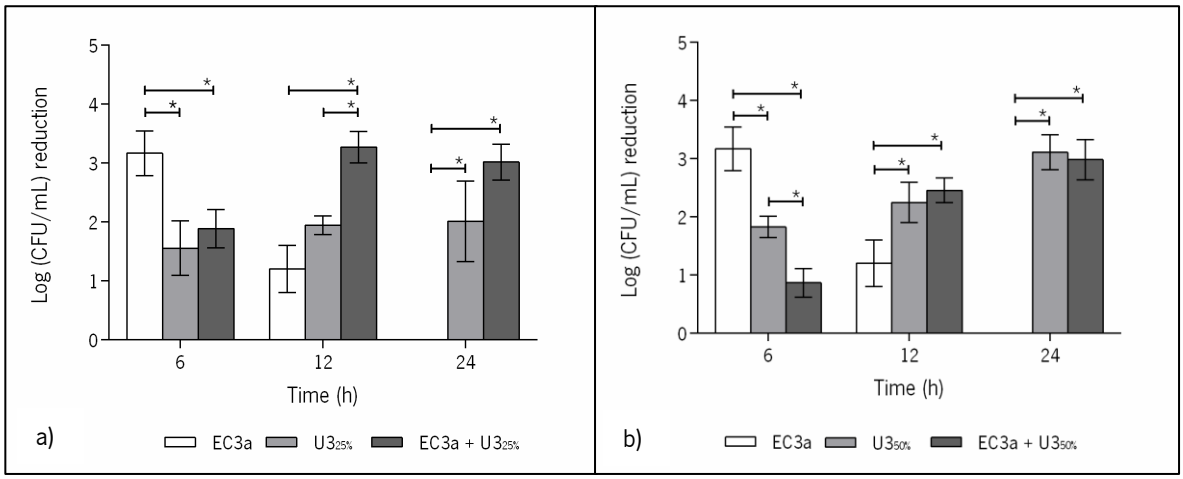


Figure 17. Antibiofilm effect of phage EC3a, honey U3 and of the phage-honey combination on 24 h old *E. coli* biofilms. a) 25% (w/v); b) 50% (w/v). Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

Regarding biofilm treatment in 24 h old biofilms, EC3a revealed a decreasing antibiofilm activity along the time of treatment, with the maximum reduction after 6 h, where achieved a 3.2 log viable cell reduction, diminishing significantly its activity ($p < 0.05$) until 24 h of treatment, where no antibacterial effect was observable. Oppositely, honey activity increased during the treatment. In fact, 6 h after treatment, U3$_{50\%}$ produced a biofilm viable cells reduction of 1.82 log that was similar to U3$_{25\%}$ (reduction of 1.56 log). Both reached a reduction of approximately 2 log at 12 h, achieving the maximum antibiofilm effect after 24 h of treatment, where honey concentration was a differential factor as significant differences were observed ($p < 0.05$): at this time point, a reduction superior to 3 log was observable with U3$_{50\%}$. As so, considering single approaches, it is concluded that, relatively to antimicrobial activity, at 6 h, phage was significantly better than honey (with any of the concentrations) ($p < 0.05$), tendency that was inverted at 12 h and 24 h where honey U3$_{50\%}$ was more efficient (2.25 and 3.11 log viable cell reduction, respectively).

The combinatory approach phage-U3$_{25\%}$ revealed diminished efficacy ($p < 0.05$) at 6 h of treatment when compared to phage alone, producing similar effect to honey 25% (w/v) alone. Although achieving a viable cell reduction of approximately 3 log after 24 h of treatment, significant differences were not observed compared with U3$_{25\%}$ alone. Nevertheless, in the intermediary point, the combination produced an additive effect ($p < 0.05$) compared to the treatment with each components individually. When using the most concentrated honey, combination of agents showed a tendency to increase the efficacy of biofilm viable cell reduction along the treatment, however it did not produce a synergistic effect in every time points. In fact, similar reductions were achieved with the combination of phage – U3$_{50\%}$ and U3$_{50\%}$ alone at 12 h and 24 h post treatment.

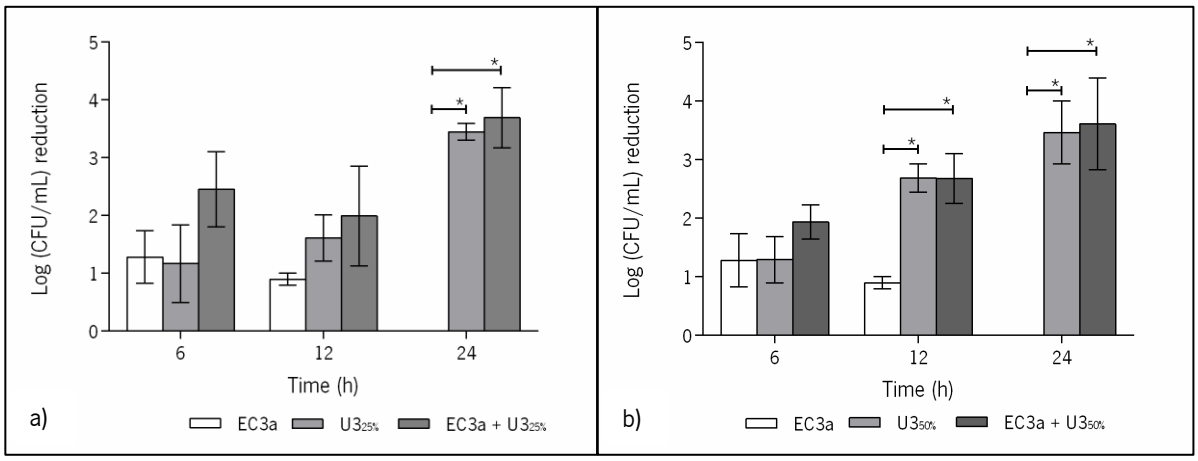


Figure 18. Antibiofilm effect of phage EC3a, honey U3 and of the phage-honey combination on 48 h old *E. coli* biofilms. a) 25% (w/v); b) 50% (w/v). Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

Regarding more mature biofilms (48 h), the treatment with phage EC3a revealed the same tendency as in 24 h old biofilm: the biofilm maximum reduction was achieved at 6 h of treatment (1.28 log viable cell reduction) which decreased over time until no influence of treatment was noticed 24 h after treatment. Efficiency was significantly lower in 48 h old biofilms ($p < 0.05$). The effect of both honey U3 concentrations in 48 h old biofilms was similar ($p > 0.05$) at 6 h and 24 h, achieving maximum reduction at the latter time point (3.44 and 3.46 log viable cell reduction in U3$_{25\%}$ and U3$_{50\%}$, respectively). At 12 h, however, the efficacy of U3$_{50\%}$ was higher ($p < 0.05$) than U3$_{25\%}$.

The combination phage-U3$_{25\%}$ was efficient at 6 h, where an additive effect was achieved. In the other time points, the combination resulted in similar viable cell reduction as the honey U3$_{25\%}$ alone. This tendency was also registered for the combination phage–U3$_{50\%}$. Of notice is the fact that, in both 24 h old and 48 h old biofilms, antibacterial effect produced by the combinatory approach seems to result from the action of honey alone, for both concentrations.

3.7 Flow cytometry analysis of *E. coli* biofilms

The outcome of the treatments administered on *E. coli* 24 h old biofilms was also evaluated by Flow cytometry using LIVE/DEAD staining (Figure 19). At this phase, PF2$_{25\%}$ was also used based on previous results obtained with this honey in *E. coli* biofilms[190]. As expected, the majority of *E. coli* control biofilm (Figure 19a) cells were viable (SYTO+/PI-) with a small fraction of compromised/injured cells (SYTO+/PI+). When honey was used individually, an increase of the SYTO mean fluorescent intensity was verified. PF2$_{25\%}$ and U3$_{50\%}$ both resulted in a substantially higher number of compromised cells compared to the control (Figures 19b and 19d), but U3$_{50\%}$ lead to a higher amount of cellular debris (SYTO-/PI-). On the other hand, U3$_{25\%}$ had a similar amount of compromised cells than the control biofilm (Figures 19a and 19c). Phage alone clearly moved part of the viable cell population, causing an increase of damaged cells and cell debris (Figure 19e) compared to control biofilm (Figure 19a). The amount of cell debris was greater using phage than both honeys at 25% (w/v) (compare Figures 19b, 19c and 19e) but not at all comparable to the amount of debris after treatment with U3$_{50\%}$.

The comparison of single agent treatments (Figures 19b to 19e) with the combined treatments (Figures 19f to 19h) gives us an indication of the population shifts as a result of the strategy used. The treatment with phage-PF2$_{25\%}$ revealed that i) the core population was similar to the one obtained with honey and the phage was able to infect the live cells that had the highest uptakes of SYTO; ii) an increase of cell debris compared to phage and honey single treatments; iii) there are less live cells compared to single treatments. Altogether, the treatment reveals a synergistic effect.

The combinatory approaches using honey U3 both revealed an action of phage on cells targeted by honey individually, due to the reduction of live population with a higher SYTO uptake while viable cells with less SYTO uptake remained. Additionally, the population with the lowest SYTO uptake, visible after phage single treatment, was presented in the combined strategy. Finally, the amount of cellular debris formed in both conditions was not superior than in individual treatments.

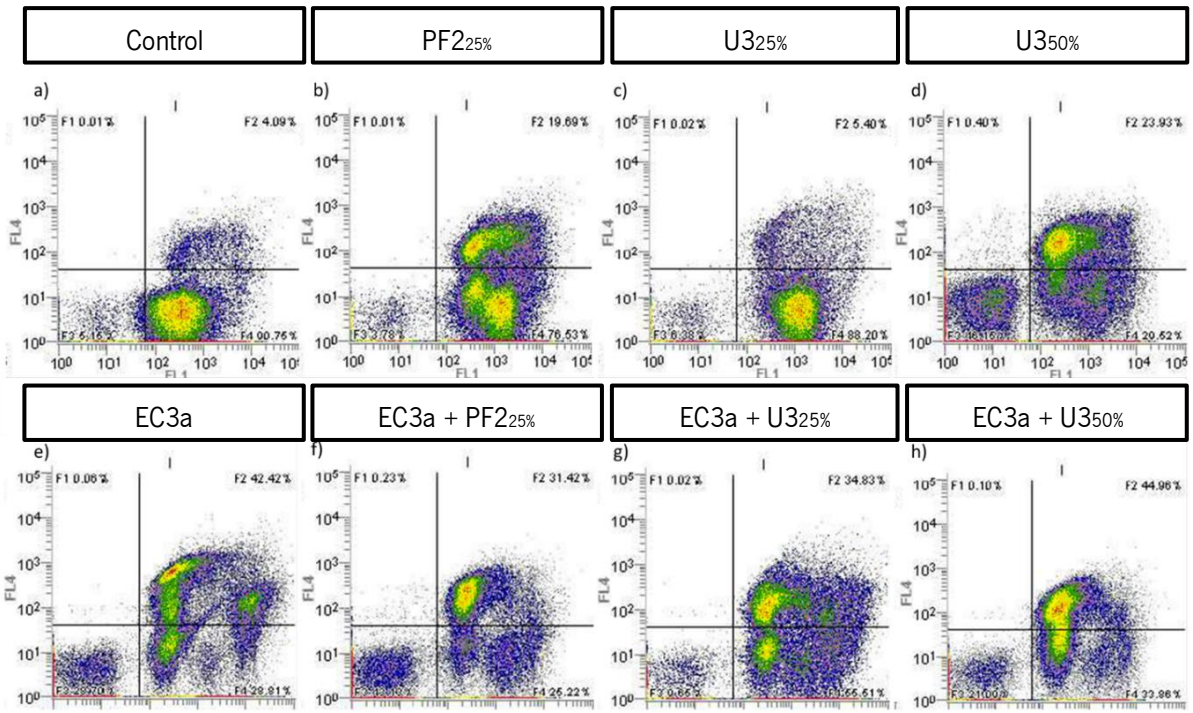


Figure 19. Flow cytometric analysis after 12 h application of single and combined treatments to 24 h old *E. coli* biofilms. Representative dot plot FL1 (xx axis) vs FL4 channel (yy axis) showing *E. coli* cells stained with SYTO BC (250 nM) and PI (20 µg/mL). a) *E. coli* control; b) PF2 25% c) U3 25%; d) U3 50%; e) EC3a phage; f) phage-PF2 25%; g) phage-U3 25%; h) phage-U3 50%. Results are a representative example of 2 independent experiments performed in duplicate.

3.8 Evaluation of antibiofilm effect of the combinatory approach on dual species biofilms

Biofilms are generally formed by polymicrobial communities. As so, it is of relevance to study the effect of the treatment in polymicrobial biofilms of *P. aeruginosa* and *E. coli.*

The study of this approach in 24 h old biofilms allowed to retrieve the following conclusions. In *P. aeruginosa*, the combination phage-U3 25% resulted in the same log reduction along the treatment (~1.4 log viable cell reduction), which was considerably lower ($p < 0.05$) than the obtained with combination phage–U3 50% in every time points. The latter led to a 2.15 log viable cell reduction after 6 h of treatment and increased until achieving a maximum of 3.84 log reduction 24 h post treatment. The presence of a second species seems to impair significantly ($p < 0.05$) the action of the treatment with U3 25% only 24 h after treatment. On the other side, the treatment with phage-U3 50% in dual species biofilms led to a significant increase ($p < 0.05$) of viable cell reduction at 6 h and 24 h after treatment when compared to the same condition in a monospecies biofilms.

In *E. coli,* the combination phage-U3$_{25\%}$ reduced the biofilm viable cells by 2.40 log after 6 h of treatment, and 2.54 log after 24 h. Phage–U3$_{50\%}$ had a stronger antibiofilm effect. After 24 h the combination caused a biofilm reduction of more than 4 logs (lower numbers than the limit of detection). Here, the effect of the combination in dual species biofilms was more pronounced ($p < 0.05$) than in monospecies biofilms. However it is important to consider that the established concentration of administered phage was 1×10^9 PFU/mL in both conditions. Since the growth of *E. coli* in dual species biofilms was more reduced than in monospecies ones, this led to a MOI of approximately 1000.

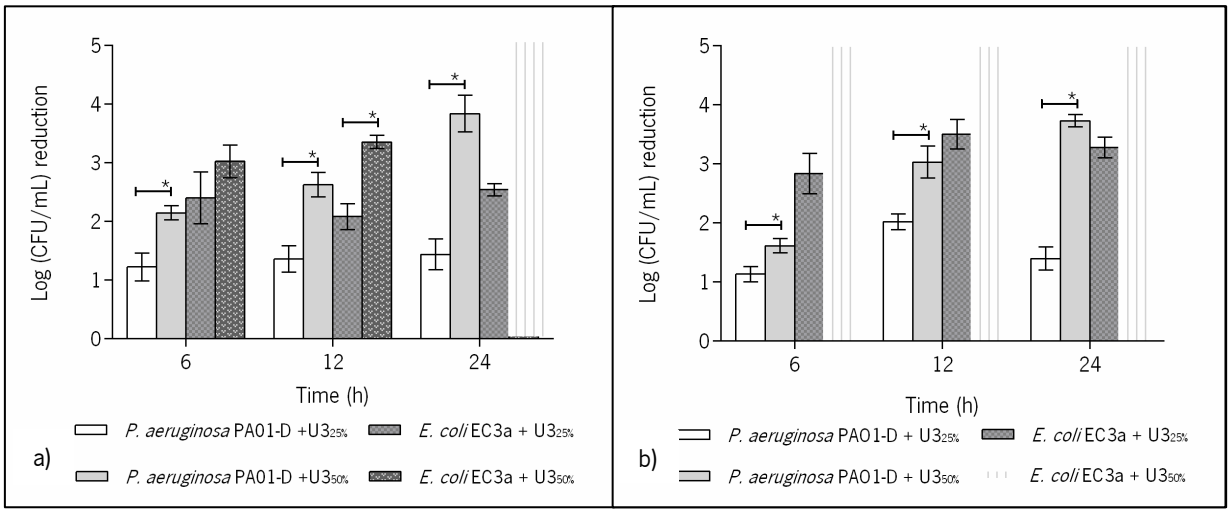


Figure 20. Antibiofilm effect of phage-honey combination on 24 h and 48 h old dual species (*P. aeruginosa* and *E. coli*) biofilms. a) 24 h old biofilms; b) 48 h old biofilms. Error bars represent standard deviations from 3 independent experiments performed in triplicate. * Statistically different ($p < 0.05$).

The biofilm development time was also considered for evaluation. In 48 h old biofilms, the combination phage-U3$_{25\%}$ on *P. aeruginosa* species achieved the maximum reduction at 12 h post treatment (~2 log reduction of biofilm viable cells). At this time, the longer development of biofilm seemed to benefit the action of the treatment ($p < 0.05$). Using the combination with more concentrated honey, the values of reduction were significantly higher than with phage-U3$_{25\%}$ ($p < 0.05$) in every time point and the tendency was similar to the one observed for 24 h old biofilms, increasing the action until a maximum of biofilm viable cell reduction of 3.72 log after 24 h of treatment, value that was not influenced by a second species (*E. coli*). Additionally, the approach was equally effective ($p > 0.05$), in spite of the longer biofilm development time. Regarding the action of this approach in *E. coli* species, the combination of phage–U3$_{25\%}$ was, similarly to what occurred to *P. aeruginosa*, more effective after 12 h of treatment, where a viable cell reduction of 3.50 log was observed. A stronger effect ($p < 0.05$) was observed when compared to the reductions in 48 h monospecies biofilms and to the reduction in 24 h dual species biofilms. The combination phage–U3$_{50\%}$ was very effective, as more than 4.3 log of viable cells (detection under limit) were eliminated since 6 h of action.

4. Discussion

Chronic wounds have become a global threat to public and health systems nowadays as numbers of incidence continue to increase due, not only to the ageing of the population, but also to the growing incidence of diseases prone to the development of wounds. One of the factors causing a delay in the efficacy of treatment is the inability of current antimicrobials to eliminate the microorganisms within the wound, associated with a rapid expansion of bacterial resistance to antibiotics due to their inappropriate use. Among the frequently isolated bacteria in chronic wound environment are *P. aeruginosa* and *E. coli*[6,28,29]. As so, novel therapeutic strategies are urgently needed. Phage and honey are two antimicrobial agents that are becoming promising approaches to eliminate bacteria. In order to have more insights of their action, this study accesses their activity individually and combined on two frequently isolated microorganisms within the wound environment: *P. aeruginosa* and *E. coli*.

The isolation of *P. aeruginosa* phages was done from two commercial phage products. Seven phages were isolated and their lytic spectra against 33 clinical isolates and 3 reference strains tested. None of the phages were able to lyse 11 previously characterized clinical isolates, of which three presented a high level of resistance (43% up to 86%) towards seven antimicrobials tested: imipenem, ciprofloxacin, ceftazidime, gentamicin, amikacin, tazobactam, tobramycin[188]. Interestingly, 5 phages completely inhibited the growth of clinical isolate 12 that presented one of the highest resistance (86%) and all phages lysed clinical isolate 19 that presented a high value of resistance (71%). The morphology of plaques of the isolated phages revealed different sizes and the presence of a halo around the phage plaques which is an indicator for the presence of phage-associated depolymerases in all phages and consequently of a probable higher antibacterial activity, since phages with this property may be more effective in eradicating biofilms[119]. The preliminary assessment of antibiofilm activity of the seven isolated phages on 24 h old *P. aeruginosa* biofilms indicated that the majority lost efficacy throughout the 24 h treatment. In fact, only PAO1-A and PAO1-D were able to reduce biofilm viable cells in, respectively, 0.87 and 1.04 log after 24 h, which can be explained due to the selective pressure exerted by phages. Similarly to the occurred in other studies[188,190,199], it seemed that bacteria acquired resistance to the other 5 phages and therefore regrowth of the biofilm cells was observed.

Due to the biggest halo size of phage PAO1-D, to its relatively good lytic spectra (41.6%) and to the results of antibiofilm assay, PAO1-D was chosen for further analysis. On the other hand, the previously isolated *E. coli* phage, infecting approximately 40% *E. coli* clinical isolates, also possessing depolymerase activity[200] and not encoding known genes associated with lysogeny or toxin proteins[201] was the *E. coli* phage from the BBiG collection chosen for antibiofilm evaluation experiments.

Both *P. aeruginosa* phage PAO1-D and *E. coli* phage EC3a were tested alone against 24 h and 48 h biofilms of their respective host strains. PAO1-D reduced viable cells in 24 h and 48 h old biofilms, after 24 h of treatment, by 1 log and 0.7 log respectively. This shows that, even in more mature biofilms, phage PAO1-D maintained antibiofilm efficacy. Although the majority of antibiofilm studies with phages report decrease of activity after an initial good control at early treatment periods, there are some studies which support the phage PAO1-D results at 24 h. For instance, in a study regarding phage activity against established biofilms of *Proteus mirabilis* and *E. coli*, a coli-proteus phage with ability to cause lysis of both species, and T4 bacteriophage, reduced by 4 log *E. coli* biofilms at 24 h of treatment[202], and *P. aeruginosa* phage phiIBB-PAA2 still revealed increased antibiofilm activity at 24 h post infection[188].

The treatment of *E. coli* biofilms with phage EC3a revealed biofilm maximum reduction at 6 h of treatment until no antibiofilm influence was perceived after 24 h and 48 h. This behavior has been frequently reported and reveal that phages, after an initial effective biofilm control allow bacterial cell regrowth at some point of treatment (due to phage resistance and changes in bacterial phenotype and genotype) which varies according to the phage-host system in study[188,190,199]. Another factor that may explain the decrease in EC3a efficacy can be the thick EPS matrix present in biofilms that is a physical obstacle that mask bacterial receptors, making them inaccessible to phages[203,204]. Another explanation rises from the physiological state of the cells in biofilms that are less metabolically active and consequently slow down the infection process[205]. Although the rising of resistance by bacterial strains to the phage is a threat to its therapeutic use, the use of cocktails with more than one phage with complementary lytic spectra and different host receptors may be an effective solution[206,207]. Nevertheless, this need to be addressed carefully as the response may vary according to the phages and bacterial strains in study[208].

The two phages used in this study presented short latent periods however considerably differences in burst size. The number of EC3a progeny released after the first cycle is approximately 13 times higher than the average release of PAO1-D *per* infected cell. This may explain the higher reductions of viable *E. coli* cells from biofilms at 6 h of treatment compared to *P. aeruginosa* cell reductions by each phage. This information needs to be addressed carefully for biofilm challenge, as phage burst size is shown to be influenced by the type of phage, the cell size, the cell cycle, the cell physiology, etc.[205,209,210].

Portugal produces 12 tons of honey per year making honey production an important contributor to the national economy[211,212]. Portuguese honeys are harvested from different regions and this reflects in their floral nectar origin. The botanical origin of many Portuguese monofloral honeys is heather, lavender, blueberry, chestnut, orchard, etc. but many others are designated as polyfloral showing no predominant type of pollen. In this work, the antimicrobial action of two Portuguese honeys, a polyfloral honey with

heather (U3) as the main floral source and a chestnut honey (C1), was evaluated. Honey MIC values, their MGO content and antibiofilm effect were used to select the honey for use in single and combinatory experiments. MIC provided information of the inhibitory effect induced by honey in the growth of *E. coli* CECT 434 and PAO1 suspensions. The MIC value for Portuguese honeys U3 and C1 was 25% for both *E. coli* and *P. aeruginosa*. Previous studies have reported 25% MIC for *P. aeruginosa* with Manuka and heather honeys from Portugal, UK, and Australia[190,213]. Lower MICs have been reported with Manuka honey against *P. aeruginosa*, such as 6.9% (v/v) against 20 strains of infected wounds isolates[214] and 9.5% (w/v) against a reference strain[215]. Previous studies have also compared MIC values of Manuka against *E. coli* and *P. aeruginosa* having showed that the MIC for *P. aeruginosa* (25%) was twice the value than for *E. coli* (12.5%)[190] but other studies have revealed similar MIC (12.5% and 40%) for both bacteria depending of the type of honey used[216,217]. In another study, *P. aeruginosa* was shown to be the least sensitive to Manuka honey, in a total of 7 studied organisms (*E. coli*, *P. mirabilis*, *P. aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *S. aureus*, *and S. pyogenes*).

The MGO content was considerably different between the honeys analyzed, with U3 possessing a higher concentration (2092.4 mg/kg)[201] that was approximately twice the concentration of C1 (1000.2 mg/kg) (data not shown) and thrice the value obtained for Manuka (756.5 mg/kg)[190]. These differences were somewhat unexpected since Manuka has been shown, in a previous study evaluating peroxide and non-peroxide honeys from different origins all over the world, to be the honey with highest non peroxide activity that was attributed to its high MGO content[218]. It was possible to conclude that MGO content and the value of MIC are not, however, strictly related since, although these Portuguese honeys presented the same MIC (25% (w/v)) value for *E. coli* and *P. aeruginosa*, they have considerably distinct MGO values. It is known that the antibacterial activity of Manuka is not fully attributed to MGO content[219]. Instead, it is due to a combination of several antimicrobial properties, as pH, MGO, hydrogen peroxide, defensing 1, sugar components, flavonoids, and more, that gives honey its antimicrobial properties[149,156,220,216].

The use of Portuguese honey at 25% (w/v) reduced cells by 1 log within the first 6 h, however, had lower effect at longer treatment periods. As so, the values obtained in MIC assay were not capable of effectively reducing biofilm, especially after 24 h of treatment, where antibiofilm effect was not perceived. This comes in accordance with previous studies, where the minimum bactericidal activity was lower for planktonic compared to biofilm embedded cells[164]. The same is usual for antibiotics where the necessary antibiotic concentrations are usually 100 or even 1000 times higher to eradicate biofilms compared to the MIC value obtained for planktonic cultures[221,222].

At 12 h and 24 h, U3$_{50\%}$ displayed a higher effect than C1$_{50\%}$. This could be due to the higher MGO content of the sample. Furthermore, the effect of honey revealed to be cumulative for both 50% (w/v) and 75% (w/v), where the highest reduction were observable after 24 h in the three honey types evaluated. Similar reductions with 2-fold diluted honey have been achieved after 24 h of exposure to Manuka[190], but the values are much lower than those obtained with another Portuguese honey, PF2, that caused a 6.1 log reduction on viable cells. Contrarily to this study where U3 maintained an inhibitory effect throughout time, the same was not achieved in a study using Manuka at 40% and 20%[223], where maximum honey inhibition (40%) was seen at 11 hours; after which the biofilm biomass started to increase. When U3$_{25\%}$ was tested against 24 h biofilms, a stronger antibiofilm effect was obtained against *E. coli* compared to the effect in *P. aeruginosa* (1.56 log *vs* 0.77 log biofilm viable cells reduction at 6 h; 2.01 log *vs* 0.02 log biofilm viable cells reduction at 24 h). However, U3$_{50\%}$, resulted in a better outcome in *P. aeruginosa* biofilms.

In 48 h biofilms, U3 was more effective against *E. coli* than *P. aeruginosa* biofilms. This is in agreement with a previous work were the concentration of Manuka honey needed to reduce *E. coli* was lower (10%) than to reduce *P. aeruginosa* (20%)[224]. It can be concluded that *E. coli* may be more sensitive to the honeys action than *P. aeruginosa*, probably due to the different cellular targets of honey in each specie. However, this needs further confirmation for instance by TEM analysis. Unlike the poor action of another Portuguese polyfloral honey PF2 in mature *E. coli* biofilms[190], U3's effect was not at all influenced by the age of the *E. coli* biofilms treated. A possible explanation for this fact might be related to the high MGO content present in U3, since this molecule holds strong antimicrobial and antibiofilm activity even in mature biofilms[225]. Another possible reason can also be the repression of quorum sensing signaling and virulence associated genes by honey which has previously been attributed to the main sugars present within honey (glucose and fructose), and not to honeys MGO and hydrogen peroxide[170].

On the other hand, the lower activity of U3 when compared to PF2 against the same organism *E. coli*, using 1× MIC in U3 and 2× MIC in PF2, could also be explained due to the botanic origin of these two honeys. PF2 presented a predominant pollen of *Castanea sativa* (56%) while U3 had no predominant pollen and had *Erica* spp. (30%) as the most dominant secondary pollen[201]. In a previous study, Chestnut honey (*Castanea sativa)* was shown to be effective against *E. coli* (13 samples) while Erica heather honey did not inhibit *E. coli* growth (1 sample)[226]. This highlights that honeys can be organism and even strain dependent with the same honeys or honey components revealing different antimicrobial and antibiofilm activity in two different strains of *E. coli*[170].

Osmotic pressure derived from the use of higher concentrations of honey (50%) seemed to cause higher reductions in viable *P. aeruginosa* and *E. coli* biofilm cells. Although this effect is variable for the bacterial species in study, *P. aeruginosa* seemed to be highly responsive to osmotic pressure[158]. In the same way, Ulmo 90 at 50% (v/v) and laboratory synthesized honey at 50% (v/v), as well, presented identical antibacterial activity, suggesting that, at this concentration, their effect was due to osmotic pressure or high sugar content[216]. Lerrer and colleagues concluded that the exact sugar content of honey may be important, as specifically fructose blocks the bacterial sugar binding protein – lectin - of *P. aeruginosa*, affecting consequently the bacterial adhesion capacity[220]. The sugar content in U3 and C1 honeys was not analyzed, however this information could have also provided evidence for the differences in the antibacterial action of the honeys[220]. Other components, such as defensin 1, revealed to strongly contribute to the antibacterial properties of honeys[142]. This clearly highlights the importance of the multifactorial nature of honey, for its broad-spectrum bactericidal activity.

The combinatory strategy of phages with other agents has been tested in several studies in order to increase the bactericidal or bacteriostatic action of each of the agents or reduce the acquired resistance (antibiotics, sugar alcohol xylitol, chemical adjuvants, etc.)[113,126,127]. In this work, an innovative combination was evaluated, and phages were associated with honey.

Even though honey evidenced strong antiviral action, phages maintained their integrity and viability during different time periods, depending on the honey concentration tested. Nevertheless, phages had, in general, enough time to adsorb and infect the host bacterium after addition to biofilms.

The combinatorial approach included the use of honey at 25% (w/v) and 50% (w/v) together with each phage. For *P. aeruginosa* biofilms, a synergistic effect was observed with $U3_{25\%}$ and phage addition after 24 h. No synergism was, however, observed with honey at 50% (w/v) or in the combinatorial approach against older biofilms. However, there was a tendency indicating that the combinatory approach was relatively more successful in reducing viable cells compared to the individual treatments.

The combinatorial therapy against *E. coli* biofilms resulted in an additive effect at 12 h. Although phage alone had no effect at 24 h of treatment, possibly its previous effect on the cell structure enhanced and facilitated honey's action at this time point. On the other hand, combination of EC3a with $U3_{50\%}$ did not seem to increase the antibiofilm effect in any of the time points of treatment. This can be due to phages' rapid destruction by honey and to the strong antibiofilm capacity of honey itself that is sufficient to cause high cell reduction rates. Relatively to more mature *E. coli* biofilms (48 h), this combination of agents did not produce a synergistic effect with any of the honey concentrations. In fact, the effect was

similar to the one with honey treatment alone, supported by the fact that phage action was poor on these 48 h old biofilms.

Flow cytometry experiments revealed no increase in intact dead cells in both microorganisms studied with every treatment administered to biofilms. This is due to cell burst to release progeny phages. The flow cytograms showed that membrane structure disruptions increased cell permeability, leading to higher uptakes of SYTO in *E. coli* biofilms and to great amounts of compromised *P. aeruginosa* cells. The shift of the initial population to intermediate (12 h) and higher states of uptake of SYTO (24 h) was possibly due to changes in membrane permeability. Flow cytometry complemented with the CFU counts showed that compromised cells were not able to be cultivated possibly because the cells were already manufacturing new phage particles or were already in early stages of lysis[171]. Flow cytometry analysis of suspended *E. coli* biofilm cells following treatment with $PF2_{25\%}$ showed that phages were able to target cells that remained viable after honey action. Further analysis by SEM revealed that phages and honey did not act on the same cells[201]. This can be seen as an advantage since honey can target cells not sensitive to phages. Reduced emergence of phage resistant cells has also been observed after combination of phage with antibiotics[117,227].

Summarizing, the synergism observed in this work might have occurred due to: i) the simultaneous application of two different agents that allowed, in one hand, phage resistant cells to be targeted by honey and, on the other hand, accordingly to flow cytometry results, cells where honey didn't act (with high SYTO uptakes) to be targeted by phages; ii) lower osmotic pressure that did not immediately disrupt the cellular membrane fully allowing phages to bind to their receptors and replicate within the cell; iii) longer phage viability when mixed with more diluted honey, which contributed to their enhanced combined action, giving them enough time to reach and infect neighbor cells.

The majority of biofilms in their natural environments exist in a consortium of species where bacteria can co-existence having synergistic or antagonistic interactions. Based on this reality, the antibiofilm effect of the combinatory approach was further studied in a dual species biofilm of *P. aeruginosa* and *E. coli.* The overall conclusion is that, in this case, multispecies biofilms do not seem to impair the effect of the treatment. In fact the combinatorial approach (phage - $U3_{25\%}$) was equally effective in young and mature biofilms and for both species of the consortium. The antibiofilm effect of the combination $U3_{50\%}$ + PAO1-D + EC3a in dual species biofilms was more pronounced for *E. coli* ($p < 0.05$) than the effect observed in monospecies biofilms. Since the concentration of the phage in all solutions tested was kept constant (1×10^9 PFU/mL) and the growth of *E. coli* in dual species biofilms inferior than in monospecies biofilms, the MOI was considerably higher (approximately 10 to 100-fold) than in

monospecies biofilms. The lower concentration of *E. coli* in mixed biofilms has been reported[228,229] and has been attributed to bacterial competition[228], faster growth of *P. aeruginosa* and its eventual production of virulence factors[230] .

5. MAIN CONCLUSIONS AND FUTURE PERSPECTIVES

The role of biofilms in failure of medical devices and chronic inflammatory conditions is increasingly being recognized. Biofilms are of difficult control and eradication, not only because this physiology confers them a protected mode of growth, but also due to the intensification of bacterial resistance to multiple drugs. Bearing in mind this issue, this work evaluated the applicability and effect of combining phages and Portuguese honey to control *P. aeruginosa* and *E. coli*, two prolific biofilm formers in wound related biofilm infections.

Phages specificity and self-replicating nature are attractive characteristics that not all antimicrobial agents possess. The first task of this work consisted in the isolation and characterization of *P. aeruginosa* phages. Phage lytic profiles against clinical isolates, holding depolymerase activity and antibiofilm efficacy were the characteristics taken into account to select the phage to be used in the further experiments. Also, the selected *P. aeruginosa* and *E. coli* phages, PAO1-D and EC3a respectively, were further characterized regarding growth cycle characteristics that showed higher bursts in EC3a phage compared to PAO1-D phage. Phage particles were analyzed by TEM and revealed that both belong to the *Caudovirales* order and are from the *Podoviridae* (PAO1-D) and *Siphoviridae* (EC3a) families. Overall phages demonstrated good antibacterial efficacy for treatments of shorter duration. Phage resistance and changes in bacterial phenotype and genotype may be influencing the effectiveness in long treatment periods.

Honey was the second agent considered and the antibacterial efficacy of two Portuguese honeys was studied. Although both presented the same MIC value (25% (w/v)) for planktonic cultures, different antibiofilm effects were observed. U3 was more effective, probably due to its higher content in the MGO molecule. Antibiofilm assays showed that, generally, honey was effective for treatments of longer duration, which can be, not only a consequence of the non-development of resistant strains, but also to the possible sequential effect of the several constituents of the honey, namely its main sugars (glucose and fructose), MGO and hydrogen peroxide.

The combinatorial approach included the use of honey at two concentrations - 25% (w/v) and 50% (w/v) - together with each host specific phage. For phage and honey at 25% (w/v), a synergistic effect was observed for *P. aeruginosa* biofilms after 24 h; and for *E. coli* biofilms an additive effect was perceived 12 h after administration. No synergism was, however, observed with honey at 50% (w/v) and phage most likely due to the rapid destruction of phages by honey and to the strong antibiofilm capacity of honey itself that is sufficient to cause high cell reduction rates. Additionally, in *E. coli* biofilms, although phage alone had no effect at 24 h of treatment, the same did not occur when phages were combined with honey. These results, obtained by viable cell counts, were confirmed by flow cytometry where

cytograms showed that the simultaneous application of the two different agents allowed, in one hand, phage resistant cells to be targeted by honey and, on the other hand, cells where honey didn't act to be targeted by phages. The lower osmotic pressure with honey at 25% (w/v) did not immediately disrupt the cellular membrane fully allowing phages to bind to their receptors and replicate within the cell. Additionally, flow cytometry complemented with the CFU counts, showed that compromised cells were no longer cultivable possibly because the cells were already manufacturing new phage particles or in early stages of lysis.

Overall, the work developed reveals that the activity of honey at 25% (w/v) is enhanced by the addition of phages when applied to 24 h and 48 h old biofilms. This result reinforces the potential use of both antimicrobials together, taking advantage of honey's antibiofilm activity, and of the phages ability to lyse specific bacteria. Furthermore, it was verified that treatment efficacy was not affected by the consortium of species, which gives support to its use in typical wound environment.

Future work can involve several approaches such as:

- inclusion of other species in the study such as *A. baumannii*, to better evaluate the behavior and response of the consortia to the treatment;

- evaluation of the effect of honey and phages on *P. aeruginosa* cell structure, by microscopic techniques, such as TEM and SEM, to gain more insights of their mechanisms of action and possible adaptations in order to improve antibacterial activity;

- characterization and antibacterial evaluation of more Portuguese honeys and evaluation of the honeys used in this study regarding their content in hydrogen peroxide;

- performance of *ex vivo* and *in vivo* evaluation to gain clinical relevance for the potential application of these agents for wound management.

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