Universidade do Minho
Escola de Engenharia

Jéssica Carolina da Silva Sousa

Evaluation of the antimicrobial activity of a bacteriophage-honey formulation in an *ex vivo* skin model

Dissertation for the Master Degree

Integrated Master in Biomedical Engineering

Clinical Engineering

Supervisors

Doctor Sanna Maria Sillankorva

Doctor Ana Cristina Afonso Oliveira

October 2017

ACKNOWLEDGMENTS

To my supervisors, Dr. Sanna Sillankorva and Dr. Ana Oliveira I want to thank this opportunity and all the support and knowledge transmitted along the year. To Dr. Sillankorva I want to express my appreciation for the availability and excellent guidance provided. To Dr. Oliveira, I want to thank the encouragement and advices that kept me motivated. I believe that this year was essential for my overall growth as a biomedical engineer and I have mainly you both to thank.

To all my colleagues of the Bacteriophage and Biotechnology Group (BBiG) who were always available to help me, especially in the beginning of this journey. As so, I want highlight the future Doctor, Henrique Ribeiro, whom I thank dearly all the knowledge, help with the protocols, and friendship. I also want to acknowledge Daniela Silva for the help with the DLS and Célia Rodrigues for the help with the RP-HPLC.

To my fellow master students in the Center of Biological Engineering (CEB), I want to thank sincerely for the support, motivation, help, and companionship. I have to acknowledge the LMA master girls, Alice Ferreira, Catarina Silva, and Rute Ferreira for the friendship and help. Also, to Ana Catarina Antunes, Hugo Dinis, my roommate Inês Silva, and every other friend that I do not mention here by name but was equally there for me I want to thank every advice, suggestion, and support.

To all my family, who are the most important people in my life, I want to thank all the motivation, which is so important for the achievement of my overall goals. I have to mention my parents for the financial and emotional support. I know that it was not easy and I sincerely appreciate everything that you have done for me and for my education. To my little brother, my baby boy, for simply being the best gift that I ever had.

Finally, I would like to generally thank everybody who was present in this period of my life for the support and for turning this experience much more fulfilling.

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte and the Project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462) and the project PTDC/CVT-EPI/4008/2014 (POCI-01-0145-FEDER-016598).

Co-funded by:

ABSTRACT

Nowadays, the main treatments used on infected chronic wounds are antibiotics. However, the incorrect and overuse of these led to the appearance of antimicrobial resistances (AMR). In fact, along time multiple bacteria acquired the ability to express properties that help them to engage in a sessile community environment in order to protect themselves from external factors. These communities are called biofilms and are known to be tolerant to biocides and to the host immune response. As so, it is important to find different strategies. This study proposes a bacteriophage-honey combination due to their potential already verified when used separately. The effect of the bacteriophage-honey formulations was tested using both *in vitro* in polystyrene plates and *ex vivo* using pig skin explants against the pathogenic bacterial strains *Escherichia coli* and *Pseudomonas aeruginosa*. The pig skin model was used since it is anatomically similar to human skin. The Portuguese honey C1 with floral origin from *Castanea sativa* revealed to be sufficient to eradicate completely the *E. coli* biofilm cells *in vitro* when used at 50% (w/v) concentration. In *ex vivo* experiments, honey alone did not demonstrate such high efficacy. In fact, 25% (w/v) concentrated honey resulted in similar biofilm cells reduction effects as 50% (w/v), and a maximum biofilm reduction (2 log) was achieved for both concentrations when combined with phage. The commercial Manuka honey was also tested against *E. coli* biofilms as a term of comparison, and generally, statistically similar results to C1 for both *in vitro* and *ex vivo* experiments were obtained. For *P. aeruginosa* biofilm cells formed *ex vivo*, the maximum reduction observed, 2.4 log at 12 h, was obtained with 50% (w/v) C1 together with bacteriophage. This experiment suggests that the surface influences the biofilm structure, leading to different bacteria-surface interactions, and consequently to different responses to the used treatment. Both strains seemed to have benefited from the presence of the other in dual-species biofilms, resulting in a synergistic effect when honey was combined with bacteriophage in the case of *P. aeruginosa*. In fact, it was obtained a maximum reduction of 3.1 log for *P. aeruginosa* after 12 h and 1.8 log for *E. coli* at 24 h when 50% (w/v) C1 was combined with bacteriophage. The synergy is believed to be due to honeys capacity to damage the bacterial cell membrane, promoting the subsequent bacteriophage infection.

Key words: chronic wounds, biofilms, bacteriophage-honey, pig skin, synergy.

RESUMO

Atualmente, os principais tratamentos utilizados em feridas crónicas infetadas são antibióticos. No entanto, o uso incorreto e excessivo destes levou ao aparecimento de resistências antimicrobianas (RAM). De facto, ao longo do tempo, múltiplas bactérias adquiriram a capacidade de expressar propriedades que as permitem envolver-se num ambiente comunidade séssil de modo a se protegerem de fatores externos. Essas comunidades chamam-se biofilmes e são conhecidas por serem suscetíveis a biocidas e à resposta imune do hospedeiro. Desta forma, é crucial encontrar diferentes estratégias. Este estudo propõe uma combinação de bacteriófagos-mel devido ao potencial de cada um já verificado quando usados separadamente. O efeito de várias formulações de bacteriófagos-mel foi testado utilizando modelos *in vitro,* em placas de poliestireno, e *ex vivo,* utilizando explantes de pele de porco, contra as estirpes bacterianas patogénicas *Escherichia coli* e *Pseudomonas aeruginosa*. O modelo de pele de porco foi usado, sendo esta anatomicamente mais semelhante à pele humana. O mel português C1 com origem floral de *Castanea sativa* revelou ser suficiente para erradicar completamente as células de biofilme de *E. coli in vitro* quando utilizadas a uma concentração de 50% (p/v). Em testes *ex vivo*, o mel sozinho não demonstrou a mesma eficácia. De facto, o mel concentrado a 25% (p/v) resultou em efeitos de redução de biofilmes semelhantes a 50% (p/v) e foi obtida uma redução máxima de biofilme (2 log) para ambas as concentrações quando combinadas com fago. O mel comercial Manuka também foi testado contra os biofilmes de *E. coli*, sendo que este mel foi usado como um termo de comparação, e, em geral, foram obtidos resultados estatisticamente semelhantes a C1 para ambos os testes *in vitro* e *ex vivo*. Para células de biofilme de *P. aeruginosa* formadas *ex vivo*, a redução máxima observada, 2.4 log às 12 h, foi obtida com 50% (p/v) de C1 juntamente com bacteriófagos. Este estudo sugere que a superfície influencia a estrutura do biofilme, sendo que esta leva a diferentes interações bactérias-superfície e, consequentemente, a diferentes respostas ao tratamento usado. No caso dos biofilmes de duas espécies, ambas as estirpes parecem ter beneficiado com a presença uma da outra, sendo que se notou um efeito sinérgico quando o mel foi combinado com bacteriófagos, no caso da *P. aeruginosa*. De facto, foi obtida uma redução máxima de 3.1 log para a *P. aeruginosa* após 12 h e 1.8 log para *E. coli* às 24 h quando 50% (p/v) C1 foi combinado com bacteriófagos. Acredita-se que a sinergia se deva à capacidade do mel para danificar a membrana celular bacteriana, promovendo a subsequente infeção por bacteriófagos.

Palavras-chave: feridas crónicas, biofilmes, bacteriófagos-mel, pele de porco, sinergia.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

Scientific article

Oliveira, A.*, Ribeiro, H.*, Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S. (2017). Synergistic antimicrobial interaction of honey and phage in *Escherichia coli* biofilms. Frontiers Microbiololy (submitted) *Equally contributing authors

Poster Presentations

Oliveira, A., Ribeiro, H., Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S.* (2017). Synergistic antimicrobial interaction of honey and phage in *Escherichia coli* biofilms. 22nd biennial Evergreen Bacteriophage conference, 6-11 August 2017, Olympia, Seattle, USA. (Accepted poster) *oral speaker

Ribeiro, H., Oliveira, A., Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S*. Synergistic antimicrobial interaction between honey and phage against Escherichia coli biofilms. CEB Scientific Annual Meeting 2017, Braga, 6 July 2017. *oral speaker

Ribeiro, H.*, Oliveira, A., Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S. Synergistic antimicrobial interaction between honey and bacteriophage against E. coli biofilms. 3rd International hands-on Phage Biotechnology course, Braga, 19-23 June 2017. *oral speaker

Ribeiro, H.*, Oliveira, A., Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S. Synergistic antimicrobial interaction between honey and bacteriophage against Escherichia coli biofilms. I Simpósio em Bioquímica Aplicada, Braga, 31 May 2017. *oral speaker

Ribeiro, H.*, Oliveira, A., Silva, A. C., Silva, M. D., Sousa, J. C., Rodrigues, C. F., Melo, L. D. R., Henriques, A. F., Sillankorva, S. Synergistic antimicrobial interaction of honey and phage in Escherichia coli biofilms. X ENEBIOQ conference, Braga, 7-10 April 2017. *oral speaker

LIST OF FIGURES

2. General Introduction

3. Materials and Methods

4. Results and Discussion

Annexes

LIST OF TABLES

2. General Introduction

3. Materials and Methods

4. Results and Discussion

Annexes

List of Abreviations and Symbols

3D	Three-dimensional
ζ-potential	Zeta Potential
AMR	Antimicrobial Resistance
ANOVA	Analysis of variance
BBiG	Bacteriophage Biotechnology Group
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
c-di-GMP	3,5-cyclic diguanylate
CEB	Centre of Biological Engineering
CFU	Colony Forming Unit
DHA	Dyhydroxyacetone
DNA	Deoxyribonucleic Acid
ECM	Extacellular Matrix
EPS	Extracellular Polymeric Substances
EPM	Extracellular Polymeric Matrix
FAS	Ferrous Ammonium Sulfate
HMF	5-hydroxymethylfurfural
LCWB	Lubbock Chronic Wound Biofilm
LOD	Limit Of Detection
LPS	Lipopolysacharides
MBEC	Minimal Biofilm Eradication Concentration
MGO	Methylglyoxal
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
OD	Optic Density
OPD	O-phenylenediamine
PEG	Polyethylene glycol
PES	Polyethersulfone
PFU	Plaque Forming Unit

QS	Quorum Sensing
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase – High Pressure Liquid Chromatography
rpm	rotation per minute
SD	Standard Deviation
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UMF	Unique Manuka Factor
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
v	volume
w	weight

1. Motivation and aim of the thesis

Chronic wounds are one of the main worldwide health problems, being differentiated from an acute wound by the fact that they remain open for extended periods of time. As so, chronic wounds fail to proceed in the several healing stages, stagnating in a permanent inflammatory state. Nevertheless, chronic wounds are more likely to be infected and colonized by several bacterial species, *P. aeruginosa* and *E. coli* being alongside with *S. aureus* as the three main bacteria that are able to infect a wound.

In medical facilities, it is often recommended the use of antibiotics or antiseptics to treat an infected chronic wound. The incorrect and overuse of antibiotics is the main cause of the development of an Antimicrobial Resistance (AMR).

Nowadays, current advances in wound care have focused on finding new treatments for wound healing. Thus, the combination of bacteriophages with honey could be an interesting option. Bacteriophages have several advantages comparing to conventional antimicrobials, such as higher specificity, diversity, easier and rapid isolation, lower production cost, and innocuity to mammalian cells. Considering honey, the low associated costs, the absence of side effects, and its antimicrobial activity are some favorable properties that encourages it use.

The main goal of this project was to develop a bacteriophage-honey formulation that could eradicate and prevent the formation of *E. coli* and *P. aeruginosa* biofilms. Also, it was important to evaluate if the combinatorial treatment approach could have beneficial (additive or synergistic) or antagonistic antimicrobial interaction.

To achieve more reliable results, an *ex vivo* skin model (anatomically more similar to human skin) was optimized and the results were compared to those obtained *in vitro*.

2. General Introduction

2.1. Chronic wounds

According to the Centers for Medicare & Medicaid Services (CMS), it is estimated that in the United States (US) chronic wounds have an annual health care cost superior to $25 billion, involving more than 6.5 million patients [1–3].

Being the interface between the environment and the body, the skin represents an important role on the protection of underlying tissues, among other functions. These functions are performed by three distinct layers: epidermis, dermis and a subcutaneous inner layer [4]. Thus, when skin suffers an injury, the human body responds immediately by releasing blood plasma and peripheral blood cells into the wound site, process nominated by homeostasis. Then, molecules are released and collagen is exposed to the blood vessel wall [5], and the inflammatory response begins when invaders pathogens are neutralized and local debris removed. Therefore, there are four phases, which can overlap in time, involved in the process of a normal cutaneous wound healing. Besides homeostasis and that initial inflammatory response there are also two other phases – a proliferative phase and a final remodelling phase [2,4,6–8]. It is known that if a wound stagnates in a permanent inflammatory state and fail to proceed the healing process, remaining open for extended periods of time, it is diagnosed as a chronic wound [2,9]. Chronic wounds are characterized by the constant and excessive infiltration of macrophages and neutrophils [2,6,9]. The causes of this accumulation are not entirely understood yet and probably depend on several factors. This phenomenon will lead to the release and consequential increase on the production of collagenases, proteases and reactive oxygen species (ROS), and subsequently to the degradation of the extracellular matrix (ECM) and healing mediators. Furthermore, the formation of the ECM and new epithelia would be hampered and the wound could not cure by itself [2,6]. The ECM is a non-cellular part of all tissues and organs, including skin, that provides structural support to the surrounding cells [10]. Poor vascularization is another factor that characterizes the appearance of chronic wounds, contrarily to a normal cutaneous wound, where the increase of the number of blood vessels promotes the delivery of oxygen and nutrients [2].

Thus, a trauma is defined as an acute wound. If this type of injury is combined with clinical complications, such as diabetes, is more prone to be colonized by several pathogenic bacteria leading to the formation of a chronic wound, also nominated as ulcer (Figure 2.1) [9,11].

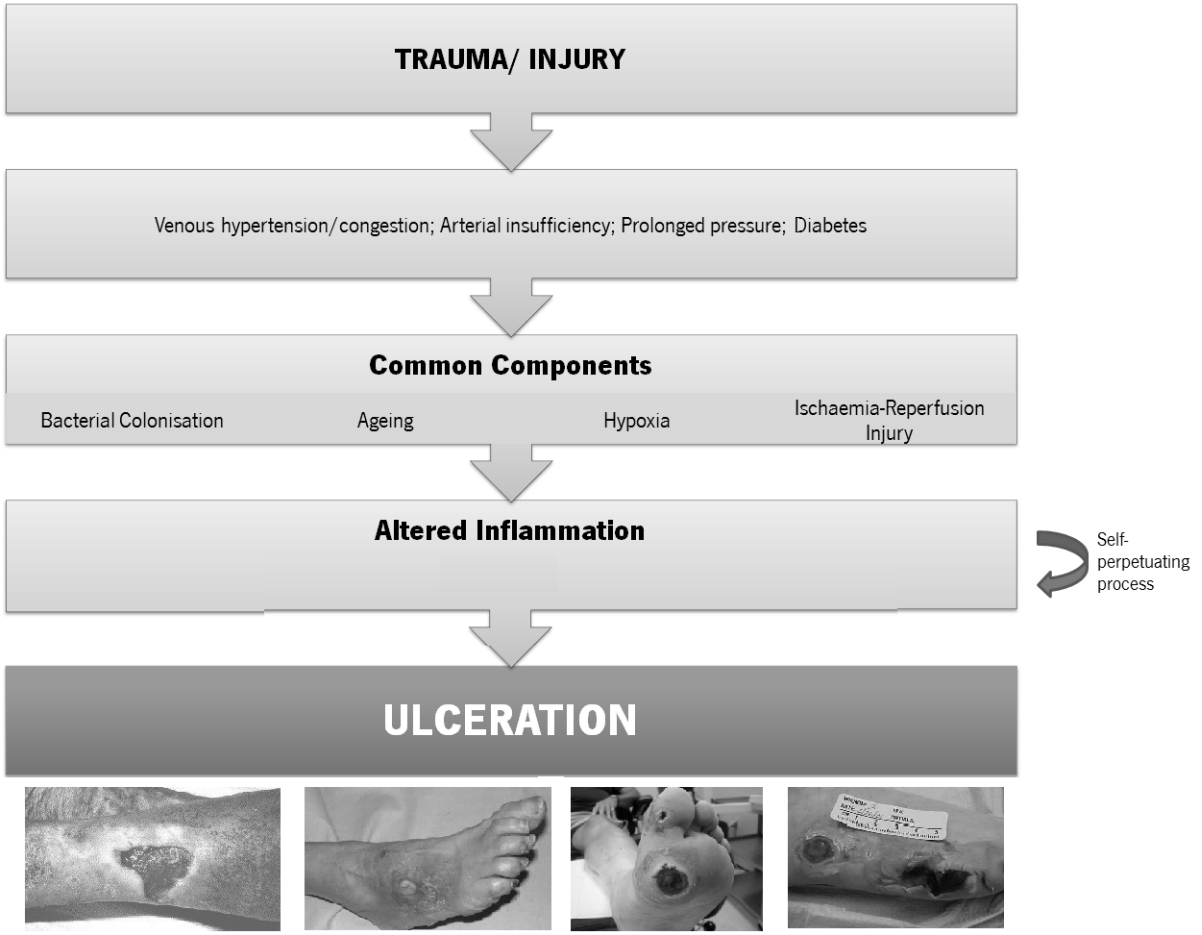


Figure 2.1 – Schematic representation of the formation of a chronic wound, being represented the role of the self-perpetuating inflammation process. Several factors can delay wound healing, turning a cutaneous injury into a chronic wound, these factors being chronic medical conditions, vascular insufficiency, diabetes, malnutrition, ageing, and bad habits. Also, local factors such as pressure, infection, edema, between others. The subsequent tissue damage locks the wound in a prolonged and heightened inflammatory state. Adapted from [9,11–15].

2.1.1. Types of chronic wounds

There are several systemic and local factors that contribute to the formation of a chronic wound. The systemic factors are age, malnutrition, obesity, chronic medical conditions, and factors derived from the misuse of medications and from bad habits, such as smoking. On the other hand, examples of local factors are neuropathy or pressure, infection of an acute wound, necrotic tissue in the wound bed, excessive wound tension, unfavorable local environment, inappropriate treatment, malignant wound, repetitive trauma, and radiation [11,16].

Furthermore, it could be defined different types of chronic wounds, the most common are arterial ulcers, venous ulcers, diabetic ulcers, and pressure ulcers [2,17,18]. Arterial ulcers are more usual in patients with hypertension, atherosclerosis, and thrombosis. These ulcers are characterized by low

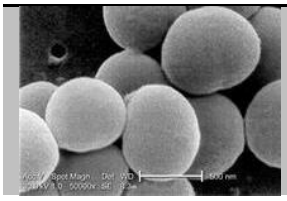
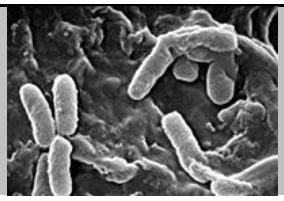
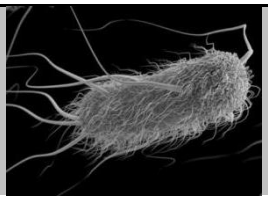
blood supply, which leads to an ischemic state. On the other hand, venous ulcers are related with deep vein thrombosis and varicose veins [2]. It is noticed that, in the US, the annual health care cost of a venous ulcer is between $2.5 and $3 billion [19]. People with diabetes mellitus are more likely to develop several complications, such as ischemia and neuropathy, complications that could lead to the formation of a diabetic ulcer [2]. According to several studies, it is estimated that diabetic neuropathy affects more than 50% of people with type 2 diabetes [20–23]. When combined with reduced blood flow and if not treated, neuropathy in the feet increase the chance of foot ulcers [24]. Diabetic foot ulcers are responsible for about 40% of all non-traumatic amputations [25], being expected that 10 to 15% of these would eventually lead to limb amputation [26,27], with half of the amputees dying within 5 years [27]. In Europe, it is speculated that there are between 1.0 to 1.4 million diabetic foot ulcers and 0.5 to 1.3 million leg ulcers [28]. Finally, pressure ulcers occur mostly in vulnerable people and are due to persistent pressure and friction from the patient weight over a specific zone. This pressure could lead to damage of skin and consequent formation of a chronic wound [2]. In the US are reported 2.5 million pressure ulcers that are treated per year only in acute care facilities, noticing that the cost for the treatment of one pressure ulcer only is about $70 million. Annually, the total health care cost of pressure ulcers, in the US, are approximately $11 billion [2]. About 60 million deaths happen per year due to complications of nosocomial pressure ulcers (ulcers acquired in a medical facility) [1,2].

There are four stages that can differentiate ulcers [29]: stage I and II are mainly superficial where stage I ulcers appear as a defined area of persistent redness in lightly pigmented skin (red, blue and purple in darker skin tones) and stage II already involve abrasion, blister or shallow crater; stage III ulcers exhibit an involvement of cutaneous and subcutaneous tissue; and stage IV show necrosis of muscles, bones, joints, and surrounding tissues [2,29,30]. Debridement of ulcers is required, and normally recommended in an operating room for stages III and IV ulcers [31]. Bacterial colonization is promoted in cases that the immune response and healing is debilitated due to loss of skin and poor circulatory conditions [32].

2.1.2 Pathogens in chronic wounds

Usually lesions can be infected with several types of microorganisms (table 2.1), which is one of the local factors that can lead to a chronic wound [16].

Table 2.1 – Examples of bacteria that are prevalent on infected wounds [33-37].

Staphylococcus aureus (*S. aureus*)	***Pseudomonas aeruginosa*** (*P. aeruginosa*)	***Escherichia coli*** (*E. coli*)	***Acinetobacter species***
			
Prevalence:	**Prevalence:**	**Prevalence:**	**Prevalence:**
93.5%	52.2%	32.6%	13.0%

Thus, it is observed that *E. coli* and *P. aeruginosa*, along with *S. aureus* are examples of the major pathogenic organisms that are able to infect a wound [37]. *E. coli* is a facultative anaerobic, Gram-negative, rod-shaped bacterial species and frequently involved in urinary, respiratory, and gastrointestinal tract infections [38,39]. On the other hand, *P. aeruginosa*, also a Gram-negative rod-shaped bacterial species, can grow both in aerobic and facultative anaerobic conditions. *P. aeruginosa* thrives when the host's normal immune defense is incapacitated, being an ubiquitous opportunistic pathogen that frequently appears in lung diseases, such as cystic fibrosis and chronic obstructive pulmonary [32,40]. Both of these organisms are a major concern in burn and chronic wounds, and highly resistant to antibiotics [32].

2.2. Biofilm Formation

In 1978, Bill Costerton presented the first definition of biofilms stating that biofilms were single cells and microcolonies, all embedded in a highly hydrated, predominantly anionic extracellular polymeric matrix (EPM) [41]. Since then biofilm science and technology has been an interesting and active field. Several bacteria have shown the potential to grow in a sessile microbial community attached to a substratum. Biofilms are tolerant to biocides, antimicrobial agents, and also show resistance to the immune defense response of the host [42,43]. Biofilm-associated organisms tend to produce extracellular polymeric substances (EPS) [44], such as polysaccharides, proteins, and nucleic acids. These substances provide to the biofilm a structural support, forming the EPM [45].

Furthermore, the formation of a biofilm entails several adhesion and dispersion events (Figure 2.2) that occur since the initial surface adhesion to the biofilm maturation. These events can be built by multiple

microbial interactions, which might not depend on growth-inhibiting molecules and/or processes. The first step to the formation of a biofilm is the bacterial attachment to the surface, noticing an initial reversible attachment followed by an irreversible strong one. The surface profile is one of the most determinant factors in this stage. The roughness and other minute flaws have a direct influence. Besides, the physiochemical properties of the surface also display an important role to the bacterial attachment. In their natural environment, microorganisms do not adhere directly to the surface but to the conditioning film, which provides nutrients to the surface, modifying its properties. To link the surface to the EPM, a few cell adhesins are activated in the bacteria [46].

Nevertheless, the biofilm development and maturation are related to determining factors such as Quorum Sensing (QS), mobility, and hydrophobicity [46,47]. Thus, QS permits the organism to sense the density of their own community, being a mechanism of intercellular communication [18,47]. After the bacterial adherence to the surface, the expansion begins and also the eventual recruitment of new microorganisms. There are physiochemical factors that characterize the influence of EPS, having an impact on the physical behavior of biofilm, such as diffusion and fluid frictional force. To form an irreversible attachment it is secreted a type of extracellular plastering substance. Also, the chemical and physical characteristics of EPS vary according to the type of bacteria, if the bacteria are Gram-negative or Gram-positive[46].

It could be counted different types of biofilm detachment, such as sloughing, erosion, and seed dispersal [47]. Generally, the dispersion process occurs due to the microcolony size but it is also related to a decrease of the levels of nutrient and other environmental cues. The detachment step begins with a spatial differentiation, according to motility. This mechanism promotes the degradation of the EPM and autolysis of a biofilm subpopulation [46,47].

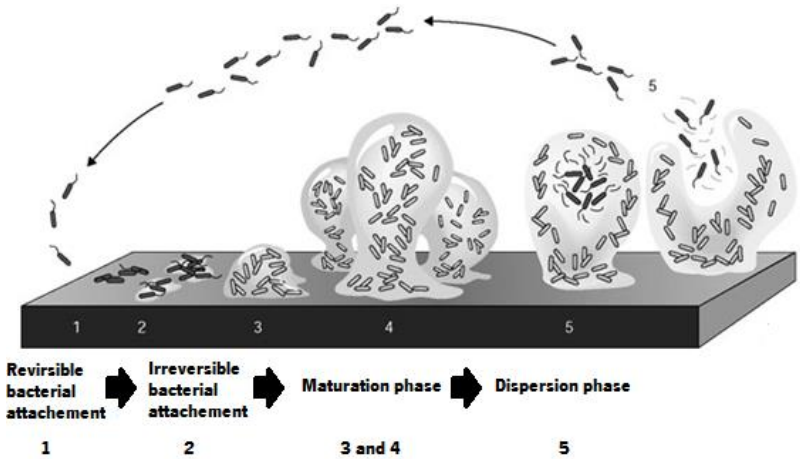


Figure 2.2 – Schematic representation of the biofilm formation process. On this process the bacterial attachment becomes irreversible due to the action of EPS as the bacteria lose their flagella-driven motility. Next it is achieved the first maturation phase and then the second maturation phase that points to a fully mature biofilms. Finally, the dispersion/detachment stage begins when single motile cells disperse from the microcolonies. Adapted from [43,45].

2.2.1. Biofilm formation by *E. coli* and *P. aeruginosa*

Both *P. aeruginosa* and *E. coli* are reported to be examples of motile bacteria having a competitive advantage, as they are able to use flagella in order to overcome hydrodynamic and repulsive forces, which is important for the initial attachment [48,49]. Besides this common property, other factors have been studied and demonstrated to be important in the formation of the biofilms of these pathogens.

E. coli is a known biofilm forming microorganism with most virulence traits being adhesins, such as Type I fimbriae (or pili), and siderophore receptors [39]. In general, different proteins may increase the transition from a reversible to an irreversible stage of cell adhesion where in *E. coli*, this is attributed to both lipopolysaccharide (LPS) and pili [50]. Furthermore, it has been demonstrated that *E. coli* cells attachment leads to a decrease in outer protein membranes, such as the outer membrane protein X (OmpX) that is known for the promotion of bacterial adhesion and for its role in the resistance against attack by the human complement system [51,52]. Also, this attachment promotes the production of EPS, altering the bacterial susceptibility [52]. The EPM constituents formed in the case of *E. coli* biofilms still require study, although cellulose was the first component identified, and was later shown to be expressed with curli in several *E. coli* isolates. Curli are amyloid fibers that are critical for the formation of pellicle biofilms [53].

Besides the use of flagella, *P. aeruginosa* is also provided with Type IV pili to mediate twitching motility in order to wade through the liquid interface, maintain adherence, and move through the attachment surface [48]. *P. aeruginosa*, is able to adjust its predisposition, whether it is in planktonic or biofilm form, and control the secretion of virulence effectors by a plethora of transcription factors, two-component systems, non-coding Ribonucleic acids (RNAs), and QS networks [54]. Moreover, *P. aeruginosa* survival in a human host at early stages of infection is supported by the secretion of toxins and virulence factors, the production of these being lower later [40]. The QS mechanisms of this microorganism have been highly studied during past years, and it has been concluded that they have a very early effect on the *P. aeruginosa* biofilms development [55]. Besides, it is known that 3,5-cyclic diguanylate acid (c-di-GMP) is a molecule that represents *P. aeruginosa* intracellular signaling system, being a major contributor to bacterial adherence, as it has been found higher levels of c-di-GMP when cells are in a sessile community environment [54,56]. As for *P. aeruginosa* EPM composition it has been extensively studied through the years, being suggested that it varies according to environmental conditions. The two primary EPS components are known to be the Pel and Psl polysaccharides [57].

Thus, *P. aeruginosa* strains are, in general, harder to treat because of its intrinsic, adaptive, and acquired resistance mechanisms, which result in a low susceptibility to an extended range of antibiotics.

2.2.2. Chronic wound biofilm models

The method most commonly used for the study of biofilms *in vitro* consists in the use of microplates where biofilms are formed in the bottom and sides of the wells [58]. There are several advantages for the use of microtiter plates, such as the fact that it is inexpensive, reproducible, and there is no need for advanced equipment besides the plate reader. However, during the washing steps of the wells to remove planktonic bacteria, the loosely attached biofilm may not be measured correctly [58]. To overcome some of the disadvantages of the use of the microtiter plate, such as the fact that using microtiter plates part of the biomass quantified may not stem from the biofilm formation process [58,59], a new microplate in which the biofilms are formed in the 96 pegs of the lids was developed (MBEC device, previously known as Calgary Biofilm Device). Besides being used in biofilm studies, this device can also be used to analyze the Minimal Biofilm Eradication Concentration (MBEC) towards different agents, such as antibiotics [59]. In 2008, Sun et al. developed the first chronic wound biofilm model *in vitro*, named Lubbock Chronic Wound Biofilm (LCWB). According to the authors, the LCWB model is more realistic and simulates the functional characteristics of chronic pathogenic biofilms [60]. Later, in 2010 another *in vitro* method was developed by Werthén, the bacteria aggregates in a collagen gel matrix with serum protein. The goal of this method is also to mimic the wound bed of chronic wounds, creating a more realistic model [61].

For the simulation of the formation of bacterial biofilms on wounds it has also been developed several *ex vivo* models, in order to achieve more reliable results. It is noticed that porcine skin is anatomically more similar with human skin, exhibiting an homologue wound healing process [62]. Analyzing the similarity of different studies, a review of several wound studies showed that the concordance between results obtained using pig skin models and human skin models is 78%, in opposed to 53% for rodents and 57% for *in vitro* studies [63]. *In vivo* models have several limitations, such as the fact that they are expensive, time consuming, and raise ethical issues. Thus, *ex vivo* models could be the most interesting option, Yang et al. developed an *ex vivo* pig skin model that combines advantages of both *in vivo* and *in vitro* models. This method guarantee the formation of a biofilm that resembles more to the biofilms developed in a real chronic wound. As so, in this study, for an optimal method of sterilization of the

porcine skin, the authors produced chlorine gas, which also allows the preservation of the properties of both epidermis and dermis [1].

2.3. Current Treatments used on Chronic Wounds

To treat an ulcer, firstly, the necrotic tissue is removed through debridement, eliminating the damaged tissue, and reducing the risk of infection [4,11,64]. Different types of debridement can be selected, surgical, enzymatic, autolytic, mechanical, and biological (table 2.2) [65].

Table 2.2 – Description of the different types of debridement. Adapted from [65].

Types of debridement				
Surgical and Sharp	**Enzymatic**	**Autolytic**	**Mechanical**	**Biological**
In an opperating room	Devitalized tissue removed by additional enzymes that digest and dissolve	Devitalized tissue naturally removed by the body's defense mechanisms	Devitalized tissue removed by physical force	Devitalized tissue removed by the use of the sterile larvae of *Phaenicia sericata*

Then, according to the characteristics of the wound and in order to maintain moisture balance different types of dressing are selected [4,11]. The methods used to treat a wound can diverse according to the guidelines of the clinic. A wound that promotes the synthesis of exudate requires an absorptive type of dressing and, on the other hand, a dry wound needs to be hydrated. The type of dressing usually changes during the process, being important that the wound remains moist in order to stimulate the epithelialization, regeneration of the tissue and consequent wound healing [4 4,66]. In general, the different types of dressings that are available are: low adherent dressings, semipermeable films, hydrocolloids, hydrogels, and alginates [67]. Furthermore, skin grafts (a piece of unharmed skin of the patient or donors relocated to the site of the injury) are able to protect the wound and can be used in a more advanced stage after the wound already exhibits enough granulation tissue [4].

Nowadays, the treatment used on an infected wound is selected according to the stage of the microbial progression. As so, on an initial stage of contamination of the wound it is only done preventive observation, especially in the case of a diabetic patient. Further, on a stage of critical colonization, the therapy used involves topical antiseptics [68]. These agents cannot be used systematically, being provided

with more than one mechanism of action and their bactericidal activity occurs faster due to being applied topically [69]. On a more advanced stage, after the appearance of systemic complications it is then used systemic antibiotics and topical antiseptics [68]. It is suggested that at an early stage of infection it is more common to find Gram-positive bacteria, and afterwards, as the infection progress, the Gram-negative bacteria start to appear. In fact, a study by Fazli et. al (2009) showed that *S. aureus* adhere mainly to the site of ulcers closest to the surface, whereas *P. aeruginosa* colonizes the deeper regions of these wounds [70]. Thus, the known or probable infecting microorganism(s) as their predisposition are the main factors taken into account for the selection of the antibiotic treatment.

According to the British Society for Antimicrobial Chemotherapy (BSAC) and European Wound Management Association (EWMA), up to 50% of all antibiotics treatments are not needed or the prescription is inappropriate. Generally, this overuse of antibiotics is the main cause of the development of an antibiotic resistance, being one of the main worldwide health problems [71]. The World Health Organization (WHO) defines an AMR as the "resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive" [72]. As so, current advances in wound care have focused on finding new treatments for wound healing.

Antibiotics have been used for biofilm control and studies have suggested using more aggressive antibiotic treatments at early biofilm stages, as mature biofilms are harder to eradicate [73].

2.4. Bacteriophages Therapy

It is understood that bacteriophages are the most common and diversified organisms that exist in the world, being considered promising options for the therapy of bacterial diseases since Félix d'Hérelle pointed them as antimicrobial agents in 1917 [74–76]. Later in 1934, Eaton and Bayne-Jones led a study that conclude that body fluids could eliminate or inhibit bacteriophages before they reach the bacteria cell [77]. Besides, in 1929, Alexander Flemming published the discovery of penicillin [78], an antibiotic with high therapeutic potential, which became the main treatment in World War II [79]. Further researches developed in 1940 by Howard Florey and Ernst Chain conclude, after *in vivo* tests in mice, rats, and cats, that penicillin is active against the bacterial organisms tested [80]. Thus, the idea of using bacteriophages for therapeutic purposes started to be dropped and antibiotics became more interesting for the control of bacterial diseases, mainly due to the fact that they were cheaper and effective. However, bacteriophages were still used in Poland and the Soviet Union. Afterwards, due to the

inadequate and excessive use of antibiotics, the appearance of multidrug-resistant bacteria started to increase abruptly [75,76].

Bacteriophages, also called phages, are viruses that infect exclusively bacteria, injecting its genetic material and using the host machinery to express their own proteins in order to replicate inside the cell with subsequent new phage release (Figure 2.3). Phages infect bacterial cells whether they are in planktonic (suspension) or in biofilm form. Also, according to their life cycles (lytic or lysogenic), phages can be divided into two different groups: virulent phages, which life cycles are strictly lytic, and temperate phages, that can follow the lysogenic cycle. Lytic phages are the ones that can be interesting for therapeutic purposes. It is known that this type of phages start their action by adsorbing to the surface of the host cell and then inject and replicate their DNA (Deoxyribonucleic acid), inducing the lysis of the host cell. Then, there are released progeny phages that will allow the beginning of another round of infection. On the other hand, temperate phages, usually, integrate their genome into the host chromosome or sometimes maintain it as a plasmid which is transmitted by cell division to the daughter cells (Figure 2.3) [75,81].

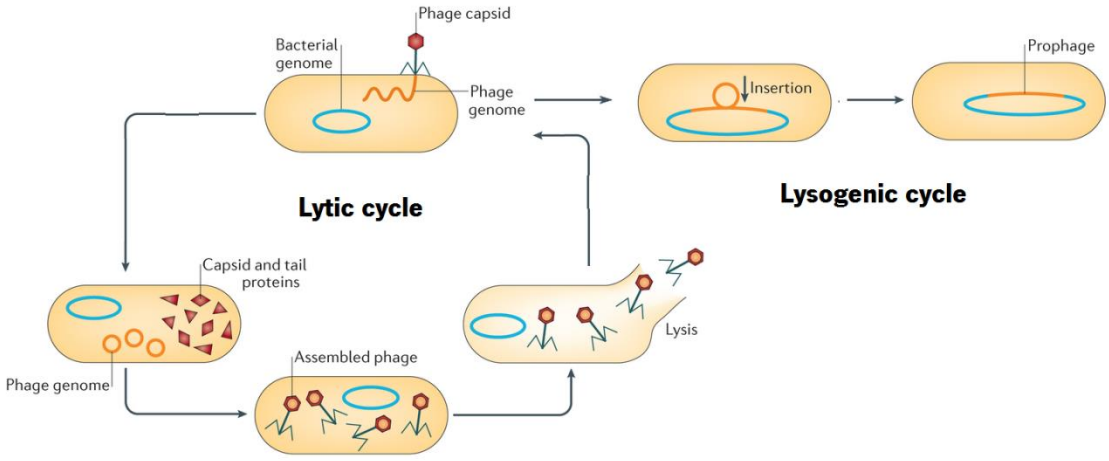


Figure 2.3 – Schematic representation of both lytic and lysogenic life cycles of phages. In case of lytic phages, phage capsid and tail proteins are produced. Also, the phage genome is replicated, inducing the lysis of the host, and then compacted into progeny phage particles, which are released after the lysis, initiating another round of infection. On the other hand, on a lysogenic cycle the genome of the phage integrates the chromosome of the host, becoming a prophage and staying at a dormant state. In this case, the bacterium reproduces normally and it is not promoted the host death or lysis by the synthesis of phage particles. Although, prophages are replicated together with the bacterial host chromosome and can switch into lytic production upon exposure to DNA damage (not shown). Adapted from [82].

According to the International Committee on Taxonomy of Viruses (ICTV), phages are classified and distributed in different orders and families [83]. Thus, the vast majority of phages publicly known (96%) are inserted in the *Caudovirales* order. *Caudovirales* order is provided by tailed bacterial viruses able to

infect *Bacteria* and *Archaea*, being counted three families that insert the order, *Myoviridae*, *Podoviridae* and *Siphoviridae* (Figure 2.4) [81,84].

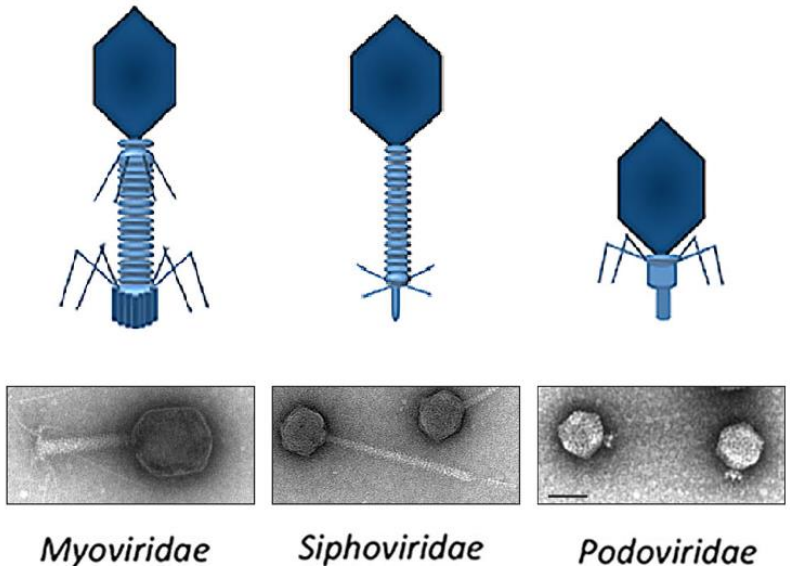


Figure 2.4 – Schematic representations and Transmission Electron Microscopy (TEM) images of the three families of the *Caudovirales* order, *Myoviridae*, *Siphoviridae* and *Podoviridae*, respectively. Adapted from [85,86].

Morphologically speaking, the virion does not have an envelope and can be divided into two segments, head and tail (Figure 2.5). The head of a *Caudovirales* phage incorporates a linear dsDNA (double stranded DNA) molecule, being considered a protein shell. On the other hand, the tail, as a protein tube, serves to transport DNA segments during the infection of the target bacterial cell, after binding its distal end to the surface receptors on the bacteria [84].

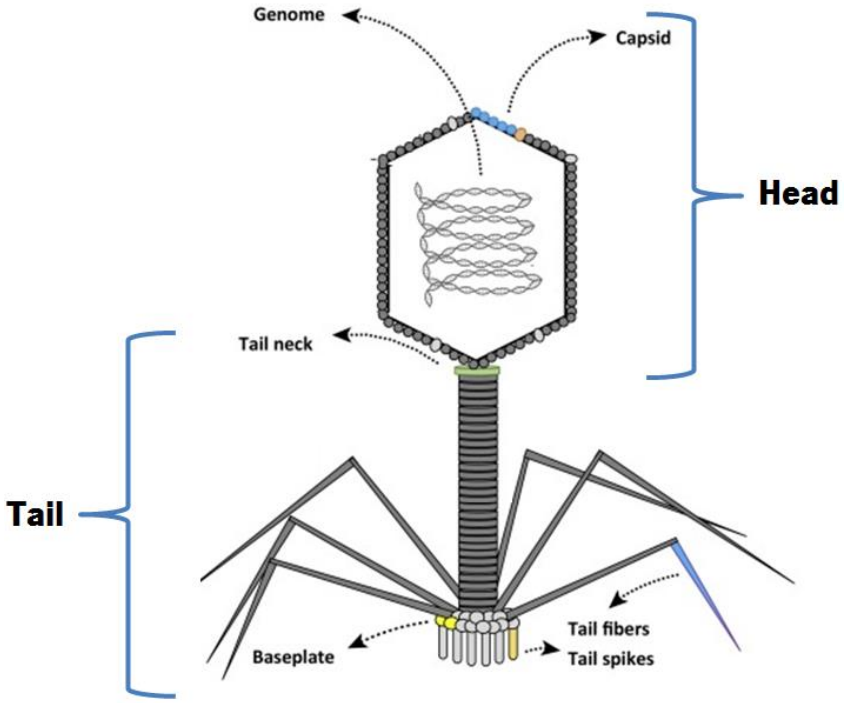


Figure 2.5 - Morphology of a phage, using *Myoviridae* from de *Caudovirale* order as an example. Adapted from [87].

2.4.1. Advantages and limitations of phage therapy

There could be counted several advantages that result from the potential use of phages in healthcare. Generally, phages infect a limited number of bacterial strains. Their high specificity in terms of infection usually guarantees the protection of the natural microbial community of the host, which is an advantage. Another property that benefits phages as an effective antimicrobial agent is their lower predisposition to induce resistance and the fact that AMR bacteria and biofilms are sensible to phages, becoming a successful solution. Phages are ones of the most common and diversified organisms in the world. Also, many of the phages described in public datasets are only a small percentage of the estimated total virions in nature [87]. Phages have other benefits, such as easy and rapid isolation, the fact that they are less expensive to develop than antibiotics, and high flexibility in terms of formulation and further application. Regarding to the safety of using phages for therapeutic purposes on humans, it is noticed that people have been exposed to phages since birth, being also constantly exposed to these organisms in the environment. Furthermore, the fact that phages have been isolated from humans suggest that its use for therapeutic purposes in the future will be well accepted [44]. Also, they need a specific bacterial pathogen to replicate, otherwise they become absent [87]. At last, in terms of their action in biofilms, Vilas Boas et al (2016) have reported that phages are able to reach all layers of biofilms in the first hours of infection [88].

However, the properties that define phages could also imply some limitations. Nevertheless, due to their specificity the treatment could be delayed, as the identification of the target pathogen and the choice of an appropriate phage is required. Also, their propagation depends on the host, replicating only at the infected area. Through generalized transduction, phages could transmit to bacteria virulent factors that they might have, such as antibiotic-resistance genes, which is another concern. However, this feature is more usual in phages with a lysogenic life cycle and this type of phages is not used for therapeutic purposes, as it was mentioned above. Another limitation is the immune system of the patient as phages can be detected as invaders and removed from systemic circulation. Strictly lytic phages could be quickly collected for some major pathogens, such as *E. coli* and *P. aeruginosa*. However, in the case of certain bacteria, like *Clostridium difficile* or *Mycobacterium tuberculosis*, their isolation could be difficult [87].

2.4.2. How phages are able to kill biofilm cells

It is understood that phages are more easily successful on the destruction of cells in the planktonic form, as they are easier to reach than cells that are in sessile community environment protected by an EPM. As so, it is known that phages can face several problems in the penetration of this EPS matrix. To overcome this problem, that is a number of mechanisms that makes some phages capable to kill biofilm cells. These mechanisms are: (i) amplified production of phages, replication within their host, releasing an increased amount of infectious progeny phages spreading into the biofilm and consequently eliminating bacteria and reducing EPS production by them; (ii) phages might carry or express depolymerases enzymes that are able to degrade EPM, some virions can induce these enzymes activity from within the host genome; (iii) sufficiently high stability of the phages, since they need to be able to survive to the travers of the EPM; (iv) phages infect bacteria but only replicate and destroy if cells are active although in case of inactivation they can be able to remain within the host until they reactivate, starting afterwards a round of infection and consequently destroying the bacterium, among others mechanisms [89,90].

2.5. Honey Therapy

Honey, as a viscous complex mixture of substances with biocidal and biostatical effects, can have an antimicrobial action in both Gram-negative and Gram-positive bacteria, and its use for medical reasons dates back to ancient times, 2200 Before Christ (BC) in both Egypt and Greece [68,91]. As so, honey could

be effective on the treatment of infected wounds. Besides, the use of honey as wound dressing could imply other advantages, such as low associated costs, safe to be used by adults and children, painless, and the fact that it is unlikely for the patient to develop an allergy [68,92]. Moreover, honey is noticed for its anti-inflammatory and antioxidant activity, for the fact that there are no reports of the development of bacterial resistance, and for its bioactivities that promote wound healing. These bioactivities lead to activation of autolytic debridement and stimulation of the tissue regeneration, promoting epithelization [68].

2.5.1. Antimicrobial properties of honey

Thus, honey has several physical and chemical antimicrobial properties. The physical factors are its acidity and osmolality. On the other hand, the chemical factors are some of its components, such as hydrogen peroxide (H_2O_2), volatiles, beeswax, nectar, pollen and propolis [93].

Further studies are needed to explore honey chemical composition and bioactivities, but it is known that honey is mainly composed by water and sugar (sucrose, glucose and fructose). Other ingredients as amino acids, wax, pollen, pigments, minerals, and enzymes are also present [94]. Some of the compounds of honey are the cause of its antimicrobial activity. In fact, it has been shown that concentrated sugar solutions display an effective role on the osmotic inhibition of bacterial growth [95]. Besides, glucose oxidase is an example of an enzyme present in honey that in full-density honey is inactive, but when diluted acts on the oxidation of glucose and on the production of gluconic acid, being responsible for the production of H_2O_2 due to the enzymatic process [93,94]. Studies have defended that the antimicrobial effect of honey is essentially due to this enzyme, more specifically due to the H_2O_2. To be effective, the concentration of H_2O_2 has to be sufficiently high, however, if the concentration is too high it might cause damage to the tissues, rising the levels of oxygen radicals [96]. According to the guideline for disinfection and sterilization in healthcare facilities approved by the Centers for Disease Control and Prevention (CDC), H_2O_2 is even often used to disinfect and sanitize medical equipment [97]. A study led by Brudzynski et al. in 2011 concluded that H_2O_2 has not only bacteriostatic action against bacteria but also a DNA degrading activity [98].

Also, the production of gluconic acid due to the same enzyme is the main cause of honey acidity. It is known that an acidic pH provides an hostile environment for microorganisms, as most microorganisms have an optimum pH between 7.2 and 7.4 [93,99]. Besides, the low pH of honey stimulates angiogenesis and consequent tissue repair [68].

The color of the honey can also be an indicative of the antibacterial activity. A study led by Araya Wasihun (2016) conclude that red honeys have higher bactericidal and bacteriostatic effects than white honeys [100]. The color of the honey, specifically its transparency, vary according to the amount of suspended particles, being related to different properties, such as botanical origin, age, and storage conditions [92].

Other honey compound that has been studied due to its antimicrobial interest is the methylglyoxal (MGO) (Figure 2.6.A) [101]. One example of honey that has been successfully used for medical purposes is the nontoxic New Zealand Manuka honey. Manuka honey has been considered highly potent against several microorganisms, being suspected that its standardized antibacterial activity is essentially due to its MGO content. In fact, a study by Mavric in 2008 have reported Manuka honey to have MGO content between 38 $mg.kg^{-1}$ to 761 $mg.kg^{-1}$, accordingly to its UMF (Unique Manuka Factor), MGO content being higher proportionally to the UMF [102]. The UMF is a quality trademark of the Manuka honey.

For the production of Manuka honey, it is collected the nectar from the *Leptospermum scoparium* bush. For the elimination of the *Clostridium botulinum* spores Manuka honey has to be sterilized with gamma irradiation [68]. Alandejani et al (2009) tested the effect of Manuka honey against *P. aeruginosa* and *S. aureus*, whether it was on biofilm or planktonic form. As so, different dilutions of the honey were made and these results were compared with the effect of several antibiotics. Results showed that bactericidal rates were higher for Manuka honey than the rates obtained for antibiotics that are usually used against *S. aureus* [103]. On the other hand, Hern Tze Tan et al. (2009) led a study that showed that at different dilutions of Manuka honey, the level of the bacterial growth inhibition was divergent [104].

A study led by Kwakman et al. (2010) presented for the first time a peptide often found in honey that has antimicrobial properties, bee defensin-1 [105]. No evidence has been found for the presence of this or other antimicrobial peptides in Manuka honey. However, Tonks et. al (2017) have identified a 5.8 kDa compound in this honey that could play a role in human monocytes cytokine induction and also on the mechanism of innate immune cells stimulation [106], being hypothesized that this component could be bee defensin-1 [107].

Although, not all honeys are interesting for wound therapy purposes as different honeys have different antimicrobial activity. Antimicrobial activity changes according to properties such as its floral source, geographic location, weather conditions, time and temperature of its storage and processing [68]. The quality of honey also varies and some of them might even be harmful, usually due to the *Clostridium botulinum* spores [94]. Moreover, honey suffers constant changes in its composition along the years during storage, which could be a limitation [99]. A component that can indicate long time of storage is the

5-hydroxymethylfurfural (HMF) (Figure 2.6.B). HMF is an organic compound present in most honeys and low levels of HMF indicate a fresh honey. Thus, HMF can be one sign of honey deterioration as it can indicate either overheating, inadequate storage conditions, old honey or even a fake honey due to the addition of invert syrup [99,108]. Besides, the sugar content, organic acids, pH, moisture, and floral source could also contribute to an alteration of the levels of HMF. HMF might even be produced at low temperatures or under acidic conditions due to the dehydration reactions of sugars [99]. The Codex Alimentarius Committee on Sugars established a maximum concentration value of 40.00 $mg.kg^{-1}$ for processed honeys and mixtures of these. In the case of honeys with a declared origin from regions with a tropical climate and their mixtures, the maximum established is of 80.00 $mg.kg^{-1}$ [109]. HMF can be produced through different pathways, being usually formed by the decomposition of monosaccharides or by the Maillard reaction that consists on the condensation of carbohydrates with free amine groups [99,110]. One other disadvantage is the fact that, despite being usually free of side effects, when used topically and directly on a wound, few patients have experienced a burning sensation, probably due to its acidity coming in contact with naked nerve endings [92].

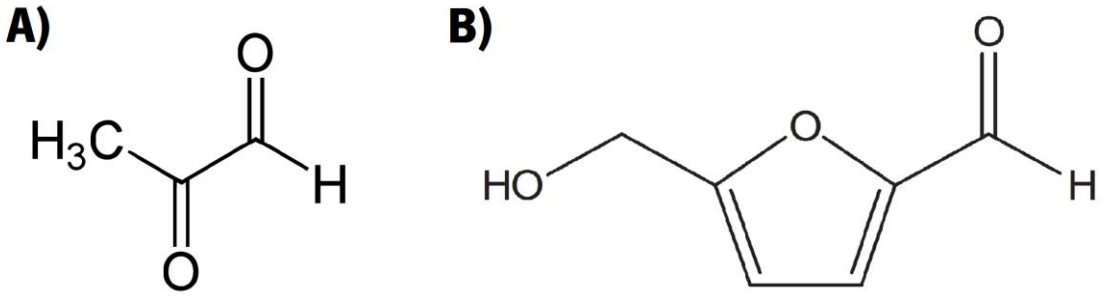


Figure 2.6 – Examples of honey compounds: **A)** MGO chemical structure; **B)** HMF chemical structure. Adapted from [111]

Synergism is defined as a cooperative interaction between two or more elements, noticing an effect that is not accomplished by one of the elements alone. On the other hand, antagonism can be defined as the annulment of the effect of one element by another [112]. Thus, combining honey and phages could be an interesting option on the therapy of skin chronic wounds, as an alternative to the use of antibiotics, being important to understand if the combination of both could enhance the antimicrobial properties of each.

3. Materials and Methods

3.1. Bacterial strains and growth conditions

The reference strain *E. coli* (CECT 434, below described as EC434) was purchased from the Spanish Type Culture Collection and used for biofilm formation. The phage host strain used for phage propagation was an *E. coli* clinical isolate, selected from a collection gently provided by Hospital Escala Braga (Braga, Portugal). Both strains were grown in Tryptic Soy Broth (TSB, Liofilchem®), Tryptic Soy Agar (TSA) – TSB containing 1.2% (w/v) agar (Fisher BioReagents™), and in MacConkey agar (PanReac Applichem), a selective medium used for the Colony Forming Units (CFU) enumeration.

The reference strain *P. aeruginosa* (DSM 22644, below described as PAO1), acquired from the German Collection of Microorganisms and Cell Cultures, was used for phage propagation and for biofilm formation. It was cultivated in TSB, in TSA, and in Pseudomonas agar base (VWR®) supplemented with 1% (w/v) of glycerol (Fisher BioReagents™), medium (the latter used for the determination of the concentration of *P. aeruginosa* by CFU enumeration).

The bacterial strains were previously stored at -80 °C in TSB supplemented with 15% (w/v) glycerol. Colonies were grown on TSA and incubated at 37 °C overnight. To obtain liquid fresh suspensions, one colony of the grown bacteria was inoculated in approximately 10 mL of fresh TSB medium and incubated overnight at 37 °C with gentle stirring (120 rpm, PSU-10i Orbital Shaker BIOSAN).

3.2. Bacteriophage Therapy

3.2.1. Bacteriophage production

The two phages used in this work were previously isolated and provided by Bacteriophage Biotechnology Group (BBiG) from the Centre of Biological Engineering (CEB) and are deposited in the CEB phage collection (Braga, Portugal). The *E. coli* phage (vB_EcoS_CEB_EC3a, referred below as EC3a) was isolated from raw sewage and its host strain was an *E. coli* clinical isolate, as it was already mentioned (table 3.1). This virulent phage belongs to the *Siphoviridae* family and has an icosahedral head of 57 nm in diameter and a non-contractile tail of 192 nm × 11 nm [113]. The other phage (vB_PaeP_PAO1, referred below as PAO1-D), a *P. aeruginosa* phage, was isolated from a Sextaphage cocktail commercialized in Russia (Microgen, ImBio Nizhny Novgorod, Russia), and the host used for the amplification was PAO1 (table 3.1). This phage belongs to the *Podoviridae* family, has a head with 56 nm × 64nm of diameter and a tail that is 12 nm long. The TEM images of both phages were provided by BBiG and are displayed in Annex I (Figure I.1.A and Figure I.1.B, respectively).

Table 3.1 – References of the phage, hosts used for amplification in this work and reference of the studies that have isolated the phages

Host	Phage designation	Reference	Isolated by
E. coli	EC3a	vB_EcoS_CEB_EC3a	Andrade, 2015 [114]
P. aeruginosa	PAO1-D	vB_PaeP_PAO1	Silva, 2017 [115]

For phage production the plate lysis and elution method was performed as described by Sambrook and Russell (2001) with some modifications [116]. Briefly, 5 µL of phage suspension were spread evenly on host bacterial lawns using a paper strip hold by forceps and the petri dishes were then incubated overnight at 37 °C. Afterwards, 3 mL of SM Buffer (5.8 $g.L^{-1}$ NaCl, Applichem Panreac, 2 $g.L^{-1}$ $MgSO_4.7H_2O$, VWR®, 50 $mL.L^{-1}$ 1 M Tris-HCl pH 7.5, VWR®) were added to each plate. The plates were then incubated overnight at 4 °C with gentle stirring (80 rpm). The liquid was then collected, concentrated with NaCl powder, incubated 1 h at 4 °C and gentle stirring (80 rpm), and centrifuged (10 min, 9000 × *g*, 4 °C, SIGMA 3-16K), the supernatant was collected and the pellet discarded. The lysate was concentrated with PEG 8000 (ThermoFisher Scientific) and incubated overnight at 4 °C with gentle stirring (80 rpm). Afterwards, another centrifugation was performed (10 min, 9000 × *g*, 4 °C), the supernatant discarded and the pellet resuspended in SM buffer. The obtained was purified with chloroform (ThermoFisher Scientific, Acros organics), mixed, centrifuged (15 min, 3500 × *g*, 4 °C), and the upper aqueous phase collected. Finally, the solution was sterilized by filtration (PES, GE Healthcare, 0.2 µm). After quantification, serial dilutions of phage stock solutions were made in SM buffer and stored at 4 °C for further use.

3.3. Honey Therapy

3.3.1. Honey samples

The Portuguese honey used in this work, C1 (92% *Castanea sativa*) was compared with a commercial honey, Manuka honey (Medihoney®, Derma Sciences) (table 3.2). Honeys were stored at 4 °C for further use, in order to prevent changes in the physicochemical properties, as the increase on HMF concentration. The pollinic analysis of C1 honey is described in Annex II (table II.1).

Table 3.2 – Description of the different honeys used in this work (origin and batch year)

Honey	Origin	Obtained from	Year
Manuka	*Leptospermum scoparium*	Commercial honey	-
C1	*Castanea sativa* (92%)	Regional beekeeper	2017

3.3.2. Physicochemical Characterization of honey – pH Determination

In order to determine the pH of honey, it was followed the method described by the International Honey Commission [117]. Briefly, honey was mixed with distilled water in the same proportion and the pH of this 50% (w/v) honey solution was measured using the proper equipment (Hanna® Instruments, HI 2210). Three independent assays were made and the average was estimated.

3.3.3. Physicochemical Characterization of honey – Color Determination

For the color determination of the honey, the optical densities (OD) of the samples previously prepared were measured at 560 nm (Biotek Synergy™ HT). Thus, 50% (w/v) honey solutions were made and the OD_{560} values obtained were then multiplied by 2. This last step was made in order to obtain the total absorbance value without dilution. Finally, according to the standards already established by the United States Department of Agriculture (USDA) the color of the honey samples was evaluated (table 3.3) [118].

Table 3.3 – USDA color standards designations of extracted honey according to the ODs. Adapted from [118]

Color Standarts Designations	OD_{560}
Water White	0.0945
Extra White	0.189
White	0.378
Extra Ligth Amber	0.595
Ligth Amber	1.389
Amber	3.008
Dark Amber	>

3.3.4. Physicochemical Characterization of honey – MGO content quantification by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The method used for MGO quantification was adapted from the protocol described by Adams et al. (2008) [119], and the concentration was obtained by RP-HPLC (Shimadzu®, Nexera X2) using a 4.0 × 250 mm C18 column (Purospher® STAR RP-18 endcapped (5 µm) LiChroCART®). A Shimadzu LC-30AD solvent delivery unit, a Shimadzu SPD-M20A Photo Diode Array Detector (PDA), and a Shimadzu SIL-30AC sample injector were used for the RP-HPLC run. Derivatization steps were first performed, O-phenylenediamine (OPD) was added in order to induce a condensation reaction and obtain MGO and dihydroxyacetone (DHA). A solution of 30% (w/v) honey was prepared and then, the solution was mixed with a 2% (w/v) OPD solution (3:11 v/v). The 2% (w/v) OPD solution was prepared by dissolving the OPD in a 0.5 M phosphate buffer, pH of 6.5. Afterwards, samples were incubated in the dark, overnight (16 h), at room temperature, and then were filtered-sterilized. For the RP-HPLC, the flow rate selected was 0.3 $mL.min^{-1}$ and the temperature was 30 °C. For the mobile phase solvent A and solvent B were used in the same proportion: solvent A: 0.075% (v/v) acetic acid (Fisher Chemical); solvent B: 80% (v/v) methanol (Biochem Chemopharma, MeOH). Initially the gradient was 10% (v/v) of solvent B for 4 min. Then, during 31 min, the gradient was gradually increased to 100% (v/v) of solvent B, being held there for 3 min. Afterwards, during a period of 6 min, the gradient decreased back to 10% (v/v) of solvent B. For the assays, 20 µL of solution were injected and the absorbance of ultraviolet (UV) radiation was measured at 312 nm in order to detect the peaks, using the Shimadzu® LabSolutions 5.71 software. In order to obtain the calibration curve for the quantification of the MGO, a standard solution with a purity grade solvent of MGO of 35-40% (v/v) (Alfa Aesar) was used (Annex III, Figure III.1). MGO was eluted after 21 min and DHA after 13 min.

3.3.5. Physicochemical Characterization of honey – Protein content Determination

The protein content was determined by colorimetry using the BCA (Bicinchoninic Acid) Protein Assay Kit (Thermo Scientific™ Pierce™). This Kit aims the colorimetric detection of the water-soluble BCA/copper complex, that with the increase of protein concentrations exhibits a proportional stronger purple-colored reaction measure by spectrophotometry, OD_{562} [120]. As so, this method was used to quantify the total protein content in honeys and did not have a role in the identification of possible antimicrobial peptides. The protocol was performed according to the manufacturer's instructions. Briefly, on a 96-well plate (Orange Scientific®), solutions of 50% (w/v) honey were successively diluted in distilled water. Then,

20 µL of protein sample were added to each well and OD read at 562 nm (Bio-tek Synergy™ HT). The samples were measured in triplicate.

The protein concentration of the honey was calculated through the equation of the standard Bovine serum albumin (BSA) line previously obtained (Annex III, Figure III.2) and used as a reference in the determination of protein concentration patterns (0-2 $mg.mL^{-1}$).

3.3.6. Physicochemical Characterization of honey – HMF content Determination by White's method

The HMF content of the honey was determined by White's method (1979) [121]. Briefly, honey was diluted in distilled water (1:26 w/v) and then Carrez solution I (150 $mg.mL^{-1}$ potassium ferrocyanide) was added (1:52 v/v). The solution was then homogenized and Carrez solution II (300 $mg.mL^{-1}$ zinc acetate) was then added and mixed (1:53 v/v). Afterwards, distilled water was added to the solution (53:100 v/v). After being homogenized, this solution was sterilized by filtration. Two new solutions were then made: (i) distilled water was added to the solution previously prepared in the same proportion; (ii) 0.2% (w/v) sodium bisulfite solution was added to the solution previously prepared in the same proportion. Sodium bisulfite is an HMF decomposing substance. The solutions were mixed and the absorbance read in triplicate at both 284 nm and 336 nm (VWR® UV-3100PC).

The absorbance at 284 nm corresponds to the sample with water and the absorbance at 336 nm corresponds to the blank with 0.2% (w/v) sodium bisulfite. The HMF was calculated using the equation (3.1).

Equation 3.1

HMF(mg.kg^(-1)) = [(A284-A336)*149,7*5]／[sample weight (g)]

3.4. Evaluation of the antimicrobial effect of honey – Determination of the Minimum Inhibitory Concentration (MIC) of honey

The MIC values were determined according to the guidelines of the Institute of Clinical and Laboratory Standards (2003) [122]. Briefly, after the growth of the bacteria strain in TSB, the OD_{620} of the solution was adjusted to 0.13. Afterwards, it was made a 30-fold dilution in TSB (approximately 10^7 $CFU.mL^{-1}$). The determination of the MICs was performed in a 96-well plate, where 200 µL of 50% (w/v) honey were displayed on the first well. Then, using a concentration range from 50% (w/v) to 3,125% (w/v), serial dilutions were made. The final volume of the several honey dilutions tested was 100 µL. Afterwards,

5 µL of the bacterial suspension were added to each well. For the positive control, the same was done but instead of honey, TSB was used. For the negative control, the respective wells were filled with only TSB. For the evaluation of each honey effect on the strain tested, five independent assays in triplicate were made. Plates were incubated for approximately 20 h at 37 °C with gentle stirring (120 rpm). In order to determine the MICs, ODs were measured by turbidimetry at 620 nm, confirming the results obtained visually.

3.5. Phage viability on honey

To test phage viability on honey, a phage concentration of 10^9 PFU.mL^{-1} was used. Both 50% (w/v) and 25% (w/v) concentrated honeys combined with a phage solution were tested. The solutions were incubated at 37 °C with no stirring and samples were analyzed at different time points up to 24 h. Phage counting (PFU.mL^{-1}) was performed using double agar overlay technique by Kropinski et al. (2009) [123]: 100 µL of the bacterial host were mixed with 3 mL of TSB containing 0,6% (w/v) of agar (TSB top agar) into a petri dish already containing a layer of TSA. Afterwards, serial dilutions of the incubated solutions were made and plated. The plates were incubated overnight at 37 ◦C and the PFU counted. Three independent assays were carried out for each condition.

3.6. Preparation of the pig skin – Designing an *ex vivo* porcine skin model

Fresh porcine skin from the abdomen was generously supllied by the Instituto de Investigação em Ciências da Vida e Saúde (ICVS; Braga, Portugal). Porcine skin samples were stored in vacuum at -20 °C for further use, the maximum time of storage at -20 °C was one month, and samples were thawed only once.

For the several assays, the skin pieces were cut into 2 × 2 cm samples. Afterwards, they were sterilized. For the sterilization several methods were tested (table 3.4).

Table 3.4 – Description of the sterilization methods tested

Method	Description
A	(i) 96% (v/v) ethanol (Dimor) during 1 min.
B	(i) 70% (v/v) ethanol (Dimor) during 10 min; (ii) 70% (v/v) ethanol vapors during 1 h at 37 °C.
C	(i) 10% (v/v) commercial bleach for 5 min; (ii) Chlorine gas vapors during 45 min at 37 °C; (iii) 10% (v/v) commercial bleach during 5 min. **Notes:** The chlorine gas was obtained by adding acetic acid with bleach. Afterwards, the pig samples were washed once with bleach and thrice with Phosphate Buffered Saline diluted 1x (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO$_4$, VWR®, 2 mM KH_2PO_4., VWR®, PBS 1x). They were then dried using a filtration system [1].
D	(i) 70% (v/v) ethanol for 20 min (ii) 70% (v/v) ethanol vapors for 1 h and 45 min at 37 °C (iii) UV radiation for 30 min (iv) Chloroformed vapors for 30 min. **Notes:** Afterwards, the samples were washed with sterile distilled water.

The method D) was selected to accomplishment sterilization as both methods A) and B) were ineffective. As for Method C) by Yang et al. [1], it did in fact guarantee sterilization, but, probably due to the bleach added, strains tested did not adhere to the pig skin. Due to the complexity and time spent on the method, after sterilized the samples were stored at 4 °C overnight and used in the next day. Briefly, and according to the model developed by da Costa et al. (2015), after disinfected, the pig skin samples

were placed between two stainless steel metal plates with an o-ring to delimit the infection region and adjusted using the upper metal plate coupled with wing nuts (Figure 3.1) [124].

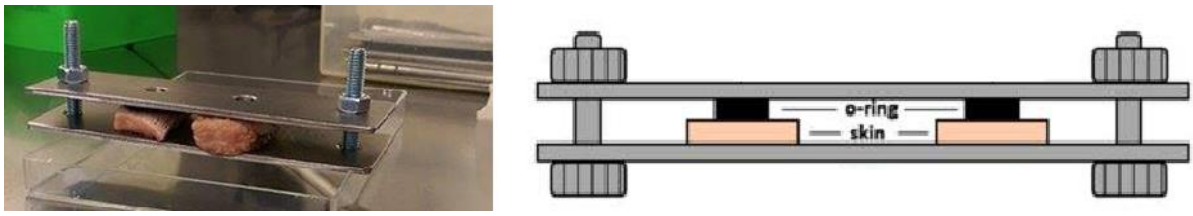


Figure 3.1 – Model developed (real and schematic representation) Adapted from [124].

3.7. Control of 24 h-old biofilms

3.7.1. Effect of the phage-honey formulations on 24 h-old biofilms – *in vitro*

Several suspensions of honey and phage combined or separated were prepared: i) in order to evaluate the effect of honey 50% (w/v) in biofilm control, a solution of TSB 4× (TSB solution four times more concentrated) was prepared and mixed with SM buffer in an equal proportion and then, this solution was mixed with the same volume of honey; ii) to prepare 25% (w/v) honey, TSB 4× was mixed in the same proportion with honey again and then, SM buffer was added, also in the same proportion; iii) for the formulation of honey 50% (w/v) combined with phage solution, an equal volume of honey and TSB 4× was mixed before the phage solution was added ($2x10^9$ $PFU.mL^{-1}$), in the same proportion; iv) for honey 25% (w/v) combined with phage, after preparing a solution by adding TSB 4× with SM buffer in a 100:82 proportion, honey was added to the solution previously prepared and afterwards, $1,2×10^{10}$ $PFU.mL^{-1}$ phage was 1:10 (v/v) mixed; v) to evaluate the effect of phage on biofilm control, phage solution was diluted in SM buffer to obtain a Multiplicity of infection (MOI) = 10. The MOI was obtained according to (3.2)

Equation 3.2

MOI=[Phage](PFU.mL^(-1))/[Host](CFU.mL^(-1))

For *in vitro* 24h biofilm formation, 96-well polystyrene plates were used. The bacterial cultures were grown for about 16 h (overnight) at 37 °C with 120 rpm. The OD_{620} of the culture was adjusted to 0.13 (approximately $3×10^8$ $CFU.mL^{-1}$) and then diluted 10-fold in TSB. Each well was inoculated with 200 µL of this bacterial suspension and the plates were incubated for 24 h at 37 °C, 120 rpm.

24 h after the infection, all the wells were washed twice with TSB. Afterwards, 200 µL of TSB were added to the control wells. In the treatment wells, the same volume of the several treatment solutions was placed. At the several and respective time points (0 h, 6 h, 12 h, and 24 h), the suspensions were discarded and the wells washed twice with 0.9% (w/v) saline solution (NaCl). Then, 200 µL of 0.9% (w/v) NaCl were dispensed in each well, the biofilm manually grafted with a tip, removed and finally stored in eppendorfs. Bacteria quantification was performed by serial dilutions in 0.9% (w/v) NaCl containing 1 mM ferrous ammonium sulfate (FAS, Applichem Panreac), concentration selected according to Ribeiro (2016), and plated in TSA using the microdrop technique [125,126]. The FAS virucide solution was used in order to inactivate phage action for biofilm quantification, being also used on the dilutions of bacterial suspensions of biofilms that were not treated with phages in order to lower the error. Three independent assays were performed for each condition *in vitro*.

3.7.2. Effect of the phage-honey formulations on 24 h-old biofilms – *ex vivo*

To achieve the desired bacterial concentrations, the same adjustments of the initial bacterial suspensions were made as described for *in vitro*. Afterwards, 80 µL of the bacterial suspension were placed in the area designed for infection (detailed in 3.7). The mechanical system (Figure 3.1) was placed in previously sterilized desiccators and incubated for 24 h at 37 °C.

At the several time points (0 h, 6 h, 12 h, and 24 h), the solutions on the systems holes were discarded and the holes washed twice with 0.9% (w/v) NaCl. The infected areas were grated with a swab humidified with 0.9% (w/v) NaCl. The collected biofilm was mixed with 1 mL of 0.9% (w/v) NaCl and then centrifuged (8000 × *g*, 10 min). The supernatant was discarded and the pellet resuspended in 1 mL 0.9% (w/v) NaCl. Finally, CFU were counted, by performing serial dilutions as described previously for the *in vitro* assay. Three independent assays were carried out for each condition *ex vivo*.

3.8. Zeta Potential of 24 h-old biofilm cells

The Zeta Potential of 24 h-old biofilm cells was measured by dynamic light scattering (DLS) using a Malvern Zetasizer, NANO ZS (Malvern Instruments Limited). The timepoints tested were 6, 12 and 24 h after treatment and the treatment solutions made and analyzed were (i) phage solution with a MOI of 10, (ii) 25% (w/v) honey solution, (iii) 50% (w/v) honey, (iv) 25% (w/v) honey combined with phage, and (v) 50% (w/v) honey combined with phage. Three independent assays were carried out for each condition.

The Zeta Potential values were then determined by applying the Smoluchowski equation described by Hunter (1981) [127]. Briefly, biofilms were formed and treated, and after each treatment, wells were washed thrice with 0,9% (w/v) NaCl, and surfaces scratched in order to detach biofilms. Samples (1 mL) were collected into a 2 mL tube, vortexed and diluted 10-fold in sterile Milli-Q™ water prior to each analysis.

3.9. Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 6. In general, for three independent assays, the mean and standard deviation (SD) were determined and the results were presented as mean ± SD. The results were compared using the tools: One-way ANOVA and Two-Away ANOVA, with Turkey's post-test. The differences between the several conditions were considered statistically significant when p-value ≤ 0.05 (95% confidence interval).

4. Results and Discussion

4.1. Physicochemical Characterization of honey

Honey is a complex mixture that involves antimicrobial properties that are not completely understood yet. Previous studies have suggested several pathways that different honey compounds are able to use in order to damage or destroy microorganisms. In order to discuss the effect of the honeys tested against *E. coli* and *P. aeruginosa* biofilms, assays of physicochemical characterization of honeys were carried out. As so, the physicochemical properties of the commercial honey Manuka are displayed in table 4.1 along with the properties of the Portuguese honey C1.

Table 4.1 – Physicochemical properties of C1 and the commercial honey Manuka

Honey	pH	Color	MGO (mg.kg^{-1})	Protein (mg.kg^{-1})	HMF (mg.kg^{-1})	Reference
C1	5.4	White	1000.2	81.7	-110.8	This work
Manuka	3.5	Ligth Amber	756.5	60.9	189.4	Ribeiro, 2016 [125]

The pH of honeys is known to vary in a wide range of approximately 3.2 and 6.1 [109,128,129], which is in accordance with the results obtained for both C1 (5.4) in this work and Manuka honey (3.5) in a previous project (Ribeiro, 2016) [125]. This is highly important for wound therapy purpose, since acidic honeys provide a more hostile environment for microorganisms and inhibit microbial growth, as it was already mentioned [93]. For instance, in 1997, Greenwood studied the minimum pH values for the growth of some pathogenic bacterial species, and found that a minimum pH of 4.3 and pH of 4.4 are needed for the growth of *E. coli* and *P. aeruginosa*, respectively [130]. Manuka honey is known for its low pH, lower than pH of the C1 Portuguese honey. As it was already stated, the acidity in honeys is mainly due to the conversion of glucose into gluconic acid, by glucose oxidase [131]. This enzyme is activated when a honey is diluted and also mediates the production of H_2O_2. As so, a low pH could be an indicative of a higher H_2O_2 concentration, which might be favorable because this compound has antimicrobial properties. However, it has been shown that non-peroxide honeys, honeys that do not rely on H_20_2 as an antimicrobial agent, are also able to demonstrate antimicrobial potential due to other factors, such as MGO. This type of honeys are usually associated with floral sources commonly derived from *Leptospermum* species, such as Manuka honey [132].

According to a study led by Jervis-Bardy in 2011, honeys that contain a MGO concentration greater than 530 mg.kg^{-1} demonstrated biofilm-cidal activity while non-MGO honey presented no biocidal effect at any tested concentration [133]. It is noted that both honeys used in this work revealed a high MGO content,

with C1 honey having the highest (1000.2 $mg.kg^{-1}$). This result is in accordance with the literature that has shown that MGO can vary in a wide range, being even absent is some cases or having residual values of approximately 5 $mg.kg^{-1}$ to up than 2092.4 $mg.kg^{-1}$ [102,113,134]. As so, in theory, the higher MGO concentration of C1 suggests that C1 would have higher biofilm-cidal activity.

Differences in physicochemical properties among these two honeys, might be due to the botanical origin, age, and storage conditions [131,135]. Being a honey derived from the *Castanea sativa,* as it was revealed by the pollinic analysis (Annex II, table II.1), it was expected that C1 honey would be darker in color [136]. Although, both C1 and Manuka are fairly light, this is probably a consequence of higher water content. Accordingly to literature, darker honeys may have higher sugar content, having less water and more organic compounds, and in the case of lighter honeys, such as the ones used in this work, the opposite. For wound treatment in particular, the high sugar content of dark and light honeys makes them hygroscopic, which means that this compound not only has the ability to reduce the wound moisture, but also dehydrate bacteria, causing osmotic stress and their shrinkage [137]. Besides this bactericidal effect, darker honeys demonstrate an antioxidant activity due to a higher concentration of antioxidants due to higher phenolic contents such as flavonoids [138]. Furthermore, the conductivity of honey is related with ash and mineral content and darker honeys are usually provided with an electrical conductivity value slightly higher than lighter honeys [139]. In the case of C1 honey, the electrical conductivity obtained was of 1534 $\mu S.cm^{-1}$, which is in accordance with the normal values defined by the Codex Alimentarius (between 200 and 1800 $\mu S.cm^{-1}$) [109]. Besides, a study by Oddo et al. (1995) reported an electrical conductivity of 1410 $\mu S.cm^{-1}$ for a honey derived from the same floral source which corroborates this result [140].

C1 total protein content (81.7 $mg.kg^{-1}$) was demonstrated to be higher than Manuka's (60.9 $mg.kg^{-1}$), C1 protein content was between the wide range between 0.75 $mg.kg^{-1}$ and 5000 $mg.kg^{-1}$ as reported by several studies [141–143], . The protein content measurements did not assured the existence of antimicrobial peptides, such as bee defensin-1. As so, further studies in order to identify eventual antimicrobial proteins are required. As it was already mentioned, another parameter used to characterize honeys is their HMF content, since high levels of this content are a sign of deterioration. The HMF concentration obtained for C1 honey was below the detection limit of the equipment, as so it was not possible to determine the exact HMF value of this honey. However, it is possible to conclude that it is in the limits defined by the Codex Alimentarius, in opposed to Manuka honey (189.4 $mg.kg^{-1}$) as it was previously reported [125]. The HMF content result is not directly related with honey antimicrobial activity, although, as it was already stated, higher HMF content could be a consequence of overheating. As so,

higher HMF could be an indicative of higher MGO content in some cases, since overheating also leads to the conversion of DHA to MGO in a non-enzymatic reaction [144]. However, this was not observed in this study being highlighted that C1 honey was stored at low temperature in order to preserve its properties. Many characteristics of honey can be used to select a particular honey for antimicrobial studies since its properties can have different impacts on different bacteria.

4.2. Minimum Inhibitory Concentration (MIC) of honeys

Although there is commonly no correlation between MIC values (planktonic) and antimicrobial concentrations necessary to inhibit biofilms [145], it is important to analyze which range of concentrations is effective using the standard antimicrobial methodology and use results as indicators for honey antimicrobial potential. For this, the MIC of honeys were determined (table 4.2) against *E. coli* (EC434) and *P. aeruginosa* (PAO1), respectively.

Table 4.2 – MIC of the Portuguese C1 and the commercial Manuka honey on *E. coli* and *P. aeruginosa*, measured in this work

	MIC	
Strain / Honey	**EC434**	**PAO1**
C1	12.5% (w/v)	25% (w/v)
Manuka	25% (w/v)	25% (w/v)

It is known that the MIC is the lowest concentration of a compound that prevents at least 99% of bacterial growth, which means that the lower the MIC, more potent is the compound against the growth of the organisms tested [146]. In this work, the C1 honey exhibited the lowest MIC against *E. coli* (12.5% (w/v)) while for *P. aeruginosa* the MIC obtained with both tested honeys was 25% (w/v). These results are in accordance with another study evaluating the antimicrobial role of honey, where *E. coli* showed lower MIC than *P. aeruginosa* [100]. Nevertheless, unlike this, other studies have reported lower MICs for Manuka honey that ranged between 8.75-20% against *S. aureus*, *E. coli* and *P. aeruginosa* [104,147]. Also, a study led by George and Cutting (2007) ranked the order of sensitivity of bacteria to the antibacterial activity of standardized Manuka honey, and among them were several strains of *E. coli* and *P. aeruginosa. P. aeruginosa* strains demonstrated the highest MIC, between 12% (v/v) and 14% (v/v).

As for *E. coli*, the MIC was between 6% (v/v) to 8% (v/v) [148]. These studies were, however, performed using different strains than the ones used in this work, which can lead to different MIC values than the ones that were obtained.

4.3. Phage viability in honey

In this work, two previously isolated phages were used – phage EC3a for *E. coli* and phage PAO1-D for *P. aeruginosa* [114,115] and their viability on C1 and Manuka honeys at 25% (w/v) and 50% (w/v) was assessed in this work (Figures 4.1 and 4.2).

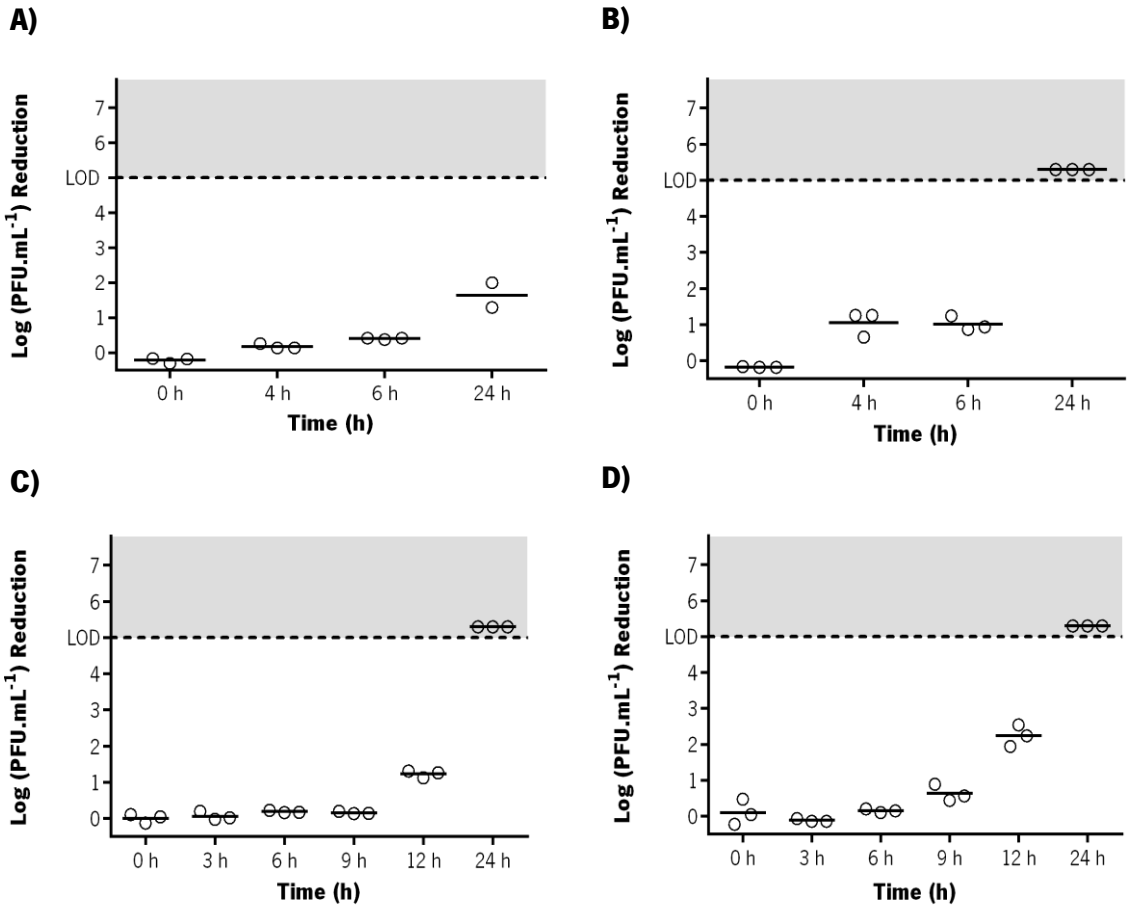


Figure 4.1 – Viability of phage EC3a on Manuka and C1 honeys along 24 h. The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of EC3a viable particles. The honey concentrations tested were: **A)** Manuka at 25% (w/v); **B)** Manuka at 50% (w/v); **C)** C1 at 25% (w/v); **D)** C1 at 50% (w/v). Data shows each independent assay (o) and mean (-). LOD (Limit Of Detection) = 5 log

The viability of phage EC3a in 25% (w/v) Manuka decreased up to 1.7 log at 24 h, remaining still active phages (3,00E+07 PFU.mL^{-1}). A concentration of 50% (w/v) Manuka led to complete destruction of phages after 24 h, considering the detection limit of 1.0E+04 PFU.mL^{-1}. The same phenomenon was observed for the C1 honey with 25% (w/v) and 50% (w/v). In the case of this honey, at 25% (w/v) and

50% (w/v), considering 12 h and 9 h in, respectively, honey start to interfere with phage viability, and 1.2 and 0.6 log reductions were obtained for these timepoints at these concentrations, respectively.

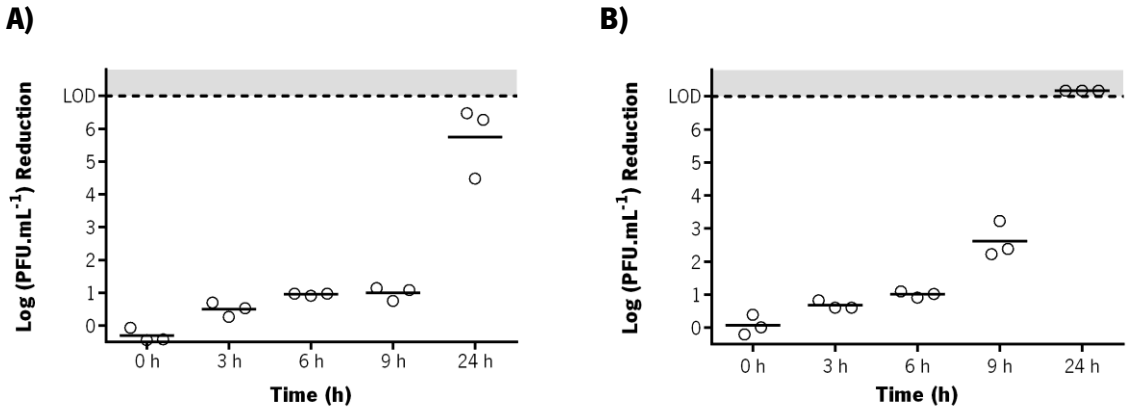


Figure 4.2 – Viability of phage PAO1-D on C1 through time along 24 h. The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of PAO1-D viable particles. The honey concentrations tested were: **A)** C1 at 25% (w/v); **B)** C1 at 50% (w/v). Data are shown as each independent assay (o) and mean (-). LOD (Limit Of Detection) = 7 log

The viability of the *P. aeruginosa* phage PAO1-D was only tested in C1, as Manuka was not tested against *P. aeruginosa* biofilms combined with this phage. Thus, 25% (w/v) C1 did not completely inactivated the phage, and after 24 h an average of 1.7E+04 PFU.mL^{-1} still remained viable. Furthermore, up to 9 h of phage contact with C1, viability reduction was less than 1.5 log. Phage contact with 50% (w/v) C1 honey led to the complete inactivation of phages, considering the detection limit of 100 PFU.mL^{-1}.

Comparing the activity of honey C1 on both phages, EC3a and PAO1-D (Figures 4.1.C, 4.1.D and Figure 4.2), it can be noticed a similar effect.

In a previous study, 50% (w/v) of another Portuguese multifloral honey (U3 honey) on EC3a, caused a complete loss of phage viability within 1 h upon contact [113,149]. Furthermore, the same U3 honey was also tested against phage PAO1-D and 50% (w/v) also inactivated the PAO1-D phage after 2 h of exposure (data not shown). C1 and Manuka tested herein were not able to inactivate as fast as U3 the *E. coli* and *P. aeruginosa* phages. Thus, it is always important to assess the effect of different honeys in the viability of phages since these can have different antiviral efficacies. Nevertheless, it can be concluded that, similarly to the results observed by Ribeiro (2016) [125] and Oliveira (2017), higher concentrations of honey induce a faster decrease in phage viability that has been previously shown to be, in the case of EC3a, a consequence of the destruction of phage capsids, observed by TEM [113,149]. It might be inferred that the same phenomenon occurred in this study, after both phages were exposed to C1 and Manuka. Despite the fact that phages tend to have high resistance to adverse environments, they are normally stored at neutral pH, whether in solution or dried. Thus, the inactivation of EC3a and PAO1-D phages

can be due to multiple factors, such as the acidity of the honeys used, the presence of high sugar concentrations that might induce osmotic shock [95,150], the temperature used for the experiment (37 °C) might not be optimum [151], or the presence of protein-degrading enzymes present in honeys [152]. García et al. (2009) demonstrated that the titer of *S. aureus* phages was reduced in 2 log between 4 h and 6 h when the pH of a solution decreased from 6.19 to 5.38 [153]. Furthermore, Jepson (2004) tested the viability of a phage suspension according to the temperature, and the results obtained showed that phage viability started to decrease at higher temperatures, remaining viable only 3.4 days at 37 °C [154]. Moreover, coliphage T4 was shown to be highly sensitive to osmotic shock and subsequent exposure to low monovalent salt concentrations [150]. In fact, osmotic shock is a familiar means for rupturing viral capsids and releasing the intact unprotected phage genomes [155]. In honeys, sucrose might have a similar effect than salt, and thus, as it was previously mentioned, the darker the honey the less water and more organic compounds they have. If we compare the antiviral action of C1 and Manuka with the previously characterized Portuguese U3 honey [125] just based in color, the later honey (U3) caused damage to phage particles more rapidly since it is dark amber in color which reflects in its higher sucrose content [113,115].

It is important to point out that phages respond differently to the same environment, as the optimum pH and temperature that each phage can withstand can vary greatly [151].

4.4. *In vitro* control of monospecies biofilms

In vitro studies have several advantages already verified, such as low cost, no need for advanced equipment, high reproducibility, etc. *In vitro* tests are performed using microorganisms outside their usual biological context and can be useful as a primary study. The microtiter plate device is well known for its applications, being useful for screening for biofilm formation capacity and for tests of different anti-biofilm compounds [58]. As such, phage, honey and their combination were tested against monospecies *E. coli* 24 h-old biofilms using the commonly used 96-weel microplate high throughput *in vitro* model.

4.4.1. Control of *E. coli* monospecies biofilms *in vitro*

Antibiofilm effect of phage EC3a combined or not with C1 and Manuka honey (Figure 4.3), and the effect of honeys individually were tested against *E. coli* biofilm cells.

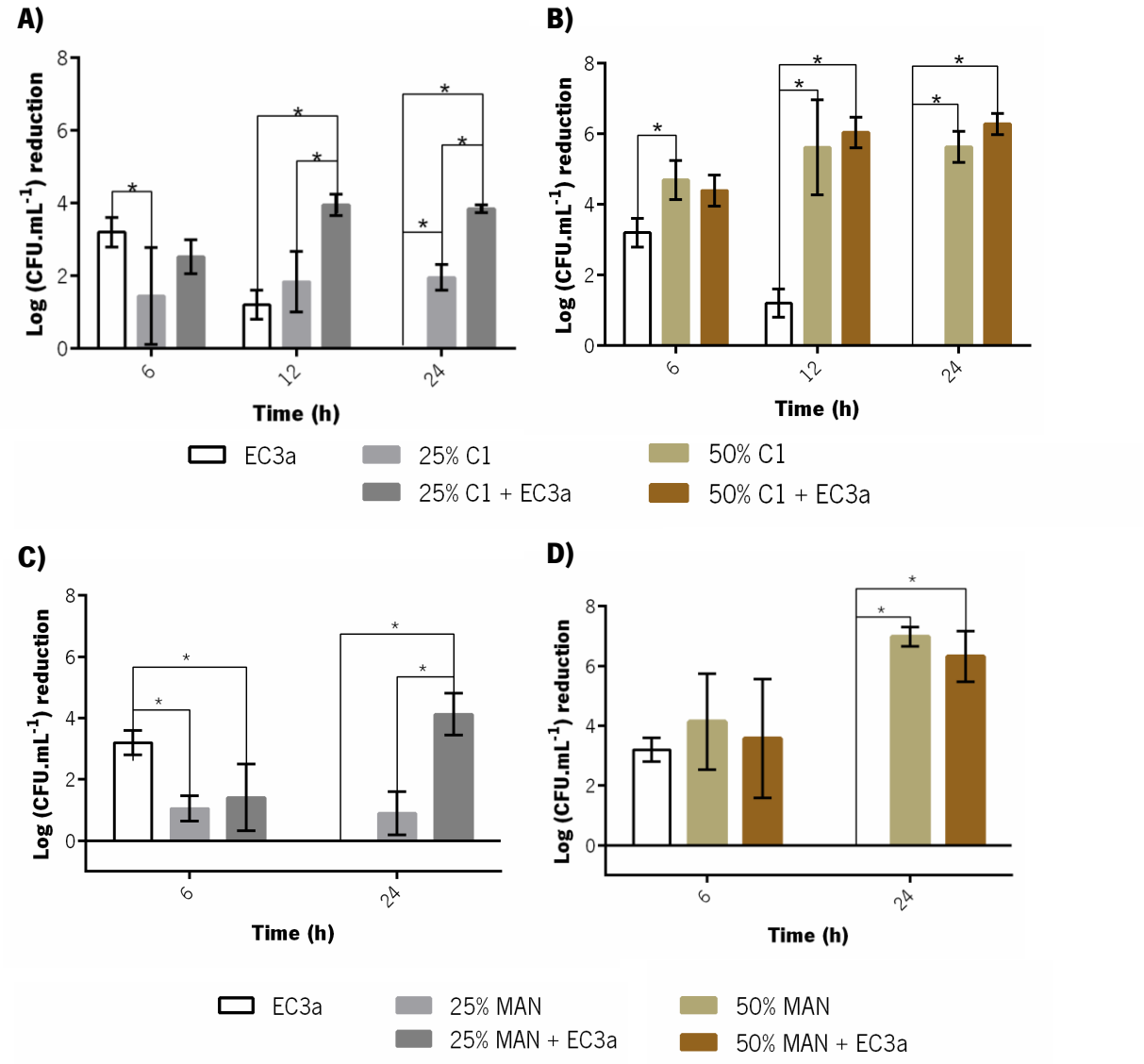


Figure 4.3 – Antibiofilm effect of EC3a phage, honey, and honey-phage combinations against 24 h-old monospecies *E. coli* biofilms formed *in vitro.* The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of *E. coli* viable cells. The honey concentrations tested were: **A)** C1 at 25% (w/v); **B)** C1 at 50% (w/v); **B)** Manuka (MAN) at 25% (w/v); **D)** Manuka (MAN) at 50% (w/v). Data are shown as mean ± SD and results were considered statistically different if $p \leq 0.05$ (*).

In general, phage reduced the highest number of biofilm cells in a short (6 h) period and was more effective than low (25% (w/v)) C1 and Manuka honey concentrations at this timepoint. However, 50% (w/v) C1 honey concentration alone, at 6 h, was able to cause a higher, and statistically significant ($p \leq 0.05$) biofilm destruction than phage alone. The same observation was not, however, registered for Manuka, as similar reductions ($p > 0.05$) were obtained comparing with phage. Prolonging the treatment period to 12 h and 24 h led to a reduction in phage efficacy and an overall improvement of the action of honey in decreasing the number of biofilm cells. Nevertheless, there was no statistically significant difference ($p > 0.05$) between the log reductions achieved after 12 h and 24 h either 25% (w/v) or 50% (w/v) C1 honey concentrations.

Results and Discussion

Several works have pointed that phage therapy is more effective in shorter periods of time, since extended treatments can lead to regrowth of bacteria in biofilms [156,157]. For instance, Chibeu (2012) tested the effect of three different phages on the control of 48 h-old established biofilms of an uropathogenic *E. coli* strain and observed that for all the phages tested the largest percentage reduction of biofilm biomass occurred after 8 h of incubation [158]. Regrowth of *E. coli*, observed in this work, beyond 6 h of phage treatment can be due to the emergence of phage-resistant variants, which has been pointed as one of the major disadvantages of phage therapy [159]. Possible explanations for the appearance of phage resistance are bacterial dynamic adaptation or the development of bacterial tactics to avoid phage infection. These tactics are based on the inhibition of the initial stage of phage infection, during adsorption to the host receptors probably explained by changes on the receptors of the bacterial cell surface. Another reason can be the occurrence of changes in biofilm three-dimensional (3D) conformation, as insensitive mutants continue to thrive and secrete EPS, masking the host receptors [160].

Comparing the results of C1 with Manuka on *E. coli* biofilm cells, for each timepoint, they were statistically similar ($p > 0.05$), despite the fact that a maximum reduction of 7 log was achieved when Manuka was used alone at 50% (w/v). These results led to the conclusion that the active compounds in both C1 and Manuka honey were able to diffuse through the EPS matrix of established *E. coli* biofilms reaching and causing damage to the bacterial cells as reported previously [113,149]. For instance, Lee et al. (2011) demonstrated that even at low concentrations honey was able reduce the colonization and virulence of a pathogenic *E. coli* strain. In that study, it was also reported that honey did not harm the commensal *E. coli* strain, unlike antibiotics [161]. Besides, a study by Rabie et al. (2016) tested the ultrastructural effect of MGO at different concentrations (0.5, 1, and 2 mM) on *E. coli* morphology. The results obtained showed that 1 mM MGO was sufficient to reduce substantially the fimbriae and flagella, not completely eliminating them, although in some cases the flagella was even lacking. Moreover, MGO at 2 mM led to fimbriae and flagella disappearance, bacteria shrinkage, and loss of membrane integrity. As so, these results suggest that MGO can be responsible for modifications in the architecture of both bacterial fimbriae and flagella, limiting bacterial adherence and motility [162]. As it was already stated, both C1 and Manuka honey demonstrated a high MGO concentration (table 4.1), being highlighted that both concentrations were higher than 2 mM, which is approximately 80.61 $mg.kg^{-1}$.

Thus, the higher efficacy of higher concentrations of C1 and Manuka honeys (50% (w/v)) is in accordance with the expected, as, there is a greater exposure of the bacterial cells to the antimicrobial

substances of honey, such as MGO. For instance, the same was observed previously by Cooper et al (2009) for Manuka honey, although against *P. aeruginosa* biofilms [163].

Regarding combination of C1 honey with phage, synergistic interaction between 25% (w/v) honey and EC3a were found at 12 h and 24 h. 25% (w/v) C1 combined with EC3a led to higher biofilm biomass reductions (4.0 log) at 12 h. Phage together with 50% (w/v) C1 led to a maximum reduction of 6.3 log at 24 h. Phage combined with both Manuka concentrations achieved maximum reductions at 24 h, approximately 4.1 and 6.3 log reduction, with 25% (w/v) and 50% (w/v), respectively. Synergistic interaction was also found between 25% (w/v) Manuka and EC3a at 24 h.

In general, for the higher honey concentrations no synergy between honey and phage was observed, indicating that honeys antimicrobial properties did not profit with the phage addition and vice versa, as 50% (w/v) achieved alone the almost total eradication of *E. coli* biofilm cells after 24 h of treatment. Conversely, this synergistic effect was observed with 25% (w/v) honey when combined with phage, being suspected that honey allowed the biofilm cells to be more accessible to EC3a. Both honeys might be damaging cells by compromising the cell membrane and consequently facilitating phage action as observed previously by TEM and flow cytometry [113,149].

4.5. *Ex vivo* control of monospecies biofilms

Despite the advantages that *in vitro* studies provide, they could also imply limitations being the main one being poor reliability, since the conditions used and the environment adopted are not similar with the reality. As so, *ex vivo* tests using a pig skin model in order to mimic the human skin, reducing the error of *in vitro* models were performed using the phages, and honey individually or combined. The formulations prepared were tested against *E. coli* and *P. aeruginosa* biofilms, both separately and co-cultured.

4.5.1. Control of *E. coli* monospecies biofilms *ex vivo*

Antibiofilm effect of phage EC3a, C1, and Manuka was tested against *E. coli* monospecies biofilms *ex vivo* (Figure 4.4).

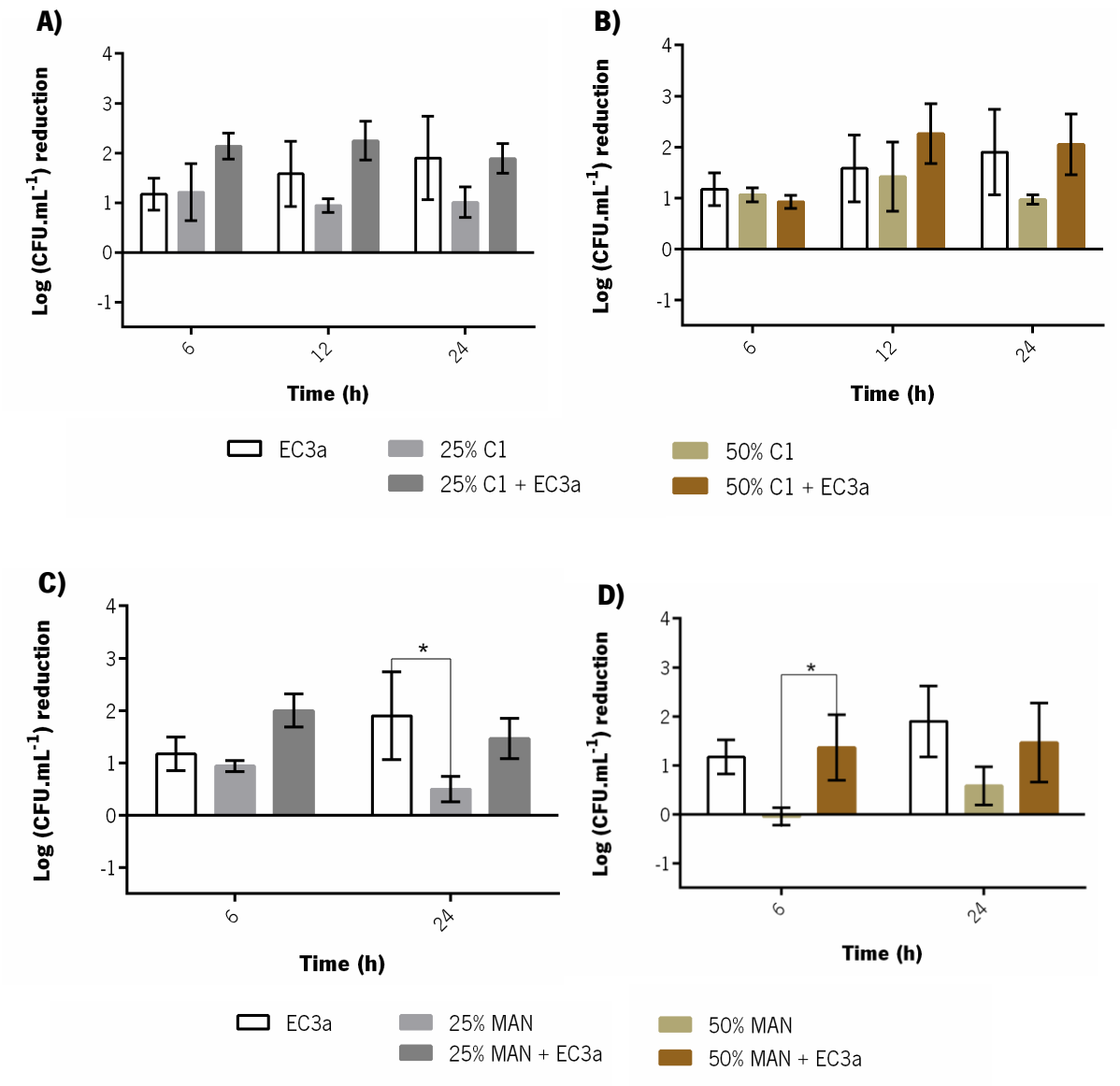


Figure 4.4 – Antibiofilm effect of EC3a phage, C1 and Manuka honeys, and honey-phage combinations against 24 h-old *E. coli* monospecies biofilms formed *ex vivo* .The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of *E. coli* viable cells. The honey concentrations tested were: **A)** C1 at 25% (w/v), **B)** C1 at 50% (w/v); **C)** Manuka (MAN) at 25% (w/v); **D)** Manuka (MAN) at 50% (w/v). Data are shown as mean ± SD and results were considered statistically different if $p \leq 0.05$ (*).

Using C1 honey, at both 25% (w/v) and 50% (w/v) concentrations resulted in similar biofilm biomass reduction though time, as differences were not significant ($p > 0.05$). As for Manuka honey, the results with 25% honey decreased slightly from 6 h to 24 h but these differences in log reductions were not significant ($p > 0.05$). In the case of 50% (w/v) Manuka, marginal antibacterial effect was observed at 6 h which only slightly increased to 0.6 log at 24 h ($p > 0.05$). These results contradict significantly with the ones obtained *in vitro* suggesting that honey might not be able to diffuse properly in the lack of media, such as observed in the microplates. Alternatively, the biofilm structure in pig skin can be different from the 3D biofilm structure formed in microplates with a different degree of matrix that can impair the diffusion of honey through the biofilm to reach and destroy the cells. Furthermore, surface

roughness is known to play a role on the protection of biofilms with bacteria living in irregularities avoiding mostly mechanical forces [164] . Although these surface irregularities could also be protecting biofilm cells towards chemical agents, such as honey.

Figure 4.4 only shows the log reduction of biofilm cells, which is calculated taking into account the number of cells in the control samples at each timepoint and therefore does not show the overall colonization of the pig skin by *E. coli*, which was 100 fold higher than in the microplates (data not shown). This suggests that the difference in surface roughness and hydrophobicity was sufficient to alter the mechanisms of gene expression (including genes related with motility and attachment) [165], secretion of EPS, among other factors which consequently led to different interaction between the antimicrobial compounds in honey and *E. coli*. These changes will influence the entire morphology of the biofilm and cell behavior, determining whether the bacterium is more repulsed or attracted to the substrata. The surface roughness is an important factor since more rough materials tend to promote bacterial adhesion, as microorganisms tend to adhere to the irregularities, as it was already mentioned [166]. Therefore, it was already anticipated that colonization would be better to porcine skin, a surface with more irregularities, than to polystyrene microplates. Despite this difference in colonization of pig skin and polystyrene (microplates), the MOI was maintained unaltered in both models used.

Contrarily to the *in vitro* results with phage alone, where a substantial effect was perceived at 6 h but decreased over time, the antibiofilm effect of phage EC3a against *E. coli* in the *ex vivo* model increased slightly throughout time, from approximately 1.2 log at 6 h to 1.9 log at 24 h. Although the reduction *in vitro* at 6 h was higher, in the *ex* vivo experiments after 24 h phages continued to play a role in decreasing the viable cells. The absence of liquid media, present in *in vitro* assays, where detached biofilm cells proliferate and due to a possibly lower *E. coli* growth rate in the *ex vivo* model between 6 h and 24 h after treatment are possibly limiting the emergence of phage insensitive phenotypes. As a result, phages continue to kill and control biofilm cells at all timepoints.

As for the combination of EC3a with both concentrations of C1, although the results were not statistically different ($p > 0.05$) compared to the other conditions tested, using both 25% (w/v) and 50% (w/v) C1 combined with EC3a resulted in overall reductions higher than the action of each agent individually. Apart from the result obtained at 6 h with 25% (w/v) Manuka honey and phage, there was no advantage in the use of a combinatory approach since the reduction values obtained were lower than phage alone. It needs however to be highlighted that even though 25% (w/v) concentration combined with phage was not statistically different from 50% (w/v) combination with phage ($p > 0.05$) the overall conclusion is that honey can be diluted without losing antibacterial efficacy.

Although the source and size of the pig skins were maintained throughout the experiments, the thickness was not possible to be maintained. Skin, as a biological surface, differs from individual to individual and also differs in virgin and damaged skin accounting for differences in nanohardness, elastic modules, stratum corneum thickness, etc. [167]. In this work, skin samples from several pigs were used, and, despite that all parts of the pig whole skin used were from the abdomen, the thickness was not always the same due to the fact that the cut was made more deeply at one times than others which also contributed to the differences observed between experiments. This high variability between pig skin samples could be counted as one disadvantage for the *ex vivo* model, since this leads to different biofilms structures between assays, being impossible to form equal biofilms between experiments. However, when comparing with the reality, human skin also varies from individual to individual, which is one factor that should to be taken into account in the laboratory.

Some studies have suggested that hydrophobicity can even have a higher influence than surface roughness in surface colonization. In general, hydrophilic materials are favorable for cell attachment when bacteria have larger surface energy than the liquid in which they are suspended. However, the contrary is more common to happen, as bacterial surface energy is normally inferior to the surface energy of the liquids. This mismatch leads to cell adhesion preferentially to hydrophobic materials [165]. According to Elkhyat et. al (2004), human skin contact angle is hydrophobic (91°) [168] and therefore it is expected that pig skins will also by hydrophobic. Polystyrene, on the other hand, is generally more hydrophilic than skin having a contact angle between 73° and 90° [169–171]. Besides these factors, different growth conditions were used for the *ex vivo* model in comparison with the *in vitro*. *Ex vivo* experiments were performed in a humid environment to promote biofilm formation [172], and were performed in a lesser media volume than *in vitro* studies.

4.5.2. Control of *P. aeruginosa* monospecies biofilms *ex vivo*

Ex vivo assays with *P. aeruginosa* were performed using phage PAO1-D, C1 honey and their combination (Figure 4.5).

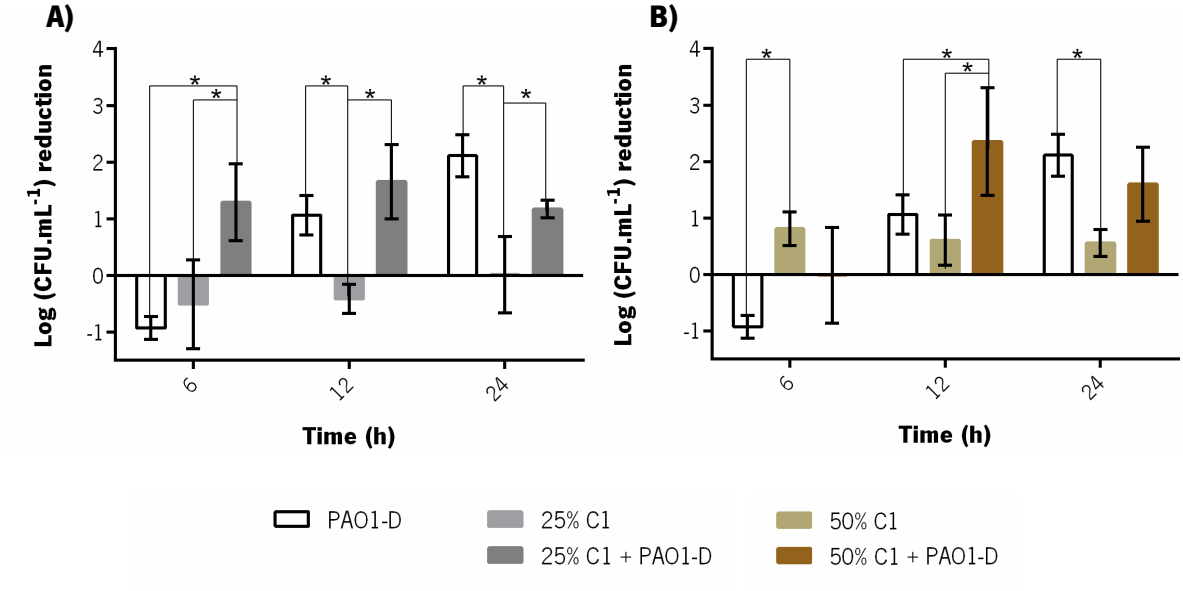


Figure 4.5 – Antibiofilm effect of PAO1-D phage, honey, and honey-phage combinations against 24 h-old *P. aeruginosa* monospecies biofilms formed *ex vivo*. The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of *P. aeruginosa* viable cells. The honey concentrations tested were: **A)** C1 at 25% (w/v); **B)** C1 at 50% (w/v). Data are shown as mean ± SD and results were considered statistically different if $p \leq 0.05$ (*).

Both 25% (w/v) and 50% (w/v) C1 alone did not reduce significantly ($p > 0.05$) the biofilm biomass through time. In fact, the maximum biofilm reduction with C1 honey (0.8 log) was achieved for 50% (w/v) after 6 h of treatment. These results are in accordance with a previous study that demonstrated that Medihoney did not have a significant effect on PAO1 biofilm cells adhered to pig skin explants 24 h after exposure [173]. In general, *P. aeruginosa* biofilms were harder to control with honey than the *E. coli* biofilms. The low sensitivity of *P. aeruginosa* pathogenic strains to honey might be a cause of the bacterial nature. It is known that *P. aeruginosa* cell wall has low permeability to antimicrobial compounds. Also, this bacterial species has the genetic capacity to express resistant mechanisms and mutation in chromosomal genes which regulate resistance genes [100]. Besides, reported multidrug resistant bacteria are usually provided with an intrinsic ability to withstand the effects of MGO, as they are often able to repair their DNA and possess sufficiently high levels of detoxification enzymes [133]. The two detoxification enzymes are believed to be the metalloenzymes GlxI and GlxII (Glyoxalases I and II) [174]. Furthermore, this hypothesis is supported by the detection of three fully functional GlxI homologs, instead of one, on *P. aeruginosa* genome [175]. Moreover, it is believed that *P. aeruginosa* bacterium is able to grow in an environment with higher MGO levels. A study led by Kilty in 2011 tested the effect of different MGO concentrations on different strains of *P. aeruginosa* biofilms, with a range of 1800 $mg.kg^{-1}$ to a range of 7300 $mg.kg^{-1}$. According to these tests, to reduce the biofilm biomass of some of the *P. aeruginosa* strains it was required a MGO concentration of 3600 $mg.kg^{-1}$ and others even needed 7300 $mg.kg^{-1}$ [176]. A study led by Lu in 2013 supports this hypothesis, where

Results and Discussion

P. aeruginosa had a higher tolerance to MGO than *Bacillus subtilis*, *E. coli*, and *S. aureus* [177]. As it was reviewed in the section 2.5 of the General Introduction, MGO is able to interact with bacterial cells, altering their architecture, causing membrane leakage and oxidative stress, which consequently leads to cell death [101]. Besides, it has already been mentioned in section 4.4, to explain the effect of honey in *E. coli* biofilms, that MGO can modify the architecture of both bacterial fimbriae and flagella, limiting bacterial adherence and motility [162]. In fact, Manuka honey was shown to induce *P. aeruginosa* de-flagellation, decreasing its swarming and swimming motility [178]. Despite these reports, several other studies have shown that *P. aeruginosa* strains are sensitive to different honeys. For instance, Merckoll et. al (2009) tested the effect of two honeys in biofilms of different strains that appear on chronic wounds, among them a *P. aeruginosa* strain. The results obtained showed that biofilm-embedded *P. aeruginosa* was susceptible to both honeys, although a higher concentration of the Norwegian forest honey than the commercial honey tested was required to obtain bactericidal effects [179].

Opposingly to *E. coli* biofilms, *P. aeruginosa* similar biofilm cell concentrations were obtained *ex vivo* and *in vitro* (approximately 10^8 CFU.ml^{-1} [115]). *P. aeruginosa* is known to be provided with different properties and mechanisms than *E. coli*. These properties, reviewed in the section 2.2.1, leads to a different biofilm structure comparatively to *E. coli*.

PAO1-D phage antibiofilm effect against PAO1 increased through time, akin experiments with the *ex vivo* treatment of *E. coli* with EC3a phage. At 6 h phage did not have a significant effect on biofilm cells nevertheless at 24 h a significant ($p \leq 0.05$) (2.1 log) biofilm cells reduction was achieved.

Regarding the combination of PAO1-D with 25% (w/v) C1, a maximum effect was achieved at 12 h (1.7 log), however these results were not statistically significant throughout time ($p > 0.05$). On the other hand, the combination of 50% (w/v) C1 with PAO1-D led to a maximum reduction of approximately 2.4 log at 12 h ($p \leq 0.05$). According to previous experiments performed *in vitro*, the efficacy of *P. aeruginosa* phages tend to decrease over time due to the emergence of phage resistant phenotypes with defective LPS production (mutations in the *galU* and *pil* genes) in phages that use LPS as primary receptor [156,159,180]. However, in the experiments *ex vivo* the emergence of phage resistant phenotypes might be decreasing as already observed in the *E. coli* experiments.

In general, the results of this study point to a better *P. aeruginosa* biofilm control using either combination of honey-phage or only phage (the results between these formulations were normally not significant ($p > 0.05$)). A synergistic effect was only noticed at 12h with 50% (w/v) C1 combined with phage. This suggests that the bactericidal effect of honey formulations against *P. aeruginosa* depend on the honey concentration used.

4.6. Zeta Potential of 24 h-old biofilm cells formed *in vitro*

Zeta potential (ζ-potential) can be measured in order to characterize the electric properties of bacterial cells that are suspended in liquids, being defined as the electrical potential between the interface of the cell and the stationary layer of this surrounding fluid attached to the bacterial cell [181]. As the envelope of a bacterial cell behaves in order to maintain the cell physiological functions, acting as a barrier and providing selective permeability, ζ-potential variation was considered in this work, an indicator of membrane damage, predicting the cell subsequent death. [182]. In fact, Soni et al. measured the ζ-potential of several strains grown at different conditions (nutrient-starved, grown in different media and even dead bacteria) and concluded that surface charge is a function related to the viability and nutrient state of the cells [183].

Measurements of ζ-potential were carried out in 24 h-old biofilm cells of both *E. coli* and *P. aeruginosa* (Figure 4.6) after 6, 12 and 24 h of treatment with honey and phage.

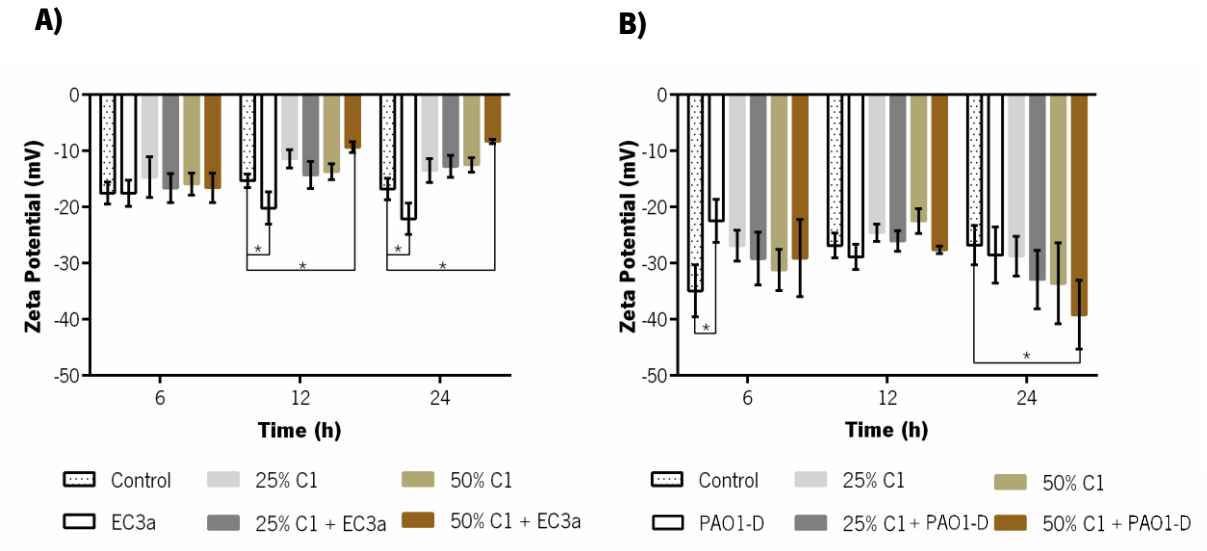


Figure 4.6 – ζ-potential (mV) of both untreated and treated (phage, C1 honey and phage-C1 combination) biofilms. The xx axis presents the different timepoints evaluated and the yy axis presents the ζ-potential of viable biofilm cells of: **A)** EC434 strain; **B)** PAO1 strain. Data are shown as mean ± SD and results were considered statistical different when $p \geq 0.05$ (*).

The contribution of phage and C1 honey to the background conductivity of both strains biofilm cells was found to be negligible with a variation of no more than 0.3 $mS.cm^{-1}$ (data not shown). All ζ-potential measurements were performed immediately following sample dilution (1:10) in Milli-Q™ water.

At 6 h, the average ζ-potential of untreated *E. coli* and *P. aeruginosa* strains was found to be -17.5 mV and -34.9 mV, respectively. The negative values are attributed to the negatively charged LPS of Gram-negative bacteria [182,184] particularly to the presence of anionic groups and O-antigen in the LPS

membrane [182]. The difference in electronegativity between untreated *E. coli* and *P. aeruginosa* was maintained throughout time.

In the case of the *E. coli* strain, at 6 h, both control and phage treated samples had statistically similar ($p > 0.05$) negative ζ-potential values. Both concentrations of honey showed a surface charge neutralizing effect ($p > 0.05$). With the exception of phage-treated biofilm cells, a slight increase of ζ-potential (to less negative values) after 12 h of treatment was observed. Statistical differences along time between treatments were obtained for phage and combined phage-honey (50% (w/v)) treated biofilm cells ($p \leq 0.05$). This suggests that *E. coli* biofilm cells are less sensitive to phages than to honey.

In the case of 24 h-old *P. aeruginosa* biofilm cells, at 6 h, untreated samples had more negative ζ-potential values. Also, at this timepoint it was noticed significantly similar ($p > 0.05$) results, except for phage treated samples that demonstrate a significant increase of ζ-potential values ($p \leq 0.05$). The two concentrations of honey tested (25% (w/v) and 50% (w/v)) also impaired similar surface charge neutralizing effect as observed for *E. coli*. With the exception of phage-treated biofilm cells, samples analyzed after 12 h of treatment yielded a slight increase of ζ-potential (less negative values), although statistically similar ($p > 0.05$). After 24 h of treatment, statistical differences ($p \leq 0.05$) in comparison to the previous timepoints were only obtained for phage and combined phage-honey (50% (w/v)). With the exception of phage, all other samples resulted in a decrease of ζ-potential to more negative values. As the bacterial envelope has the role to maintain the membrane structure in order to prevent cell death and compromising bacterial functions, a lower ζ-potential for the treatment samples at 24 h could mean that *P. aeruginosa* membrane was more successful in the homeostasis process than *E. coli*.

The synergistic effect observed in the biofilm experiments is corroborated by the ζ-potential measurements, that showed less negative values when phage is combined with honey, which leads to the hypothesis that the bacterial envelopes are being compromised, affecting bacterial adherence and easing the process of damaging and destroying *E. coli* cells.

4.7. Control of multispecies biofilms *ex vivo*

Bacterial physiology, morphology, metabolism, and architecture in simple biofilm cells can result in misleading conclusions. Interactions between different species involved in the same microbial community may trigger different bacterial mechanisms that can be either advantageous to both strains,

such as conjugation, coaggregation, and antimicrobial protection or harmful to one or more populations, like pH variations or toxin production [185]. Here, the antibiofilm effect of phage, C1 honey and their combination was tested *ex vivo* against dual-species 24 h-old *E. coli* and *P. aeruginosa* biofilms and the effect are reported in terms of log reductions on each bacterium individually (Figures 4.7 and 4.8, respectively).

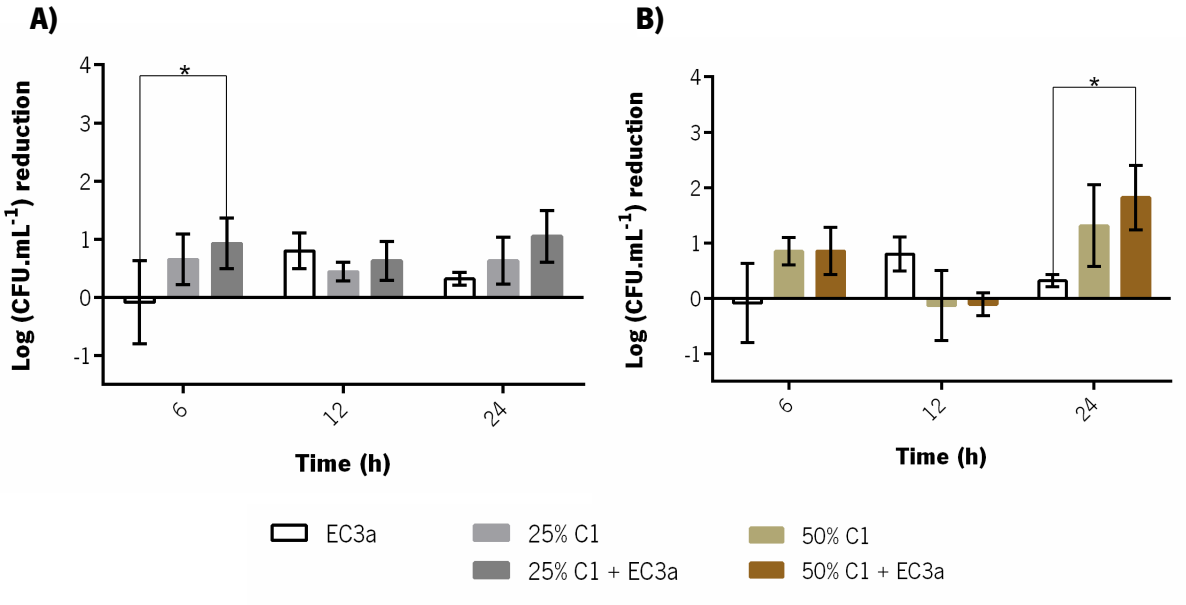


Figure 4.7 – Antibiofilm effect of EC3a phage, C1 honey, and honey-phage combinations against dual-species 24 h-old biofilms formed *ex vivo*. The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of *E. coli* viable cells. The honey concentrations tested were: **A)** C1 at 25% (w/v); **B)** C1 at 50% (w/v). Data are shown as mean ± SD and results were considered statistically different if $p \leq 0.05$ (*).

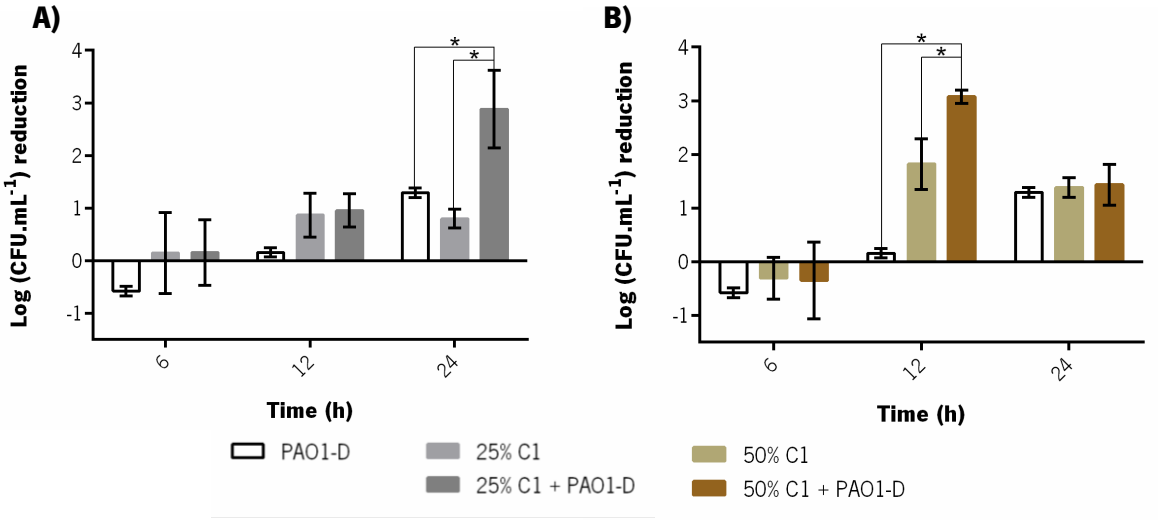


Figure 4.8 – Antibiofilm effect of PAO1-D phage, C1 honey, and honey-phage combinations against dual-species 24 h-old biofilms *ex vivo*. The xx axis presents the different timepoints evaluated and the yy axis presents log reductions of *P. aeruginosa* viable cells. The honey concentrations tested were: **A)** C1 at 25% (w/v); **B)** C1 at 50% (w/v). Data are shown as mean ± SD and results were considered statistically different if $p \leq 0.05$ (*).

Results and Discussion

Untreated *E. coli* biofilm cells did not profit when co-cultured with *P. aeruginosa*, as the average log was around 7.5 for simple biofilm at 6 h in comparison with 4.5 log for mixed biofilms (data not shown). On the other hand, PAO1 appeared to have a better adaptation against the presence of another bacterium, as the concentration did not differ significantly (data not shown). This observation is in accordance with other studies that point out that any types of interspecies relations analyzed are usually beneficial for *P. aeruginosa* strains, promoting its survival [186,187]. For instance, a study by Cerqueira et al. (2013) observed that *E. coli* and *P. aeruginosa* biofilms had comparable cultivability values when mono-cultured while when mixed, *E. coli* revealed a clear cultivability decrease [185]. The hypothesis that explain these results are nutrient competition between strains, effect of QS regulators, other secondary products, and bacteriocins [187].

In terms of viable cell reductions in *E. coli*, 25% (w/v) C1 alone did not reduce significantly the biofilm viable cells ($p > 0.05$) throughout time, while with 50% (w/v) C1 alone, the biofilm cells decreased by 1.3 log at 24 h, having only a significant effect at this timepoint ($P \leq 0.05$). On the other hand, the antibiofilm effect of EC3a phage against *E. coli* in mixed biofilms achieved the maximum action at 12 h (0.8 log reduction), although this value was not significant in comparison with the other tested timepoints. The combination of phage with C1 honey at 25% (w/v) reduced between 0.9 log and 1 log throughout the whole experiment ($p > 0.05$). The combination of phage with 50% (w/v) C1 led to the maximum biofilm reduction of 1.8 log at 24 h, after a previous lower antimicrobial effect at 12 h where the biofilm cell numbers increased (0.1 log) instead of decreasing. Overall and contrarily to the monospecies biofilm results, in mixed biofilms, no synergistic effect of combined therapy was observed that targeted *E. coli*.

Regarding viable cell reductions of *P. aeruginosa* when present in dual-species biofilms, 25% (w/v) C1 alone did not have a significant antibiofilm effect (<1 log reduction of viable cells), 50% (w/v) C1 alone caused maximum biofilm reduction (1.8 log) at 12 h, that was statistically significant from the other timepoints ($P \leq 0.05$). On the other hand, PAO1-D phage effect against *P. aeruginosa* biofilm cells increased significantly through time until 24 h (1.3 log) ($P \leq 0.05$), similarly to the result observed for monospecies *P. aeruginosa* biofilms cells treated with phage. For the combination of phage with C1 honey at 25% (w/v), the reductions started with approximately 0.2 log at 6 h that increased up to around 2.9 log after 24 h and these results were statistically significant between the different timepoints ($p \leq 0.05$). For 50% (w/v) C1 combined with phage, at 6 h the antibiofim action was negligible, and the maximum reduction was obtained at 12 h (3.1 log), decreasing to less than 1.5 log at 24 h. A comparison of these results (Figure 4.8) with the monospecies biofilm results with the same agents

(Figure 4.5) show that both honey and phage were more effective in controlling *P. aeruginosa* when the bacterium formed biofilms together with *E. coli*. Moreover, synergistic effects were observed for both honey concentrations combined with phage although at different time points (24 h for 25% (w/v) and 12 h for 50% (w/v)). These results suggest that in this context, honey and phage are enhancing each other's antimicrobial properties.

When analyzing log reduction of biofilm cells, it is important to consider the recovery rate of bacteria. In mathematical terms, 1 log reduction is equivalent to a 90% reduction of the biofilm biomass, as, for example, from 10^8 to 10^7 CFU.mL^{-1} corresponds to a loss of 9×10^7 CFU.mL^{-1} of bacteria. This reduction is considered insignificant in a biological position, since, for example, *P. aeruginosa* have an average generation doubling time of approximately 40 to 50 minutes. As so, full recovery would be accomplished in less than 3 hours [173]. However, multiple applications of the proposed treatment along time are likely to overcome this problem.

5. Main Conclusions and Suggestions for Forthcoming Work

5.1. Main Conclusions

One of the main goals of this project was to develop a phage-honey formulation that was successful in the eradication of *E. coli* and *P. aeruginosa* biofilm cells, being important to evaluate the eventual synergistic effect between these compounds. As so, after the access of *in vitro* and *ex vivo* experiments, it was found that both phages used in this work (EC3a and PAO1-D) demonstrated potential in the treatment of *E. coli* and *P. aeruginosa*, respectively. When the pig explants were used as surface for bacterial colonization, these phages showed higher potential against biofilms. However, honey used against *E. coli* biofilms showed a higher and more significant biofilm cells reduction *in vitro*. Despite honey not being as effective against biofilm cells as desirable in pig skin explants, honey still provides other properties that are interesting for wound treatment purposes, such its role in tissue regeneration.

In average, 12 h after *ex vivo* treatment, results demonstrated better biofilm biomass reductions when using honey-phage combination for *P. aeruginosa*. The antibiofilm *ex vivo* effect of C1 at both concentrations with PAO1-D against dual-species 24 h-old biofilms resulted in a synergistic effect.

The comparison of the Portuguese honey used in this work (C1) with the commercial Manuka honey was performed to find a non-commercial honey that imparted similar or even better antimicrobial properties. As so, it was concluded that C1 honey demonstrated high antibiofilm potential when comparing its results with the results obtained for Manuka. In some cases C1 honey demonstrated an even higher antibiofilm effect against *E. coli*.

In order to have a synergistic effect when phage is combined with honey it is important to guarantee that the phage remains viable for at least a certain period of time. Some of the properties of honey, such as its acidity and sugar content might contribute to the destruction of phages capsids. As so, it is concluded that it is important to find a balance on the selection of the honey. Both phages tested demonstrated a relatively high tolerance to honey when comparing the results with previous studies, since, in general, even after 12 h upon contact there was still a high phage concentration remaining viable.

Finally, it is concluded that this work provided novel insights into alternative strategies to control chronic wounds infected with both *E. coli* and *P. aeruginosa* pathogens using phage-honey formulations. Multiple applications of the proposed wound treatment can possibly be even more effective. Thus, honey-phage combinations have the potential to be used for therapeutic purposes.

5.2. Future work

Despite the conclusions noted from this work, it is highlighted that there are still some forthcoming experiments that need to be developed:

- In order to find a better phage-honey combination, it is believed that more *in vitro* and *ex vivo* tests are required using different honeys and phages. Thus, isolation of new phages and physicochemical characterization of other honeys are required;
- Strategies in order to prevent phage inviabilization by honey should be found. For instance, phage encapsulation strategies or the product should be commercialized in separate;
- As it was previously mentioned other bacterial species are common to appear on chronic wounds, such as *Acinetobacter baumanii* or *S. aureus*. Thus, it is important to also test the effect of the phage-honey formulations against both mono and multispecies biofilms;
- It is needed to test *ex vivo* older biofilms (48 h, 72 h, among others) since it is more usual for the patient to start the treatment at later stages of biofilms;
- The development of an *in vivo* model (e.g. mice model) should also be relevant, in order to considerate for instance, the effect of the immune response, wound fluid, among other factors.
- the role of honey on tissue regeneration and on the inflammatory response should also be evaluated and taken into account using an *in vivo* model;
- Tests in microaerophilic (low oxygen concentrations) conditions should be relevant, since the development of biofilms in deeper tissue structures where the amount of oxygen is reduced is common.

REFERENCES

1. Yang, Q. *et al.* Development of a novel ex vivo porcine skin explant model for the assessment of mature bacterial biofilms. *Wound Repair Regen.* **21,** 704–714 (2013).
2. Estronca, L. & Ferreira, L. in *Stem Cell Manufacturing* (eds. Cabral, J. M. S., da Silva, C. L., Chase, L. G. & Chase, M. M.) 291–312 (Elsevier, 2016).
3. Brem, H. *et al.* Molecular markers in patients with chronic wounds to guide surgical debridement. *Mol. Med.* **13,** 30–39 (2007).
4. Snyder, D. L., Sullivan, N. & Schoelles, K. M. *Skin Substitutes for Treating Chronic Wounds*. *Skin Substitutes for Treating Chronic Wounds* (Agency for Healthcare Research and Quality, 2012).
5. Stroncek, J. D. & Reichert, W. M. in *Indwelling Neural Implants: Strategies for Contending with the In Vivo Environment* (ed. Reichert, W. M.) (CRC Press/Taylor & Francis, 2008).
6. Garcia-Orue, I. *et al.* LL37 loaded nanostructured lipid carriers (NLC): A new strategy for the topical treatment of chronic wounds. *Eur. J. Pharm. Biopharm.* **108,** 310–316 (2016).
7. Diegelmann, R. F. & Evans, M. C. Wound healing: an overview of acute, fibrotic and delayed healing. *Front. Biosci.* **9,** 283–289 (2004).
8. Frykberg, R. G. & Banks, J. Challenges in the Treatment of Chronic Wounds. *Adv. wound care* **4,** 560–582 (2015).
9. McCarty, S., Jones, E. M., Finnegan, S., Woods, E. & Cochrane, C. A. in *Biofilms in Infection Prevention and Control* (eds. Percival, S. L., William, D., Cooper, T. & Randle, J.) 339–358 (Elsevier, 2014).
10. Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *J. Cell Sci.* **123,** 4195–4200 (2010).
11. Zhao, R., Liang, H., Clarke, E., Jackson, C. & Xue, M. Inflammation in Chronic Wounds. *Int. J. Mol. Sci.* **17,** E2085 (2016).
12. O'Meara, S. *et al.* in *Cochrane Database of Systematic Reviews* (ed. O'Meara, S.) (John Wiley & Sons, Ltd, 2014).
13. Primary Care Dermatology Society. Leg ulcers. (2016). Available at: http://www.pcds.org.uk/clinical-guidance/leg-ulcers. (Accessed: 28th April 2017)
14. Foot Clinic of South Carolina. Diabetic Foot. Available at: http://www.footclinicsc.com/foot-disorders/diabetic-foot/. (Accessed: 28th April 2017)
15. Kirman, C. N. Pressure Injuries (Pressure Ulcers) and Wound Care. (2017). Available at: http://emedicine.medscape.com/article/190115-overview. (Accessed: 28th April 2017)
16. Morton, L. M. & Phillips, T. J. Wound healing and treating wounds. *J. Am. Acad. Dermatol.* **74,** 589–605 (2016).
17. Deufert, D. & Graml, R. Disease-specific, health-related quality of life (HRQoL) of people with chronic wounds—A descriptive cross-sectional study using the Wound-QoL. *Wound Med.* **16,** 29–33 (2017).
18. Clinton, A. & Carter, T. Chronic Wound Biofilms: Pathogenesis and Potential Therapies. *Lab. Med.* **46,** 277–284 (2015).
19. McGuckin, M. & Kerstein, M. D. Venous leg ulcers and the family physician. *Adv. Wound Care* **11,** 344–6 (1998).
20. Dyck, P. J. *et al.* The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. *Neurology* **43,** 817–824 (1993).
21. Young, M. J., Boulton, A. J., MacLeod, A. F., Williams, D. R. & Sonksen, P. H. A multicentre study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. *Diabetologia* **36,** 150–154 (1993).
22. Kumar, S. *et al.* The prevalence of foot ulceration and its correlates in type 2 diabetic patients: a population-based study. *Diabet. Med.* **11,** 480–484 (1994).

References

23. Cabezas-Cerrato, J. The prevalence of clinical diabetic polyneuropathy in Spain: a study in primary care and hospital clinic groups. Neuropathy Spanish Study Group of the Spanish Diabetes Society (SDS). *Diabetologia* **41,** 1263–1269 (1998).
24. Weledji, E. P. & Fokam, P. Treatment of the diabetic foot - to amputate or not? *BMC Surg.* **14,** 83 (2014).
25. Boulton, A. J. M., Gries, F. A. & Jervell, J. A. Guidelines for the diagnosis and outpatient management of diabetic peripheral neuropathy. *Diabet. Med.* **15,** 508–514 (1998).
26. Schaper, N. C., Apelqvist, J. & Bakker, K. The international consensus and practical guidelines on the management and prevention of the diabetic foot. *Curr. Diab. Rep.* **3,** 475–479 (2003).
27. Armstrong, D. G., Lavery, L. A. & American Diabetes Association. *Clinical care of the diabetic foot*. (American Diabetes Association, 2005).
28. Posnett, J., Gottrup, F., Lundgren, H. & Saal, G. The resource impact of wounds on health-care providers in Europe. *J. Wound Care* **18,** 154 (2009).
29. Shea, J. D. Pressure sores: classification and management. *Clin. Orthop. Relat. Res.* 89–100 (1975).
30. Staas, W. E., Cioschi, H. M. & Cioschi, H. M. Pressure sores–a multifaceted approach to prevention and treatment. *West. J. Med.* **154,** 539–544 (1991).
31. Schiffman, J. *et al.* Operative debridement of pressure ulcers. *World J. Surg.* **33,** 1396–1402 (2009).
32. Mulcahy, L. R., Isabella, V. M. & Lewis, K. Pseudomonas aeruginosa biofilms in disease. *Microb. Ecol.* **68,** 1–12 (2014).
33. CDC. General Information| E.coli | CDC. *Centers for Disease Control and Prevention* (2015). Available at: https://www.cdc.gov/ecoli/general/index.html. (Accessed: 30th March 2017)
34. CDC. Acinetobacter in Healthcare Settings | HAI | CDC. *Centers for Disease Control and Prevention* (2010). Available at: https://www.cdc.gov/hai/organisms/acinetobacter.html. (Accessed: 30th March 2017)
35. CDC. Pseudomonas aeruginosa in Healthcare Settings | HAI | CDC. *Centers for Disease Control and Prevention* (2014). Available at: https://www.cdc.gov/hai/organisms/pseudomonas.html. (Accessed: 30th March 2017)
36. CDC. Staphylococcus aureus in Healthcare Settings | HAI | CDC. *Centers for Disease Control and Preventation* (2011). Available at: https://www.cdc.gov/hai/organisms/staph.html. (Accessed: 30th March 2017)
37. Gjødsbøl, K. *et al.* Multiple bacterial species reside in chronic wounds: a longitudinal study. *Int. Wound J.* **3,** 225–231 (2006).
38. Rodriguez-Bano, J. *et al.* Risk Factors and Prognosis of Nosocomial Bloodstream Infections Caused by Extended-Spectrum- -Lactamase-Producing Escherichia coli. *J. Clin. Microbiol.* **48,** 1726–1731 (2010).
39. Leimbach, A., Hacker, J. & Dobrindt, U. in *Curr. Top. Microbiol. Immunol.* **358,** 3–32 (2013).
40. van 't Wout, E. F. A. *et al.* Virulence Factors of Pseudomonas aeruginosa Induce Both the Unfolded Protein and Integrated Stress Responses in Airway Epithelial Cells. *PLOS Pathog.* **11,** e1004946 (2015).
41. Costerton, J. W., Geesey, G. G. & Cheng, K. J. How bacteria stick. *Sci. Am.* **238,** 86–95 (1978).
42. Gomes, L. C., Carvalho, D., Briandet, R. & Mergulhão, F. J. Temporal variation of recombinant protein expression in Escherichia coli biofilms analysed at single-cell level. *Process Biochem.* **51,** 1155–1161 (2016).
43. Sauer, K. The genomics and proteomics of biofilm formation. *Genome Biol.* **4,** 219 (2003).
44. Donlan, R. M. Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends*

Microbiol. **17,** 66–72 (2009).
45. Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. Biofilms as Complex Differentiated Communities. *Annu. Rev. Microbiol.* **56,** 187–209 (2002).
46. Kumar, P., Mishra, S. & Singh, S. Advanced acuity in microbial biofilm genesis, development, associated clinical infections and control. *J. des Anti-infectieux* **19,** 20–31 (2017).
47. Lee, K. & Yoon, S. S. Pseudomonas aeruginosa biofilm, a programmed bacterial life for fitness. *J. Microbiol. Biotechnol.* (2017).
48. O'Toole, G. A. & Kolter, R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Mol. Microbiol.* **30,** 295–304 (1998).
49. Pratt, L. A. & Kolter, R. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* **30,** 285–293 (1998).
50. Chao, Y. & Zhang, T. Probing Roles of Lipopolysaccharide, Type 1 Fimbria, and Colanic Acid in the Attachment of Escherichia coli Strains on Inert Surfaces. *Langmuir* **27,** 11545–11553 (2011).
51. Vogt, J. & Schulz, G. E. The structure of the outer membrane protein OmpX from Escherichia coli reveals possible mechanisms of virulence. *Structure* **7,** 1301–1309 (1999).
52. Otto, K. & Hermansson, M. Inactivation of ompX causes increased interactions of type 1 fimbriated Escherichia coli with abiotic surfaces. *J. Bacteriol.* **186,** 226–234 (2004).
53. Kostakioti, M., Hadjifrangiskou, M. & Hultgren, S. J. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb. Perspect. Med.* **3,** a010306 (2013).
54. Klockgether, J. & Tümmler, B. Recent advances in understanding Pseudomonas aeruginosa as a pathogen. *F1000Res.* **6,** 1261 (2017).
55. Omar, A., Wright, J. B., Schultz, G., Burrell, R. & Nadworny, P. Microbial Biofilms and Chronic Wounds. *Microorganisms.* **5,** (2017).
56. Valentini, M. & Filloux, A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *J. Biol. Chem.* **291,** 12547–12555 (2016).
57. Colvin, K. M. *et al.* The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. *Environ. Microbiol.* **14,** 1913–1928 (2012).
58. Azeredo, J. *et al.* Critical review on biofilm methods. *Crit. Rev. Microbiol.* **43,** 313–351 (2016).
59. Ceri, H. *et al.* The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* **37,** 1771–1776 (1999).
60. Sun, Y., Dowd, S. E., Smith, E., Rhoads, D. D. & Wolcott, R. D. In vitro multispecies Lubbock chronic wound biofilm model. *Wound Repair Regen.* **16,** 805–813 (2008).
61. Werthén, M. *et al.* An in vitro model of bacterial infections in wounds and other soft tissues. *APMIS* **118,** 156–164 (2010).
62. Ganesh, K. *et al.* Chronic Wound Biofilm Model. *Adv. wound care* **4,** 382–388 (2015).
63. Sullivan, T. P., Eaglstein, W. H., Davis, S. C. & Mertz, P. The pig as a model for human wound healing. *Wound Repair Regen.* **9,** 66–76 (2001).
64. Steed, D. L. Debridement. *Am. J. Surg.* **187,** S71–S74 (2004).
65. Gokoo, C. A primer on wound bed preparation. *J. Am. Col. Certif. Wound Spec.* **1,** 35–39 (2009).
66. Abbade, L. P. F. & Lastoria, S. Venous ulcer: epidemiology, physiopathology, diagnosis and treatment. *Int. J. Dermatol.* **44,** 449–456 (2005).
67. Jones, V., Grey, J. E. & Harding, K. G. Wound dressings. *BMJ.* **332,** 777–780 (2006).
68. Simón, M. Active Leptospermum Honey: A Strategy to Prevent Chronic Wounds. *J. Nurse Pract.* **12,** 339–345 (2016).
69. Roberts, C. D., Leaper, D. J. & Assadian, O. The Role of Topical Antiseptic Agents Within

Antimicrobial Stewardship Strategies for Prevention and Treatment of Surgical Site and Chronic Open Wound Infection. *Adv. Wound Care* **6,** 63–71 (2017).

70. Fazli, M. *et al.* Nonrandom Distribution of Pseudomonas aeruginosa and Staphylococcus aureus in Chronic Wounds. *J. Clin. Microbiol.* **47,** 4084–4089 (2009).
71. Lipsky, B. A. *et al.* Antimicrobial stewardship in wound care: a Position Paper from the British Society for Antimicrobial Chemotherapy and European Wound Management Association. *J. Antimicrob. Chemother.* **71,** 3026–3035 (2016).
72. World Health Organization. About AMR. (2017). Available at: http://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance/about-amr. (Accessed: 24th March 2017)
73. Wu, H., Moser, C., Wang, H.-Z., Høiby, N. & Song, Z.-J. Strategies for combating bacterial biofilm infections. *Int. J. Oral Sci.* **7,** 1–7 (2015).
74. D'Hérelle, F. *Sur un microbe invisible antagoniste des bacilles dysentériques*. (Comptes rendus hebdomadaires des séances de l'Académie des Sciences, 1917).
75. Pires, D. P., Vilas Boas, D., Sillankorva, S. & Azeredo, J. Phage Therapy: a Step Forward in the Treatment of Pseudomonas aeruginosa Infections. *J. Virol.* **89,** 7449–7456 (2015).
76. Broxmeyer, L. Bacteriophages: Antibacterials with a future? *Med. Hypotheses* **62,** 889–893 (2004).
77. Eaton, M. D. & Bayne-Jones, S. Bacteriophage Therapy. *J. Am. Med. Assoc.* **103,** 1769 (1934).
78. Fleming, A. On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenzae. *Br. J. Exp. Pathol.* **10,** 226–236 (1929).
79. Shama, G. in *Advances in Applied Microbiology, vol.69* (eds. Laskin, A. I., Sariaslani, S. & Gadd, G. M.) 133–158 (Elsevier, 2009).
80. Chain, E. *et al.* Penicillin as a chemotherapeutic agent. *Lancet* **236,** 226–228 (1940).
81. Sillankorva, S. & Azeredo, J. in *Microbial Biofilms. Methods in Molecular Biology (Methods and Protocols), vol 1147* (ed. Donelli, G.) 277–285 (Humana Press, 2014).
82. Feiner, R. *et al.* A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat. Rev. Microbiol.* **13,** 641–650 (2015).
83. Pringle, C. R. Virus Taxonomy. *Arch. Virol.* **143,** 1449–1459 (1998).
84. Ninth Report of the International Committee on Taxonomy of Viruses in *Virus Taxonomy* (eds. King, A., Lefkowitz, E., Adams, M. J., et. al) 39–45 (Elsevier, 2012).
85. Krupovic, M., Prangishvili, D., Hendrix, R. W. & Bamford, D. H. Genomics of Bacterial and Archaeal Viruses: Dynamics within the Prokaryotic Virosphere. *Microbiol. Mol. Biol. Rev.* **75,** 610–635 (2011).
86. Pires, D. P., Oliveira, H., Melo, L. D. R., Sillankorva, S. & Azeredo, J. Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. *Appl. Microbiol. Biotechnol.* **100,** 2141–2151 (2016).
87. Nobrega, F. L., Costa, A. R., Kluskens, L. D. & Azeredo, J. Revisiting phage therapy: new applications for old resources. *Trends Microbiol.* **23,** 185–191 (2015).
88. Vilas Boas, D. *et al.* Discrimination of bacteriophage infected cells using locked nucleic acid fluorescent *in situ* hybridization (LNA-FISH). *Biofouling* **32,** 179–190 (2016).
89. Chan, B. K. & Abedon, S. T. Bacteriophages and their enzymes in biofilm control. *Curr. Pharm. Des.* **21,** 85–99 (2015).
90. Harper, D. *et al.* Bacteriophages and Biofilms. *Antibiotics* **3,** 270–284 (2014).
91. Hilary, S., Habib, H., Souka, U., Ibrahim, W. & Platat, C. Bioactivity of arid region honey: an in vitro study. *BMC Complement. Altern. Med.* **17,** 177 (2017).
92. Eteraf-Oskouei, T. & Najafi, M. Traditional and modern uses of natural honey in human diseases: a review. *Iran. J. Basic Med. Sci.* **16,** 731–742 (2013).

93. Pontes, M., Marques, J. C. & Câmara, J. S. Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase microextraction-gas chromatography–quadrupole mass spectrometry. *Talanta* **74,** 91–103 (2007).
94. Stewart, J. A., McGrane, O. L. & Wedmore, I. S. Wound Care in the Wilderness: Is There Evidence for Honey? *Wilderness Environ. Med.* **25,** 103–110 (2014).
95. Chirife, J., Herszage, L., Joseph, A. & Kohn, E. S. In vitro study of bacterial growth inhibition in concentrated sugar solutions: microbiological basis for the use of sugar in treating infected wounds. *Antimicrob. Agents Chemother.* **23,** 766–773 (1983).
96. Bang, L. M., Buntting, C. & Molan, P. The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing. *J. Altern. Complement. Med.* **9,** 267–273 (2003).
97. Rutala, W. A., Weber, D. J. & The Healthcare Infection Control Practices Advisory Committee. *Guideline for Disinfection and Sterilization in Healthcare Facilities Guideline for Disinfection and Sterilization in Healthcare Facilities*. (2008).
98. Brudzynski, K., Abubaker, K., Laurent, M. & Castle, A. Re-Examining the Role of Hydrogen Peroxide in Bacteriostatic and Bactericidal Activities of Honey. *Front. Microbiol.* **2,** 213 (2011).
99. da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O. & Fett, R. Honey: Chemical composition, stability and authenticity. *Food Chem.* **196,** 309–323 (2016).
100. Wasihun, A. G. & Kasa, B. G. Evaluation of antibacterial activity of honey against multidrug resistant bacteria in Ayder Referral and Teaching Hospital, Northern Ethiopia. *Springerplus* **5,** 842 (2016).
101. He, Y. *et al.* Study on the mechanism of antibacterial action of magnesium oxide nanoparticles against foodborne pathogens. *J. Nanobiotechnology* **14,** 54 (2016).
102. Mavric, E., Wittmann, S., Barth, G. & Henle, T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium)honeys from New Zealand. *Mol. Nutr. Food Res.* **52,** 483–489 (2008).
103. Alandejani, T., Marsan, J., Ferris, W., Slinger, R. & Chan, F. Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. *Otolaryngol. - Head Neck Surg.* **141,** 114–118 (2009).
104. Tan, H. T. *et al.* The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. *BMC Complement. Altern. Med.* **9,** 34 (2009).
105. Kwakman, P. H. S. *et al.* How honey kills bacteria. *FASEB J.* **24,** 2576–2582 (2010).
106. Tonks, A. J. *et al.* A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *J. Leukoc. Biol.* **82,** 1147–1155 (2007).
107. Bucekova, M. *et al.* Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation in vitro and in vivo. *Sci. Rep.* **7,** 7340 (2017).
108. Khalil, M. I., Sulaiman, S. A. & Gan, S. H. High 5-hydroxymethylfurfural concentrations are found in Malaysian honey samples stored for more than one year. *Food Chem. Toxicol.* **48,** 2388–2392 (2010).
109. Codex Alimentarius Committee on Sugars Codex standard 12. *Codex Standard for Honey Standards and Standard Methods*. (2001).
110. Grainger, M. N. C., Owens, A., Manley-Harris, M., Lane, J. R. & Field, R. J. Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part IV – Formation of HMF. *Food Chem.* **232,** 648–655 (2017).
111. Melo, F. C. de *et al.* Synthesis of 5-Hydroxymethylfurfural from Dehydration of Fructose And Glucose Using Ionic Liquids. *J. Braz. Chem. Soc.* **25,** 2378–2384 (2014).
112. Williamson, E. Synergy and other interactions in phytomedicines. *Phytomedicine* **8,** 401–409

(2001).
113. Oliveira, A. *et al.* Synergistic antimicrobial interaction of honey and phage against Escherichia coli biofilms. *Front. Microbiol.* (2017) (accepted).
114. Andrade, M. S. Avaliação da terapia fágica em biofilmes bacterianos presentes em feridas crónicas. (University of Minho, 2014).
115. Silva, C. In vitro evaluation of an antibiofilm product: a combination between bacteriophages and Portuguese honey. (University of Minho, 2017).
116. Sambrook, J. & Russell, D. W. *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, 2001).
117. Bogdanov, S. *Harmonised methods of the international honey commission*. *International Honey Commission* (2009).
118. Houston, D. *United States Standards for Grades of Extracted Honey*. (1985).
119. Adams, C. J. *et al.* Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* **343,** 651–659 (2008).
120. Smith, P. K. *et al.* Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85 (1985).
121. White, J. W. Spectrophotometric method for hydroxymethylfurfural in honey. *J. Assoc. Off. Anal. Chem.* **62,** 509–514 (1979).
122. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* **9,** ix–xv (2003).
123. Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E. & Johnson, R. P. in *Methods in molecular biology (Clifton, N.J.)* **501,** 69–76 (2009).
124. da Costa, A. *et al.* Development of Elastin-Like Recombinamer Films with Antimicrobial Activity. *Biomacromolecules* **16,** 625–635 (2015).
125. Ribeiro, H. G. Developing an antimicrobial product with Portuguese honey and bacteriophages. (University of Minho, 2016).
126. Naghili, H. *et al.* Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Vet. Res. forum an Int. Q. J.* **4,** 179–183 (2013).
127. Hunter, R. J. *Zeta potential in colloid science : principles and applications*. (1981).
128. Carvalho, C. A. L. *et al.* Physicochemical characteristics and sensory profile of honey samples from stingless bees (Apidae: Meliponinae) submitted to a dehumidification process. *An. Acad. Bras. Cienc.* **81,** 143–149 (2009).
129. Islam, A. *et al.* Physicochemical and antioxidant properties of Bangladeshi honeys stored for more than one year. *BMC Complement. Altern. Med.* **12,** 177 (2012).
130. Greenwood, D. *Antibiotic and chemotherapy : anti-infective agents and their use in therapy.* (Churchill Livingstone, 1997).
131. Eteraf-Oskouei, T. & Najafi, M. Traditional and modern uses of natural honey in human diseases: a review. *Iran. J. Basic Med. Sci.* **16,** 731–42 (2013).
132. Majtan, J., Bohova, J., Prochazka, E. & Klaudiny, J. Methylglyoxal May Affect Hydrogen Peroxide Accumulation in Manuka Honey Through the Inhibition of Glucose Oxidase. *J. Med. Food* **17,** 290–293 (2014).
133. Jervis-Bardy, J., Foreman, A., Bray, S., Tan, L. & Wormald, P.-J. Methylglyoxal-infused honey mimics the anti-Staphylococcus aureus biofilm activity of manuka honey: Potential Implication in Chronic Rhinosinusitis. *Laryngoscope* **121,** 1104–1107 (2011).
134. Atrott, J., Haberlau, S. & Henle, T. Studies on the formation of methylglyoxal from dihydroxyacetone in Manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* **361,** 7–11

(2012).
135. El Sohaimy, S. A., Masry, S. H. D. & Shehata, M. G. Physicochemical characteristics of honey from different origins. *Ann. Agric. Sci.* **60,** 279–287 (2015).
136. Alvarez-Suarez, J. M. *Bee Products - Chemical and Biological Properties*. (Springer Science+Business Media, 2017).
137. Mandal, M. D. & Mandal, S. Honey: its medicinal property and antibacterial activity. *Asian Pac. J. Trop. Biomed.* **1,** 154–160 (2011).
138. Moniruzzaman, M., Sulaiman, S. A., Khalil, M. I. & Gan, S. H. Evaluation of physicochemical and antioxidant properties of sourwood and other Malaysian honeys: a comparison with manuka honey. *Chem. Cent. J.* **7,** 138 (2013).
139. Yadata, D. Detection of the Electrical Conductivity and Acidity of Honey from Different Areas of Tepi. *Food Sci. Technol.* **2,** 59–63 (2014).
140. Oddo, P. L., Piazza, M., Sabatini, A. & Accorti, M. Characterization of unifloral honeys. *Apidologie, Springer Verlag* **26,** 453–465 (1995).
141. Tewari, J. & Irudayaraj, J. Quantification of Saccharides in Multiple Floral Honeys Using Fourier Transform Infrared Microattenuated Total Reflectance Spectroscopy. *J. Agric. Food Chem.* **52,** 3237–3243 (2004).
142. Lee, D.-C., Lee, S.-Y., Cha, S.-H., Choi, Y.-S. & Rhee, H.-I. Discrimination of Native Bee-Honey and Foreign Bee-Honey by SDS-PAGE. *Korean J. Food Sci. Technol.* **30,** 1–5 (1998).
143. Akkiraju, P. C., Shinde, M. B. & Dhamale, K. S. Antimicrobial activity of different honey samples and their protein estimation. *Int. J. Curr. Res.* **8,** 34587–34592 (2016).
144. Adams, C. J., Manley-Harris, M. & Molan, P. C. The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* **344,** 1050–1053 (2009).
145. Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G. & Read, R. R. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.* **66,** 86–92 (2002).
146. Zainol, M. I., Mohd Yusoff, K. & Mohd Yusof, M. Y. Antibacterial activity of selected Malaysian honey. *BMC Complement. Altern. Med.* **13,** 129 (2013).
147. Sherlock, O. *et al.* Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. *BMC Complement. Altern. Med.* **10,** 47 (2010).
148. George, N. M. & Cutting, K. F. Antibacterial Honey (Medihoney™): in-vitro Activity Against Clinical Isolates of MRSA, VRE, and Other Multiresistant Gram-negative Organisms Including Pseudomonas aeruginosa. *Wounds a Compend. Clin. Res. Pract.* **19,** 231–236 (2007).
149. Oliveira, A. *et al.* Synergistic antimicrobial interaction of honey and phage in Escherichia coli biofilms - Poster Presentation. *Center of Biological Engineering* 1 (2017). Available at: https://www.researchgate.net/publication/318983593_Synergistic_antimicrobial_interaction_between_honey_and_phage_against_Escherichia_coli_biofilms. (Accessed: 31st October 2017)
150. Leibo, S. P. & Mazur, P. Effect of osmotic shock and low salt concentration on survival and density of bacteriophages T4B and T4Bo1. *Biophys. J.* **6,** 747–772 (1966).
151. Jończyk, E., Kłak, M., Międzybrodzki, R. & Górski, A. The influence of external factors on bacteriophages–review. *Folia Microbiol. (Praha).* **56,** 191–200 (2011).
152. Rossano, R. *et al.* What are the proteolytic enzymes of honey and what they do tell us? A fingerprint analysis by 2-D zymography of unifloral honeys. *PLoS One* **7,** e49164 (2012).
153. García, P., Madera, C., Martínez, B., Rodríguez, A. & Evaristo Suárez, J. Prevalence of bacteriophages infecting Staphylococcus aureus in dairy samples and their potential as biocontrol agents. *J. Dairy Sci.* **92,** 3019–3026 (2009).
154. Jepson, C. D. & March, J. B. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine* **22,** 2413–2419 (2004).

References

155. Cordova, A., Deserno, M., Gelbart, W. M. & Ben-Shaul, A. Osmotic Shock and the Strength of Viral Capsids. *Biophys. J.* **85,** 70–74 (2003).
156. Pires, D., Sillankorva, S., Faustino, A. & Azeredo, J. Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. *Res. Microbiol.* **162,** 798–806 (2011).
157. Cornelissen, A. *et al.* The T7-related Pseudomonas putida phage φ15 displays virion-associated biofilm degradation properties. *PLoS One* **6,** e18597 (2011).
158. Chibeu, A. *et al.* Bacteriophages with the Ability to Degrade Uropathogenic Escherichia Coli Biofilms. *Viruses* **4,** 471–487 (2012).
159. Pires, D. P. *et al.* A Genotypic Analysis of Five P. aeruginosa Strains after Biofilm Infection by Phages Targeting Different Cell Surface Receptors. *Front. Microbiol.* **8,** 1229 (2017).
160. Labrie, S. J., Samson, J. E. & Moineau, S. Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* **8,** 317–327 (2010).
161. Lee, J.-H. *et al.* Low concentrations of honey reduce biofilm formation, quorum sensing, and virulence in Escherichia coli O157:H7. *Biofouling* **27,** 1095–1104 (2011).
162. Rabie, E., Serem, J. C., Oberholzer, H. M., Gaspar, A. R. M. & Bester, M. J. How methylglyoxal kills bacteria: An ultrastructural study. *Ultrastruct. Pathol.* **40,** 107–111 (2016).
163. Okhiria, O. A., Henriques, A. F. M., Burton, N. F., Peters, A. & Cooper, R. A. Honey modulates biofilms of Pseudomonas aeruginosa in a time and dose dependent manner. *J. ApiProduct ApiMedical Sci.* **1,** 6–10 (2009).
164. Wang, Y., Leng, V., Patel, V. & Phillips, K. S. Injections through skin colonized with Staphylococcus aureus biofilm introduce contamination despite standard antimicrobial preparation procedures. *Sci. Rep.* **7,** 45070 (2017).
165. Tuson, H. H. & Weibel, D. B. Bacteria-surface interactions. *Soft Matter* **9,** 4368–4380 (2013).
166. Alnnasouri, M., Lemaitrea, C., Gentric, C., Dagot, C. & Pon, M.-N. Influence of surface topography on biofilm development: Experiment and modeling. *Biochem. Eng. J.* **57,** 38–45 (2011).
167. Bhushan, B., Chen, S. & Ge, S. Friction and durability of virgin and damaged skin with and without skin cream treatment using atomic force microscopy. *Beilstein J. Nanotechnol.* **3,** 731–746 (2012).
168. Elkhyat, A., Courderot-Masuyer, C., Gharbi, T. & Humbert, P. Influence of the hydrophobic and hydrophilic characteristics of sliding and slider surfaces on friction coefficient: in vivo human skin friction comparison. *Skin Res. Technol.* **10,** 215–221 (2004).
169. R.E. Baier & A.E. Meyer. *Interfacial Phenomena and Bioproducts*. (Marcel Dekker, 1996).
170. J. S. Cho *et al. Adhesion Aspects of Thin Films*. (VSP, 2005).
171. Kondyurin, A., Gan, B. K., Bile, M. M. M., Mizuno, K. & McKenzie, D. R. Etching and structural changes of polystyrene films during plasma immersion ion implantation from argon plasma. *Nucl. Instruments Methods Phys. Res. Sect. B Beam Interact. with Mater. Atoms* **251,** 413–418 (2006).
172. McBride, M. E., Duncan, W. C. & Knox, J. M. The environment and the microbial ecology of human skin. *Appl. Environ. Microbiol.* **33,** 603–608 (1977).
173. Phillips, P. L. *et al.* Antimicrobial dressing efficacy against mature Pseudomonas aeruginosa biofilm on porcine skin explants. *Int. Wound J.* **12,** 469–483 (2015).
174. Cooper, R. A. Metabolism of Methylglyoxal in Microorganisms. *Annu. Rev. Microbiol.* **38,** 49–68 (1984).
175. Sukdeo, N. & Honek, J. F. Pseudomonas aeruginosa contains multiple glyoxalase I-encoding genes from both metal activation classes. *Biochim. Biophys. Acta - Proteins Proteomics* **1774,** 756–763 (2007).

176. Kilty, S. J., Duval, M., Chan, F. T., Ferris, W. & Slinger, R. Methylglyoxal: (active agent of manuka honey) in vitro activity against bacterial biofilms. *Int. Forum Allergy Rhinol.* **1,** 348–350 (2011).
177. Lu, J. *et al.* The Effect of New Zealand Kanuka, Manuka and Clover Honeys on Bacterial Growth Dynamics and Cellular Morphology Varies According to the Species. *PLoS One* **8,** e55898 (2013).
178. Roberts, A. E. L., Maddocks, S. E. & Cooper, R. A. Manuka honey reduces the motility of Pseudomonas aeruginosa by suppression of flagella-associated genes. *J. Antimicrob. Chemother.* **70,** 716–725 (2015).
179. Merckoll, P., Jonassen, T. Ø., Vad, M. E., Jeansson, S. L. & Melby, K. K. Bacteria, biofilm and honey: A study of the effects of honey on 'planktonic' and biofilm-embedded chronic wound bacteria. *Scand. J. Infect. Dis.* **41,** 341–347 (2009).
180. Fu, W. *et al.* Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. *Antimicrob. Agents Chemother.* **54,** 397–404 (2010).
181. Kłodzińska, E. *et al.* Effect of zeta potential value on bacterial behavior during electrophoretic separation. *Electrophoresis* **31,** 1590–1596 (2010).
182. Halder, S. *et al.* Alteration of Zeta potential and membrane permeability in bacteria: a study with cationic agents. *Springerplus* **4,** 672 (2015).
183. Soni, K. A., Balasubramanian, A. K., Beskok, A. & Pillai, S. D. Zeta Potential of Selected Bacteria in Drinking Water When Dead, Starved, or Exposed to Minimal and Rich Culture Media. *Curr. Microbiol.* **56,** 93–97 (2008).
184. Lee, K.-J., Lee, M.-A., Hwang, W., Park, H. & Lee, K.-H. Deacylated lipopolysaccharides inhibit biofilm formation by Gram-negative bacteria. *Biofouling* **32,** 711–723 (2016).
185. Cerqueira, L., Oliveira, J. A., Nicolau, A., Azevedo, N. F. & Vieira, M. J. Biofilm formation with mixed cultures of Pseudomonas aeruginosa / Escherichia coli on silicone using artificial urine to mimic urinary catheters. *Biofouling* **29,** 829–840 (2013).
186. Bandara, H. M. H. N., Yau, J. Y. Y., Watt, R. M., Jin, L. J. & Samaranayake, L. P. Pseudomonas aeruginosa inhibits in-vitro Candida biofilm development. *BMC Microbiol.* **10,** 125 (2010).
187. Kuznetsova, M. V., Maslennikova, I. L., Karpunina, T. I., Nesterova, L. Y. & Demakov, V. A. Interactions of Pseudomonas aeruginosa in predominant biofilm or planktonic forms of existence in mixed culture with Escherichia coli in vitro. *Can. J. Microbiol.* **59,** 604–610 (2013).

ANNEXES

ANNEX I – EC3A AND PAO1-D PHAGES: TEM IMAGES

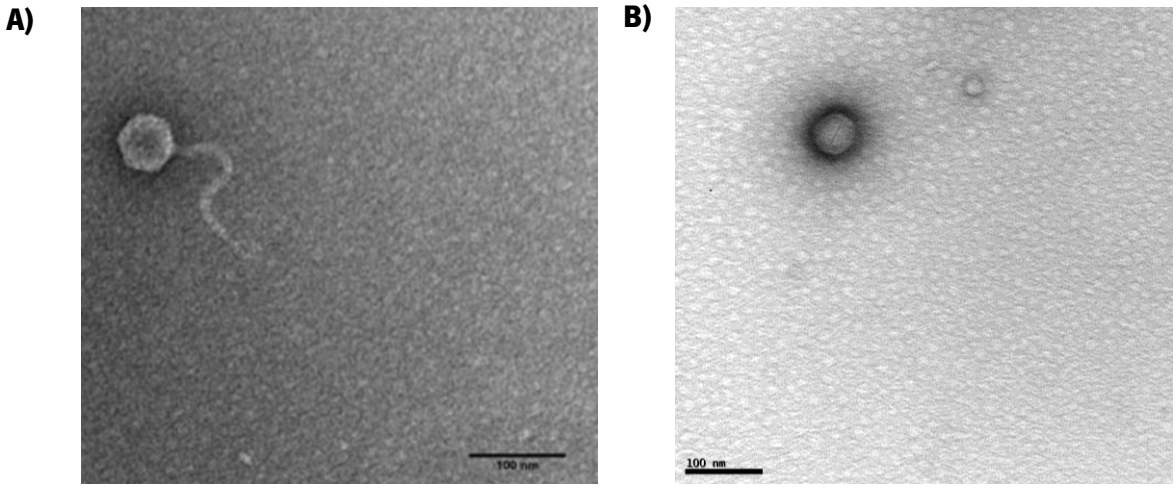


Figure I.1 – TEM images of the phages used in this work, both provided by BBiG: **A)** EC3a phage particle; **B)** PAO1-D phage particle.

ANNEX II – POLLINIC ANALYSIS OF C1 HONEY

Table II.1 – Complete pollinic analysis of the Portuguese honey C1 used in this work

	Portuguese honey C1
Predominant pollen (> 45%)	*Castanea sativa* (92%)
Secondary pollen (16-45%)	
Minoritary pollen (3-15%)	*Eucalyptus* spp. (6%)
Identified pollen (<3%)	
Nectarless pollen	

ANNEX III – CALIBRATION CURVES

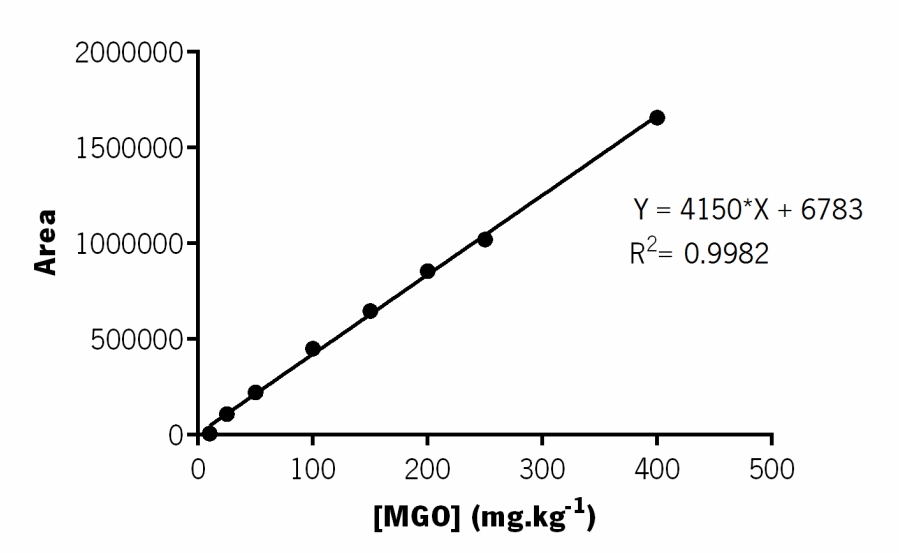


Figure III.1 – Calibration curve, determined using a standard solution with a purity grade solvent of MGO of 35-40% (v/v), used for the MGO content in honey determination; Area= 4150 [MGO] (mg.kg^{-1}) + 6783, with a correlation of R^2= 0.9982.

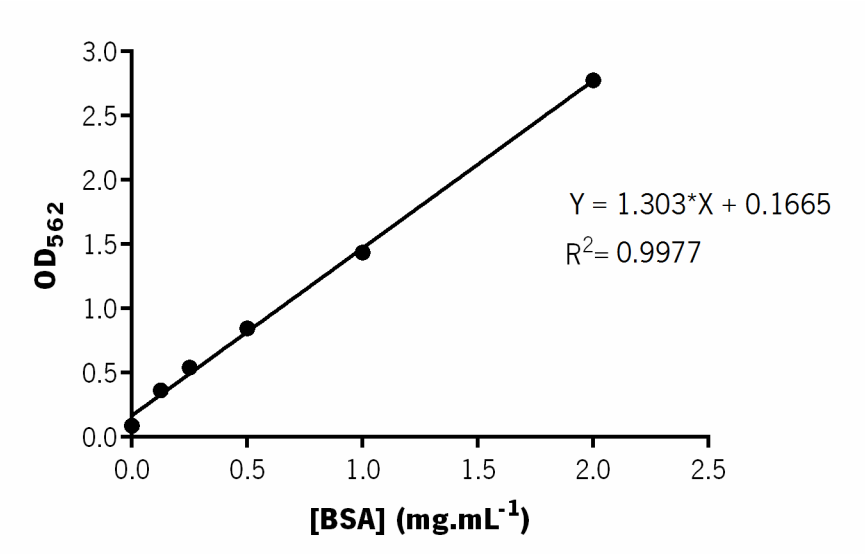


Figure III.2 – Calibration curve, obtained using a standard BSA solution, used for the protein content determination; Abs (562 nm)= 1.30 (± 0,09) [Protein (mg.mL^{-1})] + 0.17 (± 0,08), with a correlation of R^2= 0.9977.