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Development of a phage-based bioactive product to control biofilms in chronic wounds

Dissertation for the Master degree in Biomedical Engineering

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ABSTRACT

Chronic wounds are skin injuries where the normal healing process has failed and anatomical integrity is not achieved within a normal period. Worldwide, these wounds affect thousands of people, causing pain and discomfort. One of the difficulties on the healing of these wounds are the infections caused by biofilms. These polymicrobial aggregates cause serious infections, because they are persistent and highly tolerant to common therapies, namely the antibiotics. Very recently the World Health Organization published a list of priority pathogens resistant to antibiotics and emphasize the scientific community and pharmaceutical industries to focus on the development of new antimicrobials to combat these pathogens. The use of bacteriophages (phages) is seen as a possible novel treatment against antibiotic-resistant infections, namely in chronic wounds.

Therefore, the aim of this work was to develop a phage-based bioactive product to be incorporated into a wound dressing to control biofilms in chronic wounds. To achieve that, the first step of this work was to isolate phages that specifically infect *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis* and *Staphylococcus aureus* strains. After this, phages were subjected to a process of characterization that consisted in, evaluation of their host range, *in vitro* studies, morphological/genomic analysis and temperature/pH stability studies. After isolating several phages, three were selected for an in-depth characterization: *E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410. Reductions of about 2 orders-of-magnitude in biofilm cells, at 6 h were observed in *in vitro* assays, mainly when these were performed in the presence of culture media. The characterization process was also performed with the Collagen Wound Model and reductions between 1 and 2 orders-of-magnitude ($p<0.05$) were observed on 8 h post-treatment. When phages were combined to treat dual-species biofilms, the phage cocktail reduced the biofilm cells in about 2.5 orders-of-magnitude ($p<0.05$) at 3, 6 and 8 h, and in about 1 order-of-magnitude ($p<0.05$) at 24 h. These reductions were higher than any single phage application. All phages were stable after 24 h, between −20 and 37 °C, and in the pH range 5.0–11.0.

Morphological and genomic features indicated that both *E. faecalis* phages are members of the family *Siphoviridae*, while *E. faecium* phage C410 is a member of the *Podoviridae* family. Although proteins with toxic potential were not identified on *E. faecalis* phage 80-2 and *E. faecium* phage C410 genomes, *E. faecalis* phage 09-2 needs a more detailed genomic analysis, due to the presence of the hemolysin XhlA family codifying gene.

Furthermore, for the use of phages in therapy it is important to guarantee their safety. Therefore, phages solutions were purified using chromatographic approaches, namely CIMmultus® columns. However, the purest phage fraction has not been determined. Moreover, phages cytotoxic potential was also evaluated in a 3T3 cell line by Neutral Red Uptake test. The phage solutions presented no toxicity when used at a concentration of 10^7 PFU/mL. Finally, in order to evaluate the capability of phages to kill internalized bacteria, internalization studies were conducted in the same cell line. Bacterial reductions between 2 and 3 orders-of-magnitude ($p<0.05$) were observed in phage-treated cells, showing that the studied phages can infect internalized bacteria.

In conclusion, in this work, phages capable of killing enterococci were well characterized, and therefore their use in a phage-based product may be possible. These phages showed to be non-toxic and efficient for controlling the internalized bacteria in animal cells, thus exhibiting a great potential to be incorporated in a bioactive dressing to treat chronic wounds.

KEYWORDS: CHRONIC WOUNDS, PHAGES, PURIFICATION, SAFETY ASSESSMENT

SUMÁRIO

Feridas crónicas são lesões na pele, onde o processo normal de cicatrização falhou e a integridade anatómica não é alcançada num período normal. Em todo o mundo, estas feridas afectam milhares de pessoas causando dor e desconforto. Uma das dificuldades na cicatrização das feridas crónicas são as infeções causadas por biofilmes. Estes agregados polimicrobianos causam graves infeções, devido à sua persistência e por possuírem elevada tolerância a terapias comuns, nomeadamente a antibióticos. Muito recentemente a Organização Mundial de Saúde publicou uma lista de patogénicos prioritários resistentes aos antibióticos e incentivou a comunidade científica e a indústria farmacêutica a focarem-se no desenvolvimento de novos antimicrobianos, para o combate destes patogénicos. O uso de bacteriófagos (fagos) é visto como um possível novo tratamento no combate a infeções resistentes a antibióticos, nomeadamente em feridas crónicas.

Assim sendo, o objetivo principal deste trabalho foi desenvolver um produto bioativo com fagos para ser incorporado num penso, de modo a controlar infeções por biofilmes em feridas crónicas. Para atingir o objetivo, o primeiro passo foi isolar fagos que infetam especificamente estirpes de *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis* e *Staphylococcus aureus*. Após o isolamento, os fagos foram sujeitos a um processo de caracterização que consistiu na avaliação da sua gama de hospedeiros, estudos *in vitro*, análise morfológica/genómica e estudos de estabilidade à temperatura/pH. Três fagos foram selecionados para uma caracterização mais profunda: os fagos 09-2 e 80-2 de *E. faecalis* e o fago C410 de *E. faecium*. Após 6 horas de contacto com fagos, foram observadas reduções de cerca de 2 ordens-de-magnitude, em células de biofilme, principalmente quando estes ensaios foram realizados na presença de meio de cultura. O processo de caracterização foi também realizado através do modelo "*Collagen Wound Model*" e neste, foram observadas reduções entre 1 e 2 ordens-de-magnitude ($p<0.05$), 8 horas pós-tratamento. Quando os fagos foram combinados para tratar biofilmes de duas espécies, o *cocktail* de fagos reduziu as células do biofilme em cerca de 2.5 ordens-de-magnitude ($p<0.05$) às 3, 6 e 8 horas, e em cerca de 1 ordem-de-magnitude ($p<0.05$) às 24 horas. Estas reduções foram maiores do que em qualquer aplicação de apenas o fago de uma espécie. Todos os fagos apresentaram estabilidade entre -20 e 37 °C e na gama de pH 5.0-11.0, até 24 horas.

As características morfológicas e genómicas indicaram que ambos os fagos de *E. faecalis* são membros da família *Siphoviridae*, enquanto o fago C410 de *E. faecium* é membro da família *Podoviridae*. Apesar de não terem sido identificadas proteínas com potencial tóxico no fago 80-2 de *E. faecalis* e no

fago C410 de *E. faecium*, o fago 09-2 de *E. faecalis* requer uma análise genómica mais detalhada, devido à presença do gene de codificação da família de hemolisina XhlA.

Adicionalmente, para que a utilização de fagos como terapia seja possível é importante avaliar a sua segurança. Portanto, as soluções de fago foram purificadas usando abordagens cromatográficas, nomeadamente colunas CIMmultus®. Contudo, a fração de fago mais pura não foi determinada. O potencial citotóxico dos fagos foi também avaliado na linha celular de fibroblastos 3T3 através do teste "*Neutral Red Uptake*". As soluções de fago, 10^7 PFU/mL não apresentaram toxicidade. Por último, para avaliar a capacidade dos fagos para matar bactérias internalizadas, foram realizados estudos de internalização na mesma linha celular. Observou-se reduções bacterianas entre 2 e 3 ordens-de-magnitude ($p<0.05$) em células tratadas com fagos, mostrando que os fagos estudados podem infectar bactérias internalizadas.

Em conclusão, os fagos *E. faecalis* 09-2, 80-2 e *E. faecium* C410, capazes de matar enterococci foram isolados e bem caracterizados e, portanto, o seu uso num produto baseado em fagos, pode ser possível. Estes fagos mostraram ser não tóxicos e eficientes no controlo de bactérias internalizadas em células animais, apresentando, portanto, um grande potencial para serem incorporados num penso para tratar feridas crónicas.

Palavras-Chave: Feridas Crónicas, Fagos, Purificação, Avaliação de segurança.

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ABREVIATIONS

AMR - Antimicrobial Resistance

APC - Dendritic antigen-presenting cells

ATCC - American Type Culture Collection

BIM - Bacteriophage insensitive mutants

CDC - Centers for Disease Control and Prevention

CFU - Colony Forming Unit

CIM - Convective Interactive Media

CLSM- Confocal laser scanning microscopy

CsCl - Cesium Chloride

CWM – Collagen Wound Model

DCs - Dendritic Cells

DEAE – Diethylamino

DMEM - Dulbecco's Modified Eagle's Medium

DNA - Deoxyribonucleic Acid

dsDNA - Double-stranded DNA

dsRNA - Double-stranded RNA

eDNA - Extracellular DNA

EDTA - Ethylenediaminetetraacetic acid

EMA - European Medicines Agency

EPS – Extracellular Polymeric Substances

ETAR – Estação de Tratamento de Águas Residuais

EU - European Union

FAS -Ferrous Ammonium Sulphate

FBS - Fetal Bovine Serum

FDA - Food and Drug Administration

FPLC - Fast Protein Liquid Chromatography

GMP - Good Manufacturing Practice

HGT - Horizontal Gene Transfer

HPLC - High Pressure Liquid Chromatography system

ICTV - International Committee on Taxonomy of Viruses

IL - Interleukin

LB - Lisogeny Broth

LBA - Lisogeny Broth Agar

LCWB – Lubbock Chronic Wound Biofilm Model

LPS - lipopolysaccharides

LTFs - Long tail fibers

MDR – Multidrug-resistance

MIC - Minimum Inhibitory Concentration

MOI - Multiplicity of Infection

MRSA - Methicillin-resistant *Staphylococcus aureus*

MSSA - Methicillin-sensitive *Staphylococcus aureus*

MTP – Microtiter Plate

NaCl - Sodium Chloride

NK - Natural killer cells

NPUAP - The National Pressure Ulcer Advisory Panel

NR - Neutral Red

OD -Optical Density

PB - Phosphate Buffer

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate Buffered Saline

PEG - Polyethylene glycol

PFU - Plaque Forming Unit

PMNs - Polymorphonuclear Leukocytes

QA - Quaternary amine

QS - Quorum Sensing

rpm - Rotations per minute

SLS - Sodium Lauryl Sulfate

SNS - Serviço Nacional de Saúde

ssDNA - Single-stranded DNA

ssRNA - Single-stranded RNA

SWF – Simulated Wound Fluid

TEM - Transmission Electron Microscopy

TNF - Tumor necrosis factor

TSA - Tryptic Soy Agar

TSB - Tryptic Soy Broth

UK – United Kingdom

USA – United States of America

UV - Ultraviolet

VRE - Vancomycin-resistant enterococci

VRSA – Vancomycin-resistant *S. aureus*

WHO - World Health Organization

1 INTRODUCTION

1.1 Chronic wounds

The skin, the largest organ of the human body, is the main protective barrier against the external environment. Injury or illness may cause loss of skin integrity (wound), that in some cases may become chronic (Figure 1.1-1)[1].

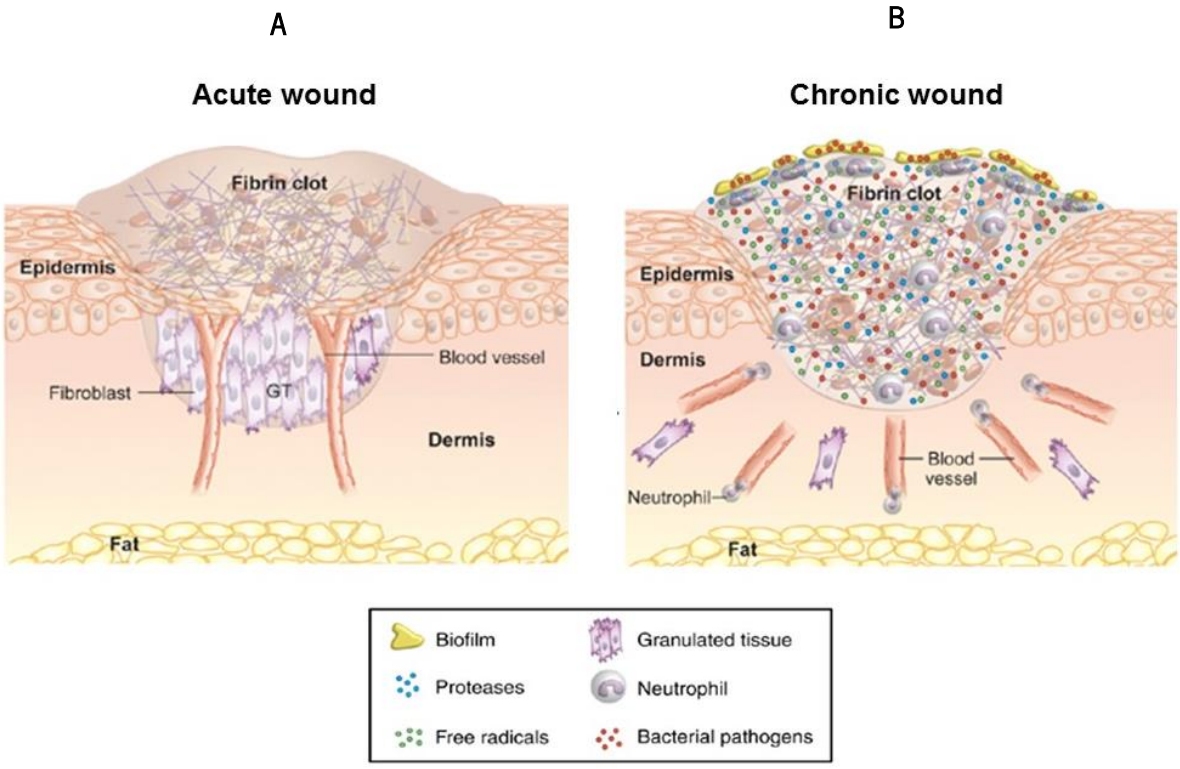


Figure 1.1-1 - Differences in reparation between acute and chronic wounds. In normal physiologic conditions, the tissue injury results in an acute wound (A), but when there is a pathology or microbial invasion these interfere with the healing process and the chronic wounds may appear (B). Adapted from[2].

Chronic wounds are defined as wounds where the normal reparative process failed and the anatomic and functional integrity is not achieved within a period of three months[1], as a consequence of a pathology or of a microbial invasion occurrence in the wound[2].

Worldwide, these wounds are responsible for considerable morbidity and significantly contribute for an increase in the health care costs. In the United States of America (USA), chronic wounds affect annually around 5.7 million people (≈ 2 % of population), and their treatment has US$20 billion of annual

expenses[1,3]. Unhealthy patients (e.g. diabetes and obesity) have shown to have more tendency to develop chronic wounds[4]. Moreover, with an increasing population aging and the lifestyle changes, in the further years it is expected an arise of new cases[5].

1.2 Types of chronic wounds

As already mentioned, a skin injury that has not healed in three months is named as chronic wound. Despite being heterogeneous, chronic wounds are generally assigned to one clinical categories: pressure ulcers, diabetic foot ulcers and venous ulcers[1].

1.2.1 Pressure Ulcers

Pressure ulcers, also named bedsores, are skin wounds that results of pressure, or pressure in combination with shear and/or friction. Pressure ulcers are divided into four stages based on the definitions of the European Pressure Ulcer Advisory Panel. On stage I the skin is red, but does not have open wounds, which might be indicative of deep tissue pressure injury. At stage II there is an open wound with exposed dermis. The wound bed is pink or red, moist, and may, also, present as an intact or ruptured serum-filled blister. Deeper tissues are not visible. At stage III there is a full-thickness loss of skin. The open wound reaches the adipose, while muscle, tendon, ligament, cartilage and/or bone are not exposed. Finally, at stage IV there is a full-thickness skin and tissue loss. The ulcer reaches the muscle, tendon, ligament, cartilage or bone (Figure 1.2-1)[6].

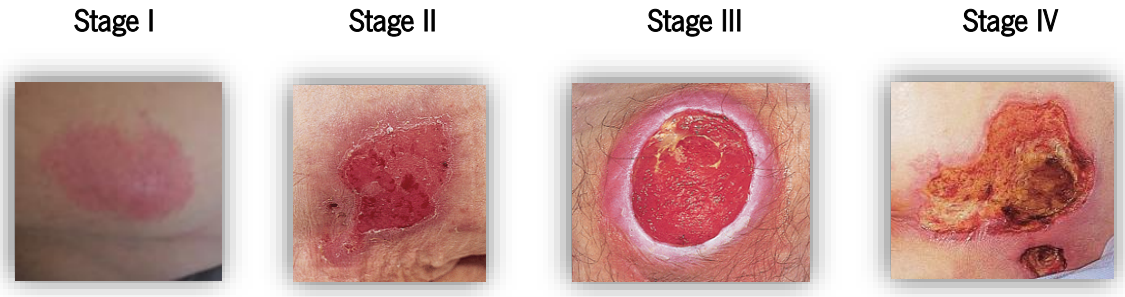


Figure 1.2-1 - Stages of pressure ulcers based on the definitions of the European Pressure Ulcer Advisory Panel[6]. Adapted from[7,8].

These wounds are common in elderly patients, stroke victims, patients with diabetes, dementia, those in wheelchairs, bedridden or suffering from impaired mobility or sensation[5].

A United States Medicare study reported the incidence of hospital-acquired pressure ulcers to be 4.5 % in hospitalized patients, with an estimated cost of US$11 billion[9,10]. In Europe the prevalence of pressure ulcers is above 20 %[5], while in Portugal a more recent epidemiological data show that the prevalence of pressure ulcers was about 11.5 %, on hospital care[11].

1.2.2 Diabetic foot ulcers

Worldwide, the prevalence of diabetes was of 8.5 %, in 2014[12]. In 2012, 29.1 million Americans, or 9.3 % of the population, had diabetes[13]. In 2014 in the European Region, there were 64 million people with this disease[12]. In 2015, the prevalence of Portuguese population between 20 and 79 years with diabetes was of 13.3 %[14].

It is estimated that 15 % of the people with diabetes develop foot ulcers that are highly susceptible to infection, which ultimately can lead to amputation. Indeed, 85 % of amputations are preceded by an ulcer[15]. In 2015, the number of patients with diabetic foot ulcers in Portugal was of 1 643 and the number of amputations by diabetes was of 1 250[14].

Patients with diabetic foot frequently have foot with deformities, leading to areas of high pressure. The lack of sensation in these areas might develop ulcers, namely in areas of bone predominance (Figure 1.2-2)[16].

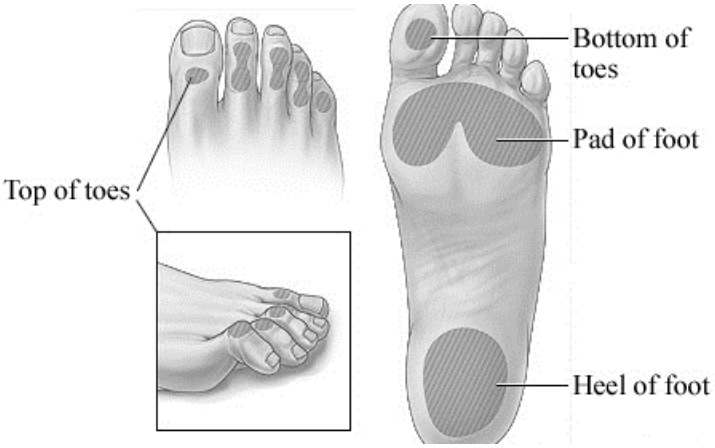


Figure 1.2-2 - Usual locations of ulcers in the diabetic foot. The diabetic foot ulcers normally occur on the bottom of the foot, but they can also appear in top and bottom of the toes. Adapted from[17].

There are various classification systems for diabetic foot ulcers. The Wagner-Meggitt classification, classifies the diabetic foot ulceration depending on the wound depth (how deep the wound is, Table 1.2-1).

Table 1.2-1 - The Wagner-Maggitt classification. This system classifies the diabetic foot ulceration depending on how deep the wound is. Adapted from[18]

Grade	Description
0	The skin is intact
1	Superficial presence of the ulcer
2	Deep presence of the ulcer
3	Deep ulcer with abscess, bone involvement or osteomyelitis
4	Gangrene in the forefoot
5	Whole foot gangrene

1.2.3 Venous leg ulcers

The venous leg ulcers, also known as varicose, are associated with venous disease, arterial insufficiency, diabetes and rheumatoid arthritis[19]. These ulcers are caused by venous reflux or obstruction[20], and represent 40-70 % of all lower extremity chronic wounds[21].

The elderlies are very susceptible to these wounds. The annual prevalence of venous leg ulcer among the elderly is 1.69 % in the United Kingdom (UK). Venous leg ulcers affect between 0.2 % and 1 % of the world's total population and between 1 % and 3 % of the elderly population in the USA and Europe, respectively[22]. In Portugal, there is an absence of recent data about the prevalence of venous ulcers. In a 1992 study, it was concluded that the prevalence of active venous ulcers was of 3.2 % in men and 3.9 % in women. In the same investigation, was concluded that in people age greater than 70 years, the prevalence of chronic venous ulcer was about 80 %[23].

1.3 Biofilms

In natural environments, microorganisms do not live as dispersed single cells, but instead accumulate at solid–liquid interfaces to form biofilms. Biofilms are an aggregation of microorganisms embedded within a self-produced polymeric matrix (Figure 1.3-1). This structure allows bacterial cells to survive in harsh environments[24].

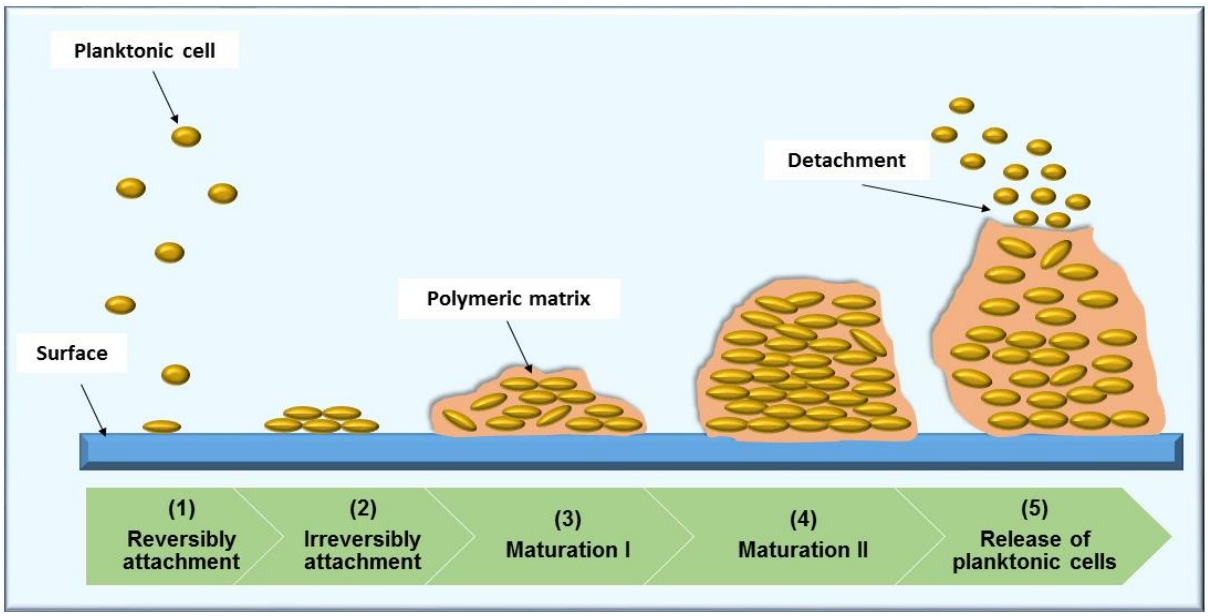


Figure 1.3-1 - Model of biofilm development. In stage 1, the planktonic cells attach reversibly to the surface. Then, in stage 2, the cells are expanded and, also irreversibly attach. In the next stage (3), the first maturation phase is reached, and there is formation of polymeric matrix. The second maturation phase is in stage 4 with fully mature biofilms. In stage 5 there is the release of planktonic cells that invade other surfaces, thus starting up a new cycle.

Biofilm formation begins when a contact is made between the surface of the cell and an interface. A reversibly attachment of planktonic cells to the surface occurs (Stage 1). Afterwards, a progressive formation of a denser monolayer of cells occur. Cellular motility ceases during this developmental stage and the surface connection is stronger - irreversibly attachment (Stage 2). Furthermore, at this stage are activated the first quorum-sensing (QS) regulation mechanisms. The next stage in biofilm development is maturation I (Stage 3). In this, matrix polymer production is detectable. Moreover, at this stage are observed biofilm architecture changes and the production of many proteins. Maximum thickness of biofilm is reached during the maturation II (Stage 4). The final stage in the development of a biofilm is the dispersion stage (Stage 5). Mechanisms of biofilm dispersal can be active or passive. Active dispersal refers to mechanisms that are initiated by the bacteria themselves; passive dispersal refers to biofilm cell detachment that is mediated by external forces such as fluid shear, collision of solid particles with the biofilm, predator grazing, and human intervention[25]. In this stage, bacteria within cell clusters are released and can colonize other surfaces, starting up a new biofilm cycle[26,27].

1.3.1 Quorum Sensing in biofilms

Bacterial cells communicate with each other, releasing, detecting and responding to small diffusible signal molecules - mechanism of QS. This characteristic has provided significant benefits to bacteria in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments[28]. Furthermore, many QS controlled activities have been involved in the virulence and pathogenic potential of bacteria[28].

The ability to express surface proteins which enable colonization and biofilm formation in humans, is a mechanism of pathogenesis in *Enterococcus* species. A family of bacterial surface proteins (MSCRAMMs) are constituted by adhesion proteins that play an important role in Gram-positive bacterial virulence by mediating adherence and colonization to host tissues[29]. The protein Ace was the first protein of this family to be discovered in enterococci[30] and the presence of this protein on *Enterococcus faecalis* cell surface is correlated with the bacterial ability to adhere to collagen[31]. A similar phenomenon was described for Cna and Acm, which are known collagen binding virulence factor of *Staphylococcus aureus*[32] and *Enterococcus faecium*[33], respectively. A study of Pinkston *et al.*[34], presented results where it was evidenced that the surface display of Ace in *E. faecalis*, was regulated by the Fsr QS system, through mediated secretion of GelE[34]. Moreover, *S. aureus*, use signal peptide-mediated systems for QS[35]. Surface-associated adhesins, hemolysin, toxins and autolysins are virulence factors involved in staphylococcal infections, that are regulated by QS via the accessory gene regulator (*agr*) system[35,36]. Studies about this system concluded that the *agr* phenotype and expression patterns may influence several aspects of biofilm phenotypes, namely cellular attachment to surfaces, biofilm dispersal, and even the chronic nature of biofilm-associated infections[36]. The expression of many virulence factors is increased by *agr* QS system, where it was shown the importance of this system for the development of invasive infections and disease progress in several infection models, such as subcutaneous abscesses, murine arthritis or pneumonia, rabbit osteomyelitits or endocarditis[35,36].

1.3.2 Biofilm matrix

The biofilm matrix serves as a scaffold that has an essential cell-to-cell connecting and structural function in biofilms. The matrix is an extracellular material, produced by the organisms themselves, and consists of a conglomeration of different types of biopolymers - extracellular polymeric substances (EPS). This contains mainly polysaccharides, proteins, and extracellular DNA (eDNA)[37,38].

Several functions of the biofilm matrix, demonstrate a wide range of advantages for the biofilm mode of life. It plays an important role in a number of biofilm processes including cell attachment, cell-to-cell interactions, and antimicrobial tolerance. Furthermore, allows the accumulation of nutrient and energy sources; the maintenance of a highly hydrated microenvironment around biofilm organisms; the presence of all components of cellular lysis available - this includes DNA, which may represent a reservoir of genes for horizontal gene transfer; the protection of microorganisms against various antimicrobial agents and immune defenses[37].

For example, *Pseudomonas aeruginosa*, produces an exopolysaccharide, alginate, in response to adverse conditions that protects bacterial cells and increases adhesion ability. Thus, alginate production is important for the development, maintenance and spread of biofilms[39].

Also, eDNA was shown to be an important adhesive molecule for biofilm attachment and stability[40], being frequently released by many species, such as *E. faecalis* and *E. faecium*. The lysis of a bacterial subpopulation (autolysis) that is controlled by QS system is the most important source of eDNA[41]. A study identified *AtlAEfm* as an important *E. faecium* autolysin, and highlighted its importance in eDNA release and in biofilm formation in this species. This autolysin is directly involved in the formation of biofilms, cell separation, cell wall hydrolysis and eDNA release[42].

1.3.3 Biofilms and chronic wounds

Biofilms are ubiquitous in nature, therefore they are present in the human body, where some are commensal, others pathogenic[43]. Pathogenic biofilms are associated with many chronic diseases, including chronic wounds[43]. These infectious biofilms are described to be present in 6 % of acute wounds, however this number increases and they are present in 90 % of chronic wounds[44].

The wound is a favourable environment for biofilm development, and the bacteria can develop inflammatory response and delay the healing process[45].

Studies identified *Staphylococcus* and *Enterococcus* polymicrobial biofilms, as the most frequent bacteria present in chronic wounds (Table 1.3-1)[46,47].

Table 1.3-1 - Classification of bacteria species most isolated from chronic wounds and their frequency (%).Adapted from[46]

Species	Classification	Frequency (%)
S. aureus	Gram +	93.5
E. faecalis	Gram +	71.7
P. aeruginosa	Gram -	52.2
Coagulase-negative staphylococci	Gram +	45.7
Anaerobic bacteria	Gram – Gram +	39.1
Enterobacter cloacae	Gram -	37.0
Escherichia coli	Gram -	32.6

In an investigation, with the aim to study the bacterial profile of chronic venous leg ulcers, 46 patients of Copenhagen Wound Healing Center (Bispebjerg Hospital, Denmark), were followed for 8 weeks, and ulcer samples were collected every second week[46]. A total of 37 bacterial species were identified from the ulcer samples. The mean number of bacterial species isolated per chronic wound was 6.3. The most common bacterial species detected in chronic wounds was *S. aureus* (93.5 %). Other species found were *E. faecalis* (71.1 %), *P. aeruginosa* (52.2 %), coagulase-negative staphylococci (e.g. *Staphylococcus epidermidis*) (45.7 %) and anaerobic bacteria (39.1 %) (Table 1.3-1)[46]. More recently, Tzaneva *et al.*[48], performed a study in the Clinic of Vascular Surgery in Trakia Hospital (Stara Zagora, Bulgaria) with 110 vascular chronic wound patients, for one year. A total of 159 strains were isolated. More than one isolate of bacterial species was detected in 30 % of the patients. The most frequently species identified were *S. aureus* (39.1 %), *E. coli* (19.1 %), *E. faecalis* (16.4 %), *P. aeruginosa* (11.8 %) and *Proteus mirabilis* (10 %)[48].

1.4 Solutions of treatment

Despite the differences between wounds, the topical and/or systemic prescription of antibiotics is the most frequent treatment in patients with chronic wounds[48]. Wound dressings are another solution, which are normally used in combination with antibiotics[49]. Rapid and aggressive treatment, such as drainage of infected tissue and debridement, is normally used to clear the area of injury prior to the application of the wound dressing[50].

1.4.1 Debridement

Debridement is a technique that allows the clearance of devitalized tissue, including necrotic and senescent cells, inflammatory enzymes, and biofilms. The aim is to create a favourable environment for the healing processes[50]. Various studies with debridement techniques have shown that these, significantly reduced bacterial burden[51].

There are various debridement techniques described in literature, namely autolytic, biologic, enzymatic, mechanical and surgical debridement[41]. The autolytic debridement is a technique wherein the wound is covered with a wet dressing retention. The objective is that the patient's enzymes and immunological cells can eliminate necrotic tissue. This is a simple technique but the patient's recovery is slow, and it can only be used in wounds with low necrotic tissue[52]. Biologic therapy, use maggots (*Phaenicia sericata*) applied directly to the wound surface, that dissolves necrotic tissue and biofilm. Maggots work selectively and quickly, secreting bactericidal enzymes, which make them effective against antibiotic-resistant bacteria, namely methicillin-resistant *S. aureus* (MRSA) and beta haemolytic streptococci[52,53]. A study with maggot therapy have shown to be effective in the treatment of diabetic foot ulcers that were unresponsive to conventional therapy[54]. In enzymatic debridement, the manufactured enzyme, such as collagenase and papain-urea are used to degreed necrotic tissue. Comparing both enzymes it can be said that papain is a broad-spectrum enzyme, while collagenase is less traumatic for viable cells[55]. Mechanical debridement is the physical removal of necrotic tissue from a wound. This technique involves methods such as wet-to-dry dressing and pressure irrigation. The first includes the use of a wet gauze in direct contact with wound surface. When the dry gauze is removed, it brings some adherent slough tissue with it. Pressure irrigation consists in the irrigation of saline solutions[56]. Despite the low cost of this method, results are slow and can cause excessive pain and bleeding to the patient[50]. In surgical debridement, the patient is submitted to surgery to remove the necrotic tissue and the biofilm. This is a fast method that allows an accurate assessment of the severity and extent of the wound. As a disadvantage, there may be removal of healthy tissue[57].

In conclusion, debridement is a technique that aims to create conditions that allow wound healing, being, normally, apply before wound dressing application. These wounds can be painful for the patient and it is important to minimize the pain, promoting comfort. For this reason, although there are several types of debridement, the selection of the appropriate methods depends on several factors, such as wound type, wound localization, and patient opinion.

1.4.2 Wound dressings

Wound dressings are an alternative method for the reduction of microbial pathogens in chronic wounds. Recent studies indicated that maintaining the moist wound environment, allows an increase epidermal cell movement and the angiogenesis, and a decrease in fibrosis. Wound dressings also work as barriers for trauma and infection[58]. The more common types of wound dressings are films, hydrocolloids, alginates, hydrogels and foams[58].

Films consist of sterile plastic sheets of polyurethane coated with hypoallergenic acrylic adhesive. These are impermeable to external fluids and bacteria, but allow atmospheric gases and water vapour to pass through. The advantage of this dressing is that is very flexible, which is good to apply in wounds located in difficult anatomical sites, allowing an easy visualization without disturbing the wound. However, they can only be potentially used in superficial lacerations and wounds that are mildly exudative[58,59]. Other dressing types are the hydrocolloids. These are gels or foams impregnated into self-adhesive polyurethane films. A systematic review demonstrated that hydrocolloid wound dressings promote chronic wound healing, by hydrating dry necrotic eschar and promoting autolytic debridement[60]. They are often composed of materials such as gelatine, pectin, and sodium carboxymethylcellulose. Hydrocolloids are impermeable to water, oxygen, and carbon dioxide. Moreover, treatment with hydrocolloids reduces wound pain and allows patients to shower. They are good for small and not excessively exudative leg wounds[58,59]. However there are some disadvantages of hydrocolloids use including, the presence of a malodorous gel and the potential for contact dermatitis. Some trials with these wound dressings have not proven their benefit, in pressure ulcers and leg ulcers[59]. Alginates are other type of wound dressings, composed of cellulose polysaccharides derived from algae or kelp. These wound dressings demonstrated positive results in the reduction of pressure ulcers area[61]. Alginates partly dissolve on contact with wound fluid to form a hydrophilic gel. They are good for exudative and deep wounds, due to their powerful absorptive properties. However, they should not be used, on wounds with little or no exudate, because one hurdle of these wound dressings is their difficulty on removal after placement on dry wounds[58,59]. Another type of wound dressings are the hydrogels that are wound dressings composed of 96 % water. They are comfortable to patients, promoting autolytic debridement[62]. Hydrogels maintain a moist environment at the wound bed, are permeable to vapours and oxygen. These wound dressings are ideal for very dry necrotic wounds, such as arterial ulcers and dry venous ulcers, but are not indicated for wounds producing high levels of exudate or where there is evidence of gangrenous tissue, which should be kept dry to reduce the risk of infection[58,59]. Finally, there are foam wound dressings composed of polyurethane or silicone foam

(hydrophobic) that have hydrophilic surface coating materials to prevent bacterial contamination. They are no adhesive and typically thicker than other wound dressings. Moreover, they are permeable to water vapour and oxygen, providing thermal insulation to the wound bed. The major advantage of foam is the ability to retain exudate, so are excellent to use over mildly to moderately exudative wounds, for example over bony areas. Some disadvantages of these wound dressings are their tendency to fail to stay in place, their ability to dehydrate dry wounds, their complete opacity and their potential to cause contact dermatitis[58,59].

In the same way as in debridement, the selection of the dressing depends on factors such as type, size and location of the wound. In addition, when there is microbial colonization, wound dressings with antimicrobial properties are used.

Wound dessings with antibicroial properties

Wound dressings with antimicrobial properties are for example, wound dressings with honey[63] or silver[64].

Honey has been used in wound investigations due to its nutritional and biomedical properties, being applied in wound care, healing acceleration[65] and treatment of infected wounds[66]. In a study with bacterial clinical isolates the efficacy of Manuka honey was studied. Results showed antibacterial activity of a hydrogel sheet loaded with Manuka honey, against bacterial infection of *S. aureus*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, *P. aeruginosa* and *P. mirabilis*[63]. Despite the referred good qualities, one disadvantage is that the antibacterial properties of the honey differ based on the region and the supply of nectar from the flora[67]. In addition, prior to therapeutic use, several purification treatments are required, because honey may contain spores of *Clostridium botulinum*[67].

Wound dressings with silver are also reported as a promising approach[64]. Silver has a long-standing history as an antimicrobial agent. Romans were using silver nitrate and other metal filings on wounds[64]. In several studies, silver had relevant antimicrobial results, including against antibiotic-resistant bacteria, such as MRSA and vancomycin-resistant enterococci (VRE)[68]. Another advantage of the silver use is its low probability of inducing bacterial resistance, in comparison with antibiotics[69]. However, wound dressings with silver also present the disadvantages of causing local skin discoloration and staining[70]. Moreover, silver can be toxic as it was shown in a study where the researchers examined the cytotoxic effects of silver on keratinocytes and fibroblasts, and they conclude that the silver and silver-based products were cytotoxic for their cells[71].

In conclusion, several researchers seem to support the use of wound dressings with silver for chronic wound treatment, because of their antimicrobial potential. However, the use of silver in clinical treatments also has drawbacks particularly with regard to metal toxicity and background levels of free radicals[67]. Therefore, additional research should be carried out in order to search for alternatives.

1.4.3 Antibiotics

Antibiotics are frequently used to treat chronic wounds, normally in combination with wound dressings. They can be use as topical and/or systemic treatment. The topical route of treatment has several advantages over systemic administration: avoidance of systemic toxicity and side effects; decreased induction of bacterial resistance; and allows a high concentration of antibacterial agent at the site of infection. However, this treatment is limited due to, local side effects such as allergic contact dermatitis, and the depth of penetration of the agent[64].

At healthcare facilities antibiotics are the antibacterial preferentially used[48]. Its appearance in the 40's supplanted all other antibacterial therapies, reducing the morbidity and mortality of patients with infections. However, the continued and inappropriate use of this therapy caused a huge problem on our societies – the antibiotic resistance[72]. The scientific community has defined a group of bacteria resistant to antibiotics called ESKAPE (*E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species), and Portugal has a high rate of resistance to antibiotics, mainly for *S. aureus* with resistance to vancomycin (VRSA), linezolid or daptomycin and for *E. faecalis* and *E. faecium* with resistance to linezolid[73]. A group of 110 patients from the Vascular Surgery Clinic at Trakia Stara Zagora Hospital was used in a study to determine the bacterial resistance to antibiotic treatment in chronic wounds of vascular origin. *S. aureus*, *E. coli*, *E. faecalis*, *P. aeruginosa* and *P. mirabilis* were the strains more predominant. The authors detected, in all bacterial isolates, 54.4 % of resistance to the five major classes of antibiotics: Beta-Lactams (Penicillin and Cephalosporins), Macrolides, Fluoroquinolones, Tetracyclines, Aminoglycosides[48].

The problem: antimicrobial resistance

According to World Health Organization (WHO), antimicrobial resistance (AMR) is the ability of a microorganism (bacteria, viruses and some parasites) to survive at exposure to antimicrobials that would normally kill them or stop their growth. Currently, antibiotic - resistant infections kill about 700 000 people per year globally[74,75].

In Portugal, by the year of 2013, it was estimated that around 4 600 deaths were caused by multidrug-resistant (MDR) bacteria[73]. Data from Direção Geral da Saúde showed that, between 2011 and 2012, the prevalence of hospital infection in Portugal was of 10.5 %. In addition, nearly half of the hospitalized patients (45.3 %) received treatment with antibiotics[73]. At the same way, the European scenario is also alarming, with MDR bacteria causing about 400 000 infections and at least 25 000 deaths in the year 2007[76]. Data from the Centers for Disease Control and Prevention (CDC), showed that in USA, at least 2 million people became infected with bacteria that are resistant to antibiotics and at least 23 000 people died, in the year 2013[77]. Besides the human costs, AMR may also represent a huge economic burden. In Europe, it is estimated that multidrug-resistant bacteria generate healthcare costs and productivity losses of 1.5 billion of Euros each year[76]. In the USA, the first two cases of VRSA were both isolated from chronic wound patients[78,79].

Very recently, WHO published a list of priority pathogens resistant to antibiotics. In the high priority list are strains such as, *E. faecium*, resistant to vancomycin and *S. aureus*, resistant to methicillin, vancomycin-intermediate and resistant[80]. Moreover, WHO emphasize the scientific community and pharmaceutical industries to focus on the development of new antimicrobials to combat AMR pathogens[80].

To sum up, it is urgent to develop new antibacterial agents and an adequate management in antibiotic usage. Currently, there is a wide variety of possible alternatives to antibiotics being researched and developed. All have different modes of action, some aim to prevent infections - vaccines -, others replace antibiotics as treatment, and still others make antibiotics more effective. Alternatives include antibodies, probiotics, lysins, stimulation of immunity, peptides and bacteriophages[75]. The use of bacteriophages as alternatives or supplements of antibiotics it is being becoming more accepted by the scientific community[81].

1.5 Bacteriophages

Bacteriophages (phages) are viruses that specifically infect bacteria. They are the most abundant entities in the biosphere, being found in every environment where their bacterial hosts are present. In 2007, over 5 500 different phages were already described[82].

The isolation of phages was observed in diverse environments, as soil, water, sewage, human and animal organisms, and food. They were first discovered in 1915 by the bacteriologist Frederick William Twort, and in 1917 the microbiologist Felix d'Herelle demonstrated that they had the potential to kill

bacteria. In that discovery, d'Herelle noticed clear spots on cultures of bacteria, suggesting that the organisms were eliminated[83,84]. He suggested that these entities were viruses and they were named bacteriophage, from the Latin words "*bacteria*" and "*phagein*", meaning "eaters of bacteria". He was the first person to use phage therapy in the treatment of bacterial dysentery. During his career, he isolated phages for bacteria responsible for several diseases, namely cholera, bubonic plague or anthrax[85]. Several promising results were presented in several studies[86–88] and several companies, as d'Herelle's commercial laboratory and Eli Lilly Company began large-scale production of phages. However, about 20 years after their discovery, Alexander Fleming revealed the first antibiotic – penicillin. The success of antibiotics along with issues on phage therapy, namely some early clinical failures, some scientific controversies and ethical concerns, dictated the end of phage therapy in the USA and in most Western European Countries. Nevertheless, research on phages was never abandoned in Eastern Europe and in the former Soviet Union. Eliava Institute, in Georgia, and the Hirszfeld Institute, in Poland, were the main centres of activities regarding phage therapeutic research and production[81]. Currently, the standards of the European Medicines Agency (EMA) or USA Food and Drug Administration (FDA) agencies have never been reached by any of the clinical phage products. However, some products were subjected to preclinical and phase I and I/II stages. Southwest Regional Wound Care Center performed a phase I clinical trial with phage products[89]. For example, the activity of phages against *S. aureus*, *P. aeruginosa* and *E. coli* in venous leg ulcer was studied and no adverse events were observed, evidencing that the phage product was safe for the patients[89]. In another study, the safety of oral administration of *E. coli* phages was also evaluated and once again no adverse effects were observed in volunteers[90]. A phase I/II clinical trial published by Biocontrol Ltd (now Ampliphi Biosciences Corporation) in chronic otitis patients showed that phage preparation Biophage-PA (*P. aeruginosa*) decreased *P. aeruginosa* bacterial counts[91]. Other safety studies with phage products are being conducted in European countries such as France, Belgium, Poland[92]. These phage-containing products are effective in several hosts, for example *E. coli*, *P. aeruginosa*, *S. aureus*, and are being tested for various clinical purposes such as wound infections and cystic fibrosis[92].

Phages are parasites that invade the bacterial cell and reproduce themselves by using bacterial machinery. With the invasion, they either form a stable association with the host or kill the cell. Moreover, phages are specific and they infect the cell using specific receptors on the cell surface. The specificity of interaction is a big advantage over antibiotics, because if the phages were not specific when inserted in the body, they would infect the commensal bacteria and would also influence the normal flora[93]. Because of their specificity, they are used towards a specific pathogen, avoiding dysbiosis and spread of resistance, being also able to tackle antibiotic- resistant bacteria. Moreover, phages have an easy, rapid and relatively

inexpensive production. Consequently, phages have being considered by the scientific community and pharmaceutical industries as promising alternatives or supplements to antibiotic therapy[94].

1.5.1 Types of phages

There are various types of phages, with different sizes and shapes, but most of them have the same basic features. Generally, they are formed by a head (capsid) and a tail. The capsid is formed by one or more proteins which protect their genetic material. The tail is a hollow tube through which the nucleic acids pass through when the phage infects a host cell[95]. Phages still have long tail fibers (LTFs) at the end of the tail that in some phages are responsible for recognizing the receptor molecules on the host surface[89].

The International Committee on Taxonomy of Viruses (ICTV) is the organ that provides the universal viral taxonomy. This Committee was established in 1966 and in 2011 released its ninth report. In that report, phages are classified according to their morphology and nucleic acid type (double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA)).

It is described that around 95 % of the viruses that infect bacteria belong to the C*audovirales* order[89]. This order consists in three families (Figure 1.5-1): *Myoviridae* (long contractile tails), *Siphoviridae* (long non-contractile tails) and *Podoviridae* (short non-contractile tails). These phages have their genetic material condensed in a molecule of dsDNA with genome sizes ranging from 18 to >500 Kbp. The percentage of G+C is the 27-72 %, generally similar to G+C % of the host cell. In its structure, phages are composed by 7–49 different structural proteins. Furthermore, some phages have non- well-characterized lipids and carbohydrates[95].

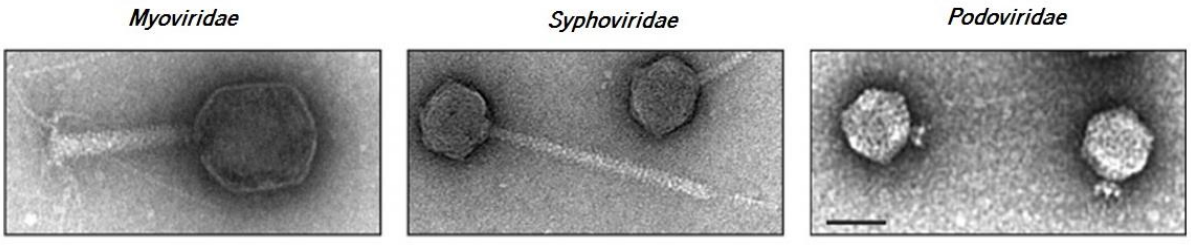


Figure 1.5-1 - Families of the *Caudovirales* order. *Myoviridae* phages have long contractile tails, *Siphoviridae* phages have long non-contractile tails and *Podoviridae* phages have short non-contractile tails. Adapted from[96].

1.5.2 Lytic and lysogenic cycle

Phages are mandatory intracellular parasites of bacteria. They have two life cycles: lytic or lysogenic. In both cases, the cycle begins with the phage interaction with receptors of the host cell. The phage binds

to the host cell, and after creating some pores on the cell envelope injects its genome. Afterwards, phage behaviour is dependent on its life cycle, i.e., if it is virulent or temperate[97] (Figure 1.5-2).

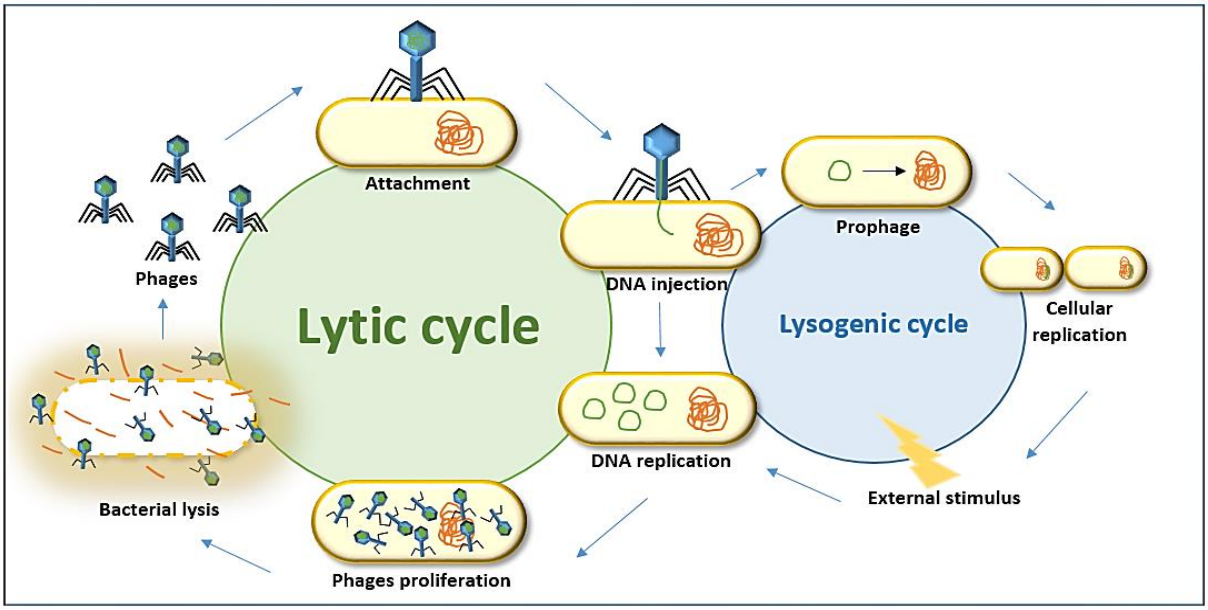


Figure 1.5-2 - Lytic and lysogenic cycles of *Caudovirales* phages. Cycles begin with the phage interacting with receptors of the host cell (Attachment), and next the phage injects its genome (DNA injection). In the lytic cycle the phages replicate inside of the cell (DNA replication) and produce new viruses (Phages proliferation). Subsequently, they destroy the bacterium (Bacterial lysis), and new phages are released. On the other hand, in the lysogenic cycle the viral genome (prophage) replicates together with the host DNA in a plasmid or integrated into the bacterial chromosome. Thus, the phage genome is passed to daughter cells, through cell division. When the cell is exposed to a certain stimulus, the phages can pass to the lytic cycle.

Virulent phages replicate inside the cell and produce new viruses. Subsequently, they destroy the bacterium, and phage progeny is released to initiate a new lytic cycle – lytic cycle (Figure 1.5-2 - left)[98]. When there is an infection with this type of phages, usually clear plaques appear, in the respective host bacterial lawns[99].

On the other hand, temperate phages can follow the lytic cycle or form a stable association with the host (as prophages), and follow the lysogenic cycle. In this cycle the viral genome is replicated together with the host DNA, in a plasmid or integrated into the bacterial chromosome. Thus the prophage is passed to progeny cells, through cell division[97]. Prophages can stay in the cell for many generations, establishing a beneficial relation with the host[89]. When the cell is exposed to certain stimuli (usually stress), like DNA damaging agents (e.g. mitomycin C or ultraviolet (UV) light), antibiotics or other chemical and physical agents with potential to harm the cell, prophages can enter into the lytic cycle[100,101] (Figure 1.5-2 - right). The problem with lysogenic cycle, is that with the insertion of phage genetic material into bacterial DNA, some transduction errors might occur and consequently phages might transmit undesirable genes (from human therapeutics point of view) to new cells, through a process named Horizontal Gene Transfer (HGT).

This process might increase the antibiotic resistance and bacterial virulence[102]. It is described that phages with lysogenic growth cycle form turbid plaques in the respective host bacterial lawns[99].

Generally, the most interesting phages for therapeutics are lytic phages, because they destroy the bacteria and do not increase the resistance and virulence of bacteria with the HGT.

1.5.3 Characteristics of therapeutic phages

The use of phages confers many advantages when compared with conventional antibiotics. Phages can only target bacteria through attachment to host cell surface receptors. Although, some phages are polyvalent, i.e., are able to infect more than one species, the majority will only target one species or in some cases a single bacteria strain - specificity. This characteristic can be seen as a dual effect. Phage specificity means that diagnosis of the bacteria involved in the infection is required before therapy can be applied. On the other hand, it warrants no influence on normal flora, because phages eradicate the targeted strain only[103]. Phages replicate only at the site of infection, lowering the probability of allergies and secondary infections, as observed after antibiotic treatment. In fact, no significant adverse reactions have been observed in several experiments[89–91]. Their lytic cycle characteristics allow an exponential growth as the virus multiplies within the susceptible bacterial host and is subsequently released. This fact allows a lower administered dose when compared to antibiotics dosage[104].

Moreover, these viruses are able to target and kill biofilms[105] and antibiotic-resistant bacteria[106]. The mechanisms developed by bacteria to resist antibiotics do not interfere with phage efficacy[106]. However, bacterial resistance to phages is also observed. This generally occurs through the loss or modification of the bacterial molecules that the phage uses as receptor[107]. However, if bacteria develop resistance to a particular phage other phages can be used, because there are different phages for the same bacteria recognizing different cell surface receptors[108]. Another solution can be the design of a phage cocktail, comprising a set of different phages targeting the same species or different species present in the same type of infection. With the use of phage cocktails, each phage bind to different surface receptors, therefore the mutations in genes encoding receptors on the bacterial cell surface are more difficult, due to phage lytic spectra complementarity[109,110]. Phage versatility and formulation are other advantages of their use. They may be used together with antibiotics[111] or other phages[112] and may be applied by liquids, creams, solids impregnated, by most routes of administration[113,114].

However, not all phages are good for therapy. Phages characterization through genomic analysis is crucial to exclude temperate phages, as only lytic phages are suggested to be used in therapeutic

treatments, because during the lysogenic cycle phages may increase resistance to antibiotics and bacterial virulence[102]. Moreover, whole-genome analysis will allow to confirm the absence of undesirable genes, such as toxins[115].

To maximally exploit the advantages of phages over antibiotics, it is important that phage products might be develop using techniques that ensure the safety and quality of the product. The development and marketing of phage medicinal products in the European Union (EU) included the use of Good Manufacturing Practice (GMP) production, preclinical and phase I, II and III clinical trials and centralized marketing authorization[116]. In that report, other requirements were described, such as: bacterial hosts used in the production process should be as safe (or least pathogenic) as feasible; the storage conditions should be monitored; bacterial endotoxins or lipopolysaccharides (LPS) should be quantified; host range on a panel of target species (reference) strains should be determined[116].

1.5.4 Phages and chronic wounds

Worldwide, there are many studies about the use of phage therapy in chronic wounds. The first reported phages application to treat human infectious diseases was published in 1921[86]. In that test, a staphylococcal-specific phage was injected into and around surgically opened lesions, to treat cutaneous furuncles and carbuncles. The researchers, reported clear evidence of clinical improvement in 48 h post-treatment, with reduction in pain, swelling, and fever in treated patients[86].

In Georgia, a wound healing product named PhageBioDerm was developed. This product consists in a degradable polymer embedded in phages, ciprofloxacin, and benzocaine. This dressing was created in 1995 and approved for commercial release in 2000. In 1999–2000, 107 patients with ulcers, were treated with PhagoBioDerm alone or in combination with other interventions. In 70 % of the patients, the ulcers were completely healed. In 22 cases, healing was associated with the reduction of pathogenic bacteria. Despite the promising results, the authors of this report suggested the development of clinical trials in order to have a better evaluation of this product[117]. More recently, studies on chronic wounds and phage treatment in *in vivo* models showed that they decrease bacterial colonization and increase the survival rate of the animals. These studies are summarized on Table 1.5-1.

Table 1.5-1 - Studies *in vivo* of effectiveness of phages against wounds pathogenic bacteria

Bacteria	Phage	Methodology	Results	Reference
Clinical isolates of *S. aureus, A. Baumannii* and *P. aeruginosa*	*S. aureus* phage cocktail (phages F44/10 and F125/10) *P. aeruginosa* phage cocktail (phages F770/05 and F510/08) *A. baumannii* phage F1245/05	Wounds were created in diabetic rats and diabetic pigs. Several groups were injected with 2.0 x 10^7 CFU/mL of *S. aureus*, *P. aeruginosa*, or *A. baumannii.* On days four, five and eight post wounding, the wounds were debrided, and each phage cocktail or single phage was topically administered. Phage doses of 10^8 to 10^9 PFU per administration were used.	After four days: - In the rat model, *S. aureus* phage cocktail, *P. aeruginosa* phage cocktail and *A. baumannii* phage, reduced respectively, about 2.5, 2.5 and 3 orders-of-magnitude the viable bacteria; - In the pig model *S. aureus* phage cocktail and *P. aeruginosa* phage cocktail reduced respectively, about 4 and 3 orders-of-magnitude the viable bacteria. No such difference was observed in *A. baumannii*.	[118]
S. aureus (2 strains)	*S. aureus* phage	Dermal wounds, were created in rabbits. After three days, wounds were inoculated with 10^6 CFU/mL of *S. aureus* for biofilm formation. Three days later, a *S. aureus*–specific phage with a MOI (Multiplicity of Infection) 1 was applied in wounds with or without debridement treatment.	After six days: - A combination of debridement followed by topical phage application, decreased in approximately 99 % the bacterial burden.	[119]
K. pneumoniae B5055	*K. pneumoniae* phages Kpn1, Kpn2, Kpn3, Kpn4, Kpn5	In BALB/c mice a burn wound was induced. After 30 min *K. pneumonia* at 10^7 CFU/mL was applied. Single phages and a phage cocktail (10^8 PFU/mL) was applied topically at the wound site, after 6 h of bacterium colonization. Bacterial load on the skin was determined for 14 days.	On the third day after treatment: - In all the single phage treated groups a significant reduction in bacterial load about 4.5 orders-of-magnitude was observed; - In animals treated with phage cocktail a significant reduction of 6 orders-of-magnitude was observed.	[112]
K. pneumoniae B5055	*K. pneumoniae* phage Kpn5	A burn was induced in adult BALB/c mice and subsequently infected with *K. pneumoniae*. All mice were treated immediately with a single injection of phage at various MOI (0.001, 0.01, 0.1, 1, 10, 100, and 200).	After 24h: - In higher phage doses (MOI>1), a 96.66 % survival rate was observed; - With MOI<1, the animals became critically ill with 0 - 53.33 % survival; - All control mice (without phage treatment) showed 0 % survival.	[120]

1.5.5 Phages and biofilms

When bacterial cells are in a biofilm, they can show higher levels of tolerance to biocides and antibiotics than planktonic cells[121]. This tolerance is also dependant on the presence/type/thickness of the biofilm extracellular matrix and the biofilm age[121]. The density of the biofilm matrix may cause limitations on phage spreading through the biofilm[122]. Moreover, it can block phage access to the specific receptors of the target cells. Also, the amount of dead cells on the biofilm increase with the biofilm age, due to this, phages can bind to dead cells (nonspecific binding) being consequently unavailable to infect susceptible hosts[122].

However, the presence of biofilm is not problem for some phages[122]. The first step of interaction between the host bacteria and lytic phages is the phage adsorption with the bacterial cell. When host bacteria are included in a biofilm, the biofilm matrix can constitute a first physical barrier to the phage. But, some phages possess polysacharide depolymerases, in the tail, that have the potential to degrade the biofilm EPS matrix[105]. This fact confers to phage an important advantage to start the infection process[123–125]. Another advantage of phages is that, they have the ability to replicate within the host cells, which results in an amplification of their number at the site of infection[105].

In general, the studies with phage therapy have positive results in decreasing bacterial colonization. With the increased interest in phage therapy, there has been a consequent interest in animal testing[112,119] and clinical trials with phage products[89,91] In a first phase the use of cell lines might be a solution, because they allow the *in vitro* study of several factors found *in vivo*[126].

1.5.6 Use of animal cell lines in the phage investigation

The use of animal cell line models, allow the study of the cellular effects of cosmetics, drugs and chemicals, for example[127–129]. These studies include, cytotoxicity, apoptosis assays, biochemical effects and enzymatic reactions[128,129]. Furthermore, the cell lines models allow the study of the administration time of a drug and the different dosages that may cause cell death[130]. The use of cell lines allow the *in vitro* study, of several influence factors found *in vivo*, excluding others, such as immune response[126]. Therefore, in the case of phages, cell lines will be important to study the cytotoxic effects of phage cocktails, to evaluate the internalization of phage particles and to study

their efficacy against a bacterial infection. A study about *A. baumannii* phage BS46 had the aim to determine the cytotoxic effects of this simple phage in 3T3 cells. The researchers concluded that phage concentrations up to 2×10^9 PFU/mL were not cytotoxic to 3T3 cells during 24 h exposure[131]. In another investigation, interactions between four phages and two cell lines (HT-29 and Caco-2) were studied[132]. Phages appeared to be non-cytotoxic for both cell lines, when they were exposed to phage concentration of 10^9 PFU/well, for 96 h. In relation to efficiency of phages, the researchers concluded that the phages, incubated with epithelial cells and the bacterial host, maintained a high killing efficiency, reducing the number of bacterial cells for 8 h. It was also observed that phages were able to actively reproduce in the presence of human cells[132].

In a small amount of data, phages did not demonstrate toxicity problems in animal cells, but the major problem of phage therapy is the potential for inducing an immune response[110].

Immune response of the skin, includes molecules of the innate or the adaptive immune system. The first is typically rapid, does not exhibit memory, and includes: antimicrobial peptides, chemokines, cytokines, the complement system (keratinocytes, fibroblasts), polymorphonuclear leukocytes (PMNs), monocytes/macrophages, dendritic cells (DCs), and natural killer cells (NK)[133]. On the other hand, the adaptive system has a high degree of specificity, memory, but it does not provide a quick response. This system includes dendritic antigen-presenting cells (APC) in the epidermis and dermis, T lymphocytes and B-cell–derived antibodies[133]. When the skin is exposed to a physical or chemical stress, keratinocytes produce and release inflammatory cytokines (interleukins (IL), tumor necrosis factor (TNF), chemotactic cytokines, growth factor, macrophage and other that generated cutaneous inflammation[133]. In literature, Mirzaei *et al.*[132] promoted a study where it was described the quantification of cytokine release *in vitro* in the presence of four *E. coli* distinct phages. Two cell lines (HT-29 and Caco-2) and peripheral blood mononuclear cells (PBMCs) were used. The authors concluded that the phages at high concentrations (10^9 PFU/well) stimulated a greater release of IL-6, IL-10 and TNF- α *in vitro*[132].

Despite the positive results in combating bacteria, a problem of the use of phages in medical therapy (topical or systemic) is that for a safe and effective application a pure and high concentration of phage suspensions is required[134].

1.5.7 Phage purification

Traditionally, techniques used to purify phages at labscale are the polyethylene glycol (PEG) precipitation and the cesium chloride (CsCl) density gradient ultracentrifugation[135]. In the early 60's a method with aqueous PEG-dextran sulfate two-phase systems was developed by Albertsson and his co-workers for concentrating and purifying various viruses[136].

PEG precipitation is a common method to concentrate and capture large proteins. The main advantage of PEG is that is uncharged, relatively inexpensive and not inflammable. Despite some difficulties in removing from protein solutions[137], a standard procedure for the phages purification consists of PEG precipitation followed by chloroform extraction of the pellet, to remove PEG[137]. With this method, phages can be concentrated from crude lysates. This method, is useful in order to concentrate phages even with very low titer lysates and it is applicable to most phages without modification. However, the final phage solution should not contain chloroform residues, which with this method is difficult to ensure[137].

CsCl density gradient ultracentrifugation has been used for the purification of a range of viruses including phages. This method of purification generally requires ultracentrifugation at more than 100 000 × *g*, that is a disadvantage because requires a costly set of ultracentrifuge, rotor and special tubes, so it is not accessible to all laboratories[137]. With the centrifugation in CsCl gradients, phage particles are separated according to their buoyant density rather[138]. This process can be used with large volumes of phage, resulting in highly purified preparations being able to remove the bacterial LPS (endotoxin) that may contaminate phage preparations obtained from Gram-negative bacterial hosts[138].

Chromatography is also an alternative for phages purification, that was first reported in 1957. Creaser and his co-workers used a cellulose anion exchange absorbent ECTEOLA, in order to purify *E. coli* phages, and they concluded that the phages are recovered in states of purity comparable with those obtained by other procedures[139]. Recently, monolithic columns named Convective Interactive Media® (CIM®) were used for phage purification. There are columns of chromatography with matrices of quaternary amine (QA) and others with diethylamino (DEAE). However, no protocols is described for phages purification. In all of them, different types of columns and conditions (equilibration and elution buffers, flow rates, detection equipment) were studied[139-142]. In general, the protocols described using monolithic columns have shown good results in purifying phages[140–143].

2 MOTIVATION AND AIM OF THIS STUDY

Chronic wounds are defined as skin injury where the normal reparative process failed and the anatomic and functional integrity is not achived within a period of three months. In the United States of America, these wounds affect annually around 2 % of population and their treatment has billion of annual expenses. With an increasing population aging and the lifestyle changes, in the further years it is expected an arise of new cases of chronic wounds.

One of the difficulties for the healing of these injuries is the emergence of a microbial invasion. *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* species and *Pseudomonas aeruginosa* are the bacteria, most frequently, isolated from chronic wounds. Normally, these microorganisms form an aggregation embedded within a self-produced polymeric matrix - biofilms, which are persistent and highly resistant to common therapies, namely the antibiotics.

Despite many strategies to combat biofilms in chronic wounds, there are still limitations in the evidence of therapeutic efficacy, particularly due to antimicrobial resistance. The World Health Organization, after publishing a list of pathogens resistant to antibiotics, suggested the urgent development of new antimicrobials to combat these pathogens.

The use of phages as a possible alternative to antibiotics is being more accepted by the scientific community. They can infect antibiotic resistant bacteria and have shown efficacy against biofilms *in vitro*. The use of phages in wound treatment is already referenced in the literature, but problems in the choice of the best phage candidates and biofilm model as well as the optimization of phage purification methods still need to be improved.

Therefore, the aim of this work is to develop a phage-based bioactive product to be incorporated in a wound dressing to control biofilms in chronic wounds. To reach the goal, this work intends to:

- ✓ Isolate phages that specifically infect *E. coli*, *E. faecium*, *E. faecalis* and *S. aureus* strains;
- ✓ Characterize phages to assess their adequacy for therapy;
- ✓ Study the effectiveness of phages in a selected *in vitro* model that mimics wounds environment;
- ✓ Purify phages using chromatographic approaches;
- ✓ Study the interactions between phages and animal cells.

3 MATERIALS AND METHODS

3.1 Phage isolation and production

3.1.1 Bacterial strains and culture conditions

The bacterial strains used for the isolation phages were clinical isolates from Hospital de Braga and collection strains. The strains were the following: six strains of *S. aureus,* six strains of *E. faecalis,* 12 strains of *E. faecium* and four strains of *E. coli.* The complete list of strains used in this study is depicted in Table 3.1-1.

Table 3.1-1 - Bacterial strains and your origin, used to isolate phages

Species	Strain	Origin
S. aureus	U104	Urine
	I366	Sputum
	I642	Bronchial Aspiration
	I097	Sputum
	C610	Skin exudate
	I334	Skin exudate
E. faecalis	I201	Urine
	I899	Urine
	I009	Urine
	I436	Urine
	I900	Blood culture
	I980	Blood culture
E. faecium	I769	Rectal exudate
	I999	Urine
	I951	Rectal exudate
	I406	Perineal exudate
	I647	Blood culture
	I459	Skin exudate
	C410	Collection
	I738	Pus
	I405	Pus
	C803	Urine
	C759	Urine
	I567	Pus
E. coli	U366	Urine
	U936	Urine
	U087	Urine
	U005	Urine

The species *E. faecalis, E. faecium, and S. aureus*, were grown in Tryptic Soy Broth (TSB, VWR), and *E. coli* was grown in Lysogeny Broth (LB, Nzytech). The solid medium was Tryptic Soy Agar (TSA) and Lysogeny Broth Agar (LBA) with 1.2 % (w/v) of agar (Thermo Scientific). The Top-agar of TSB or LB was composed by 0.6 % (w/v) of agar for *Enterococcus* strains and *E. coli,* and 0.4 % (w/v) of agar for *S. aureus.*

3.1.2 Bacterial lawns

In agar plates, 50 µL of bacterial suspension (grown overnight 16-18 h) and around 3 mL of Top-agar were added. They were gently swirled. The plates were let dry for 1-2 min.

3.1.3 Phage isolation

Wastewaters from ETAR Frossos, Braga and ETAR Paços de Ferreira, Porto were centrifuged (8 500 x *g*, 4 °C, and 10 min) (Sigma 3-16KL). Then 50 mL of centrifuged sewage were mixed with the same volume of double-strength culture media and 100 µL of each bacterial suspension (grown overnight). The mixtures were further incubated for 24 h, under agitation (120 rpm) at 37 °C (ES-20/60, Orbital Shaker-Incubator – BIOSAN). After the incubation, the solution was centrifuged (10 000 x *g*, 4 °C, 10 min) and the supernatant filtered through a 0.22 µm PES membrane filter (Whatman). Spot assays were performed against bacterial lawns to check the presence of phages. Inhibition haloes (clear or turbid), indicative of the presence of phages were further purified with toothpicks and paper to isolate all different phages. Plaque picking was repeated until single-plaque morphology was observed.

3.1.4 Phage production

Each isolated phage plaque was picked with a toothpick and it was stung in agar plates containing a bacterial lawn. A strip of paper was passed through the entire agar plates. The plates were further incubated overnight at 37 °C.

After full lysis, 3 mL of SM buffer (5.8 g/L NaCl (PanReac AppliChem); 2 g/L MgSO4.7H2O (PanReac AppliChem); 50 mL/L 1 M Tris (pH 7.5); 0.002 % (w/v) gelatin (Sigma Aldrich)) was added to each plate. Plates were further incubated with agitation (50 - 90 rpm) at 4 °C for 7 h (Panasonic).

After this period, the liquid and top-agar with the eluted phages were collected and the solution was centrifuged (5 000 x *g*, 4 °C, and 5 min) to remove all bacteria remaining. The supernatant was recovered and was filtered through a 0.22 µm PES membrane filter. Phage solution was stored at 4 °C for later use.

3.1.5 Phage enumeration

For the determination of the titer of the phage produced, the PFU (Plaque Forming Unit) method of successive dilutions was used. The concentration of phage in PFU/mL was determined by the following equation.

$$\text{Phage concentration}\left(\frac{\text{PFU}}{\text{mL}}\right) = \frac{\text{Number of phage plaques} \times \text{Dilution factor}}{\text{Sample volume}}$$

3.2 Lytic spectrum

The lytic spectrum allows to evaluate the host range of each isolated phage. For this, 10 µL of each phage solution was added in agar plates containing a different bacterial lawn. These plates were incubated overnight at 37 °C. In the next day, the plates were evaluated for the presence of lysis zone.

3.3 *In vitro* studies

The *in vitro* models allow to simulate the conditions of human body. In this work the Microtiter Plate (MTP) model and Collagen Wound Model (CWM), with single and mixed-species biofilm were used. The first allows the study of multiple parameter (composition of growth media, incubation temperatures, pH) and the second simulates wound bed with the use of collagen and a simulated wound fluid (SWF).

3.3.1 Microtiter Plate Method

Single-species biofilm formation

For the study of phage efficacy in biofilms, it was necessary, before phage infection, to study the ability of species to form biofilms. For this, 2 µL (96- well plates (SARSTEDT)) or 10 µL (24-well plates (Orange Scientific)) of bacterial suspensions (grown overnight at 37 °C and 120 rpm) and 198 µL (96-well plates) or 990 µL (24-well plates) of TSB culture medium were added in wells. The plates were incubated at 37 °C and 120 rpm for 48 h, with complete culture media renewal after 24 h. In negative control wells, only TSB medium was added. After biofilm formation, wells were washed twice with 0.9 % (w/v) NaCl, to remove the planktonic cells.

3.3.2 Collagen Wound Model

Single-species biofilm formation

This methodology was performed as previously described by Werthén *et al.*[144]. Similarly, to the previous model, the ability of bacteria to form biofilms was studied, so 350 µL (96- well plates) or 2 mL (24-well plates) of collagen from bovine Achilles tendon (50 µg⁄mL suspended in 0.9 % (w/v) NaCl, Sigma-Aldrich), were added to each well and the plates were incubated at 4 °C overnight. The coating solution was then gently removed and the wells were washed twice with 0.9 % (w/v) NaCl prior to biofilm formation.

Biofilms were formed as previously described (3.3.1) with a brief modification. Instead of using TSB, a mixture of TSB/SWF (1:1) culture medium (SWF: 50 % (v/v) Fetal Bovine Serum (FBS, Thermo Scientific) and 50 % (v/v) physiological NaCl in 0.1 % (w/v) Pepton, Amresco) was added to each well. In negative control wells, only TSB/SWF (1:1) medium was added.

Mixed-species biofilm formation

Mixed-species biofilms were formed on 24-well plates, using the collagen coating previously referenced on single-species biofilm formation chapter. Five microliters of each bacterial suspension (grown overnight at 37 °C and 120 rpm) and 990 µL TSB/SWF medium were added in collagen-coat wells. The plates were incubated at 37 °C in agitation for 24 h. Afterwards, medium was removed and 1 mL of fresh culture medium was added to each well. Plates were incubated at 37 °C in agitation for more 24 h. In negative control wells, TSB/SWF (1:1) medium was added. After this 48 h, the biofilm was washed twice with 0.9 % (w/v) NaCl, to remove the planktonic cells.

3.3.3 Phage infection

After biofilms formation, 200 µL (96- well plates) or 1 mL (24- well plates) of phages solution with MOI of 1 was added to each well. Then, the plates were incubated at 37 °C and samples were taken at 3, 6, 8 and 24 h to determine culturable bacterial counts. In each time point, biofilms were disrupted using an ultrasonic bath (Sonicor model SC-52), operating at 50 kHz for 30 min, then, the suspensions were collected and viable cells were determined.

$$\mathrm{MOI} = \frac{\text{Phage concentration} \times \text{Phage volume}}{\text{Bacterium concentration} \times \text{Bacterium volume}}$$

3.3.4 Determination of viable bacterial cells in biofilm

For the determination of viable bacterial cells in biofilm, the CFU (Colony Forming Unit) method of successive dilutions was used. The concentration in CFU/mL was determined by the following equation.

$$\text{Viable bacterial cells } \left(\frac{\text{CFU}}{\text{mL}}\right) = \frac{\text{Number of bacterial colony} \times \text{Dilution factor}}{\text{Sample volume}}$$

3.4 Transmission electron microscopy

The morphology of phage particles was observed by transmission electron microscopy (TEM), as previously described by Melo *et al.*[145]. Briefly, a solution of phage lysate was centrifuged (1 h, 25 000 ×g, 4 °C) and phage particles were collected. The pellet was washed twice in tap water using the same centrifugation conditions. Phages were further deposited on copper grids with carbon-coated Formvar films, stained with 2 % uranyl acetate (pH 4.0). Phages were examined using a Jeol JEM 1400 transmission electron microscope.

3.5 DNA isolation, genome sequencing and annotation

An adapted protocol of Melo *et al.*[146] was used for genomic DNA extraction. Purified *E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410 were treated, for 2 h at 37 °C, with 0.016 % (v/v) L1 buffer (300 mM NaCl, 100 mM Tris/HCl (pH 7.5), 10 mM Ethylenediaminetetraacetic acid (EDTA, Biochrom), 0.2 mg BSA mL^{-1}, 20 mg RNase A mL^{-1} (Sigma), 6 mg DNase I mL^{-1} (Sigma)). Next, a thermal inactivation of the enzymes for 30 min at 65 °C was performed. After this time, 50 µg proteinase K ml^{-1}, 20 mM EDTA, and 1 % (v/v) SLS (sodium lauryl sulfate) were added and a process of protein digestion was performed for 18 h at 56 °C. This was followed by phenol: chloroform: isoamyl alcohol solution (25:24:1, v/v) and chloroform extractions. Then, DNA was precipitated with isopropanol and 3 M sodium acetate (pH 4.6), it was centrifuged (15 min, 7 600 ×*g*, 4 °C), and the pellet air-dried and further resuspended in nuclease-free water (Cleaver Scientific).

Genome sequencing was performed and DNA was sequenced by STAB VIDA using a NGS Illumina MiSeq platform (Illumina Inc., San-Diego, USA) and Nextera XT to generate 250-bp fragments paired-end reads. Quality controls of DNA before being sequenced on the MiSeq System were made by Agilent

Bioanalyzer and Qubit measurements. Outputs were demultiplexed and *de novo* assembled into a single contig using Geneious 9.0 (Biomatters) and manually inspected.

MyRAST[147] was used to annotation of phages genomes. The presence of non-annotated CDSs, along with genes in which the initiation codon was miscalled, were checked manually using Geneious 9.0. BLASTX[148] was used to check potential frameshifts. For to search by homologous proteins, with an E value threshold of $<1\times10^{-5}$ and at least 80 % query, was used the BLASTP[149]. With the same cutoff parameters as used with BLASTP, the Pfam 31.0[150] was used for protein motif search. Protein pI and molecular mass were predicted using Molecule Weight and Isoelectric Point Finder at Sequence Manipulation Suite[151]. Transmembrane domains were predicted using Phobius[152] and TMHMM[153]. tRNAs were predicted using ARAGORN[154] and tRNAscan[155].

DNA homology between *Enterococcus* phages was analyzed using BLALTN[150].

3.6 pH and temperature stability

Thermal stability tests were carried out by incubating 10^8 PFU/mL of each tested phage at −20 °C, 4 °C (as control), room temperature (21 °C), 37 °C, 50 °C, and 60 °C for 24 h. Similarly, the effect of pH was also evaluated using a universal pH buffer (150 mM potassium chloride (Pronalab), 10 mM potassium dihydrogen phosphate (Panreac AppliChem), 10 mM sodium citrate (Fisher), 10 mM boric acid (Fisher) with pH adjusted to 1, 2, 3, 4, 5, 6, 7 (as control), 8, 9, 10, 11, 12 and 13, at room temperature for 24 h. In both experiments, concentration of phage in PFU/mL was determined by equation of phage enumeration.

3.7 Purification of phages

The use of phage therapy in medical (topical or systemic), applications require pure and high concentration of phage suspensions. In this work, two different phage purification methods were used. A more traditional methodology - PEG, with easy and cheap purification steps, and other more complex - chromatographic phage purification, with several advantages described and with phages recovered in better states of purity than those obtained by other procedures.

3.7.1 Polyethylene glycol precipitation

This methodology was performed as previously described by Sambrook *et al.*[156], with brief modifications. After performing the phage production procedure, NaCl (0.584 g/10 mL) was added and the solution was further incubated with agitation (50 - 90 rpm) at 4 °C for 1 h. The solution was centrifuged (8 500 x *g*, 4 °C, and 10 min). The supernatant was recovered and then PEG 8000 (1 g/10 mL, PanReac AppliChem) was added. It was gently mixed until it dissolved, and it was incubated overnight with agitation (50 - 90 rpm) at 4 °C.

The solution was then centrifuged (8 500 x *g*, 4 °C, and 10 min). The pellet was recovered and SM buffer (1/3 volume of supernatant) was added. It was incubated with agitation (50 - 90 rpm) at 4 °C for 1 h, after which, it was gently mixed and chloroform on a final concentration of 25 % (v/v) was added. Then, the solution was mixed using the vortex, it was centrifuged (3 500 x *g*, 4°C, and 15 min) and the top solution was recovered and filtered (syringe filter 0.22 µm). Finally, it was stored at 4 °C for later use.

3.7.2 Chromatography – CIMmultus® columns

The first experiments were performed on a High Pressure Liquid Chromatography system (HPLC, Nexera X2, LC-30AD - Shimadzu), consisting a manual injector (KNAEUR, HPLC 6 Port Valves) with a 5 mL sample loop (RHEODYNE) and a diode array detector (SPD-M20Aith 10 L). Second part of the experiences were performed in a Fast Protein Liquid Chromatography (FPLC, PHARMACIA) with 5 mL of sample load.

The anion-exchange chromatography columns used were the laboratory scale (1 mL) CIMmultus® QA and DEAE (BioSeperations). In HPLC system, all components were connected with a Peek Capillary Tubing 1/16'' OD, 0.010'' ID.

The loading the phages on the columns was performed using a buffer with Tris buffer (20 mM Tris–HCl, pH 7.5). For the elution, a gradient of 0 to 2 M NaCl was added to the loading buffer. The flow rate was fixed at 1 mL/min for HPLC experiences and in FPLC system flow rate was 0.5 mL/min for the solution load and 1 mL/min for elution. In both cases the wavelength was 280 nm. The HPLC software used was LabSolutions – version 5.71 SP1 (Shimadzu Corporation).

3.8 Studies in 3T3 mammalian cells

3.8.1 Cell cultures

Mouse embryonic fibroblast 3T3 cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Biochrom), supplemented with 10 % (v/v) FBS, 1 % (v/v) of penicillin-streptomycin (ThermoFisher) (complete culture medium), on tissue culture-treated flasks (Starstedt), at 37 °C, 5 % CO_2 and > 90% humidity (Heracell 150, Heraeus).

Sub-culturing was performed when cell confluence reached approximately 80 %, at a 1:2 or 1:3 flask ratio. For this, cells were washed with Phosphate Buffered Saline (PBS: 137 mM NaCl, 10 mM Sodium Phospate Dibasic (Riedel-deHaen), 2.7 mM Potassium Chloride and Potassium Phosphate Monobaisic (Sigma-Aldrich), pH=7.4) and detached using trypsin-EDTA.

3.8.2 Cytotoxicity assay – Neutral Red Uptake

Cytotoxicity of three phages (*E. faecalis* phage 09-2, *E. faecalis* phage C410 and *E. faecalis* phage 80-2) was determined according to ISO 10993- 5:2009, Annex A - Neutral Red Uptake (NRU) cytotoxicity test.

Briefly, 3T3 cells were seeded into 96-well plates, at 1×10^5 cells/mL of complete culture medium. Plates were maintained for 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity.

After 24 h incubation, the culture medium was removed and 100 µl of treatment medium, composed of complete culture medium containing the appropriate concentrations of phages (10^8 PFU/mL and 10^7 PFU/mL) was added. A positive control consisting of complete culture medium containing different concentrations (0.20 mg/mL, 0.15 mg/mL, 0.10 mg/mL and 0.05 mg/mL) of SLS (ThermoFisher) was also added to the cells. A negative control was prepared with complete culture medium, and a blank with culture medium without 3T3 cells. The 96-well plates containing these preparations was incubated for 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity.

In the third day, the culture medium was removed and the cells were washed with 150 µl pre-warmed PBS. The stock solution of Neutral Red (3-amino-m-dimethylamino-2-methlphenazine hydrochloride, NR) was supplied by ThermoFisher and prepared using 0.4 % of NR stock in 100 mL of sterile water, which was then mixed with DMEM (to a final concentration of 1.25 %). Then 100 µl of pre-filtered and pre- warmed 1.25 % NR solution were added to each well and incubated at 37 °C and 5 % of CO_2, in a humidified atmosphere, for 3 h. The NR solution was removed and the cells were washed with

150 µl of pre-warmed PBS. Subsequently, 150 µl of NR desorb solution (1 % acetic acid, 50 % ethanol and 49 % sterile water) were added to all wells and the plates were shaken for 10 min, to extract the NR from the cells and form a homogeneous solution. NR absorption was measured at an optical density of 540 nm (OD_{540}) in a spectrophotometer (Synergy HT – Bio-Tek) for further analysis of the cells viability (NR absorbance of the sample divided by NR absorbance of the negative control). The assays were performed in triplicate, and considered valid only when the IC50 of SLS is within the confidence interval of 0.070 - 0.116 mg/mL (95% confidence) and blank OD_{540} is equal or greater than 0.3 as determined by ISO 10993- 5:2009.

3.8.3 Bacterial adhesion of 3T3 cells

Two different culture media were used in this assay – Culture Medium 1: DMEM supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin; and Culture Medium 2: DMEM, supplemented with 10 % (v/v) FBS.

3T3 cells were seeded into 96-well plates at 5×10^5 cells/mL on culture medium 1. Plates were maintained for 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity. After 24 h, bacterial suspensions (grown overnight in TSB medium) of the strains in study were centrifuged (11 000 x *g*, 1 min) and washed twice with PBS, and the OD at 600 nm adjusted to 0.08-0.1 with PBS. Bacterial cells were once again centrifuged and resuspended in culture medium 2. Then, 100 µl of the bacterial suspensions were added to the 3T3 cells in the 96-well plates, previously washed with 100 µl of PBS. Negative control was prepared with culture medium 2. The 96-well plates containing these preparations were incubated for 2 h or 24 h at 37 °C, 5 % of CO_2 and > 90% humidity.

After 2 or 24 h the culture medium was removed and cells were washed twice with 100 µl of PBS. Then, 40 µl of pre- warmed trypsin were added to all wells, and the plates were incubated for 15 min at 37 °C, 5 % of CO_2 and > 90 % humidity. Sixty microliters of culture medium 2 were added to stall trypsin activity. The CFU method of successive dilutions was used for the determination of viable bacterial cell concentration. Cell death associated with bacterial adhesion was quantified with a Neubauer chamber and an inverted microscope (Leica DMIL), by the following equation.

$$\text{Viable mammalian cells } \left(\frac{\text{cells}}{\text{mL}}\right) = \frac{\text{Number of total cells} \times 10^4 \times \text{Dilution factor}}{\text{Number of squares counted}}$$

3.8.4 Internalization of bacteria and phages in 3T3 cells

Culture media used in this assay were the same of the previous assay (3.8.3).

For the bacterial internalization assay, an antibiotic unable to internalize mammalian cells was used to eliminate only the external bacterial cells. After a few susceptibility tests of the bacterial strains, vancomycin was selected and used at 150 µg/mL. Ferrous Ammonium Sulphate (FAS, Panreac AppliChem) at 10 mM was used to eliminate external phages, in the phage internalization assay.

For both assays, 3T3 cells were seeded into 96-well plates at 5×10^5 cells/mL of culture medium 1. The plates were maintained for 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity.

Bacterial internalization of 3T3 cells

Bacterial suspensions (grown overnight in TSB medium) of the studied strains were centrifuged (11 000 x *g*, 1 min) and washed twice with PBS, and the OD at 600 nm adjusted to 0.08-0.1 with PBS. Then, bacterial cells were centrifuged once again using the same conditions and resuspended in culture medium 2. One hundred microliters of the bacterial suspensions were added to the 3T3 cells in the 96–well plates, previously washed with 100 µl of PBS. A negative control was prepared with culture medium 2. Plates were incubated for 1 h at 37 °C, 5 % of CO_2 and > 90 % humidity. The culture medium was then removed and cells were washed twice with 100 µl of PBS. One hundred microliters of culture medium 2 supplemented with vancomycin at 150 µg/mL was added to wells containing bacteria, to determine the internalized bacteria. In other wells only culture medium 2 was used to determine the total number of bacteria interacting with the cells (adhered and internalized). Culture medium 2 was also added to the negative control. The 96-well plates containing these preparations were incubated for 1 h at 37 °C, 5 % of CO_2 and > 90 % humidity. One hour later, cells were washed twice with 100 µl of PBS and a solution of 0.5 % (v/v) Triton in PBS was added to all wells. Plates were incubated for 10 min at room temperature for the disruption of the mammalian cells and release of adhered and internalized bacteria. Finally, CFU method of successive dilutions was used for the determination of viable bacterial cell concentration.

Phages internalization

The wells of the 96-well plates containing 3T3 cells were washed twice with 100 µl of PBS. Then, 100 µl of phage solution (1×10^8 PFU/mL of culture medium 2), were added to the wells. A negative control was prepared with only culture medium 2. The plates were incubated for 1 h at 37 °C, 5 % of CO_2

and > 90 % humidity. After this time, the wells were washed twice with 100 µl of PBS. Then, 100 µl of culture medium 2 containing 10 mM FAS were added to wells containing phage, to determine the internalized phages. In other wells only culture medium 2 was used to determine the total number of phages interacting with the cells (adhered and internalized). Culture medium 2 was also added to the negative control. The 96-well plates containing these preparations was incubated for 1 h at 37 °C, 5 % of CO_2 and > 90 % humidity. One hour later, cells were washed twice with 100 µl of PBS and a solution of 0.5 % (v/v) of Triton in PBS was added. Plates were incubated for 10 min at room temperature for the disruption of the mammalian cells and release of adhered and internalized phages. Finally, the PFU method of successive dilutions was used for the determination of viable phage concentration.

3.8.5 Efficacy of phages in bacteria colonizing 3T3 cells

Culture media used in this assay were the same of the bacterial adhesion of 3T3 cells (3.8.3).

3T3 cells were seeded into 96-well plates at 5×10^5 cells/mL of culture medium 1. Plates were maintained for 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity. After 24 h, bacterial suspensions (grown overnight in TSB medium) of the strains in study were centrifuged (11 000 x *g*, 1 min) and washed twice with PBS, and the OD at 600 nm adjusted to 0.08-0.1 with PBS. The bacterial cells were once again centrifuged and resuspended in culture medium 2. In the microtiter plates the culture medium was removed and cells were washed twice with 100 µl of PBS. Then, 100 µl of the bacterial suspensions were added to the 3T3 cells. A negative control was prepared with only culture medium 2. Plates were incubated for 2 h at 37 °C, 5 % of CO_2 and > 90 % humidity. Cells were further washed twice with 100 µl of PBS. Then, 100 µl of phage solutions at 1 x 10^7 PFU/mL were added to the cells, and 100 µl of culture medium 2 were added to negative and non-phage treated cells. The 96-well plates containing these preparations were incubated for 6 h or 24 h at 37 °C, 5 % of CO_2 and > 90 % humidity.

After 6 or 24 h the cells were washed twice with 100 µl of PBS. Then 40 µl of pre- warmed trypsin were added to all wells, and the plates were incubated for 15 min at 37 °C, 5 % of CO_2 and > 90 % humidity. Sixty microliters of culture medium 2 were added to stall trypsin activity. The CFU method of successive dilutions was used for the determination of viable bacterial cell concentration. Cell death associated with bacterial adhesion was quantified with a Neubauer chamber and an inverted microscope.

The study of phage efficacy in killing internalized bacteria was performed as described previously with brief modifications. Bacterial suspensions were left in contact with 3T3 cells for 1 h. Then, culture

medium 2 supplemented with vancomycin at 150 μg/mL was added to the wells for an additional hour, to eliminate external bacteria. Finally, phage solutions were added to the wells for evaluating efficacy.

3.9 Statistical Analysis

All graphs were generated using GraphPad Prism 6 software (GraphPad Software). Means and standard deviations (SD) were calculated. Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni *post hoc* tests. Differences between samples were considered statistically different for *p*-values lower than 0.05.

4 RESULTS AND DISCUSSION

AMR is a serious problem in public health and requires intervention of governments and societies. Biofilms cause serious infections, because they are persistent and highly tolerant to common therapies, namely the antibiotics[157]. Moreover, biofilms-AMR increases the cost of health care in all countries[158]. Chronic wounds are defined as injuries on skin where the normal reparative process fails[1], as a consequence of a pathology or of a biofilm occurrence in the wound bed[2]. The most common bacteria observed in chronic wounds are *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis*[46,48]. Despite many strategies to combat biofilms in these wounds, there are still limitations in the evidence of therapeutic efficacy[1,159,160].

Presently, phages are seen as a possible novel treatment against antibiotic-resistant infections, namely caused by biofilms. These viruses that specifically infect bacteria are described as safe and due to its specificity, they can be used towards a specific pathogen avoiding dysbiosis and the spread of resistance[161]. Several *in vitro* studies demonstrated positive results on the use phage therapy in the treatment of biofilms[111,114,162,163]. Furthermore, using *in vivo* models, similar results on phage efficacy in chronic wound infections have also been reported[118,119,164]. It is important to highlight that on the referred studies was not reported adverse reactions to phage therapy.

The aim of the present study, was to isolate and characterize phages that could be successful in killing bacterial species found in chronic wounds. To achieve that, phages that specifically infect *E. coli*, *E. faecium*, *E. faecalis* and *S. aureus* strains were isolated. After this, phages were subjected to a process of characterization. The first parameter evaluated was host range, which should be as broad as possible, particularly including clinically prevalent bacterial species[134].

Characterization process also included *in vitro* studies for evaluating the direct interaction between phage and bacteria, which enabled the selection of the better phages. Moreover, these studies also provide valuable information for the determination of optimal posology[165].

Phages characterization in terms of morphology, genome characteristics and stability at different temperatures and pH was also analyzed. In order to ensure the safety of a phage preparation it is of utmost importance to guarantee that phages are not temperate and do not encode virulence factors[115]. This can be partially assessed by genome sequencing and annotation.

Another important point in phages for therapeutic use is their purification. It is important that the phage solutions to be used in therapy to be free from contamination[166], therefore, in the second part of this work the phages were purified using chromatography approaches.

Finally, in the third part, the interactions between animal cells and phages were assessed, namely their cytotoxic potential, their internalization ability and their efficacy against bacteria.

4.1 Isolation and characterization of phages

Phages are ubiquitous in nature and they can be found wherever their host is present, therefore it is expected to isolate them in every environment, namely soil, water, sewage, human and animal organisms, and food[167]. In this work, wastewater from ETAR Braga (Frossos) and ETAR Porto (Paços de Ferreira), strains isolated in Hospital de Braga as well as culture collection strains were used. Using these environmental sources and the referred strains, five *E. coli* phages, four *S. aureus* phages, one *E. faecium* phage and seven *E. faecalis* phages were isolated

i. *E. coli* phages

E. coli is a Gram-negative bacterium that is a common inhabitant of the animal's intestinal tract. Although most of the *E. coli* strains are harmless and part of the normal intestinal microflora, some strains are pathogenic[168]. This bacterial species is one of the most frequent causes of cholecystitis, bacteremia, cholangitis, urinary tract infection, diarrhea (due to food poisoning), neonatal meningitis, pneumonia, among others[169].

After sewage enrichment (Frossos, Braga) using ten *E. coli* strains, five *E. coli* phages were isolated. Phage plaque morphologies were clear and uniform in each respective host lawn. The same panel of strains was used to determine phages host range (Table 4.1-1).

Table 4.1-1 – Lytic spectra of five isolated phages against *E. coli* strains

	Phage	366 1	366 3	936 2	936 3	055 1
Strain	30	-	-	-	-	-
	31	-	-	-	-	-
	32	-	-	-	-	-
	33	-	-	-	-	-
	34	-	-	-	-	-
	35	-	-	-	-	-
	005	-	-	-	-	Clear
	936	-	-	Clear	Clear	-
	87	-	-	-	-	-
	366	Clear	Clear	-	-	-

Clear: clear halo; - No halo

As it can be seen in Table 4.1-1, *E. coli* phages had a narrow spectrum of activity towards the tested strains, where each phage only lysed each respective host.

For a successful application of phage therapy, it is very important that the phages display a broad spectrum of action[134]. As no phage from *E. coli* strains obeyed this parameter, they were not included on further studies.

ii. *S. aureus* phages

S. aureus is a Gram-positive bacterium, frequently found in the nose, respiratory tract and on the skin. Although, this microorganism is a common inhabitant of the human body, there are several pathogenic strains[170], being considered one of the most dangerous microbial pathogens worldwide[80]. *S. aureus* is a leading cause of bacteremia, infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections[171].

Four *S. aureus* phages were isolated from two different effluents (Frossos, Braga and Paços de Ferreira, Porto) enriched with 19 *S. aureus* strains. Although these phages were difficult to propagate, its production was optimized with 0.4 % TSB-agar plates. The size of the plaques is related to the diffusivity of the phages. On the other hand, the diffusion rate depends on the properties of the phage, such phage dimensions, but also on the top agar concentration[172]. When the agar concentration is reduced it is expected that the diffusivity of the phages and the size of the plaques will increase[173].

After production and in order to study their lytic spectrum, newly isolated phages were then tested against 19 *S. aureus* strains (Table 4.1-2).

Table 4.1-2 – Lytic spectra of four isolated phages against *S. aureus* strains

	Phage	I366 B	U104 B	I366 P	U104 P
Strain	1	Clear	Clear	Clear	-
	2	-	-	-	-
	3	-	-	-	-
	4	Clear	Clear	Clear	Clear
	5	Clear	Clear	Clear	Clear
	6	-	-	-	-
	C003	Clear	-	-	-
	C167	-	-	-	-
	C276	-	-	-	-
	741	Clear	Turbid	Turbid	-
	C517	-	-	-	-
	C117	Clear	Clear	-	-
	C483	-	-	-	-
	I097	Turbid	-	-	-
	C610	-	-	-	-
	I334	Turbid	-	Turbid	-
	I366	Clear	Clear	Clear	Clear
	I642	Turbid	-	Turbid	-
	U104	Clear	Clear	Clear	Clear

Clear: clear halo; Turbid: turbid halo; - no halo

After lytic spectrum assay, a phage of this strain was selected for use in biofilm infection experiments. Although *S. aureus* phage U104 B caused a turbid halo in the strain 741, it showed clear halos in other six *S. aureus* strains. For phage I366 P, lysis was observed in eight strains, where clear haloes were observed in five strains and turbid halos were observed in other three strains. Phage U104 P did not inhibit 15 strains, presenting clear haloes in only four *S. aureus* strains. Although phage I366 B did not cause an inhibitory effect in eight strains, it presented a clear effect in eight strains and turbid haloes in three other strains. Using the criterion of selecting the phage able to infect the larger number of strains, phage I366 B was selected for further studies as this is an important parameter for phage therapy[134].

iii. *E. faecalis* phages

E. faecalis is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. However, some *E. faecalis* strains can cause life-threatening infections in humans[174,175]. This species is associated with a variety of different infections, such bacteremia, endocarditis, in skin or soft tissue and in urinary tract[176].

A combination of raw sewage from Frossos, Braga with 16 *E. faecalis* strains was used to isolate seven phages of this species.

These isolated phages were then tested against the referred set of *E. faecalis* strains in order to study their lytic spectra (Table 4.1-3).

Table 4.1-3 – Lytic spectra of seven isolated phages against *E. faecalis* strains

	Phage	09-2	99-1	99-2	00-1 I	00-1 II	80-1	80-2
Strain	2133201	Clear	Clear	Clear	Clear	Clear	Clear	Clear
	I899	Clear	Clear	Clear	Clear	Clear	Clear	Clear
	I900	Clear	Clear	Clear	Clear	Clear	Clear	Clear
	I436	Clear	Clear	Clear	-	-	-	-
	V583	Turbid	Turbid	Turbid	-	-	Turbid	Turbid
	I009	Clear	-	-	-	-	-	-
	I980	Turbid	Clear	Clear	-	-	Clear	Clear
	CECT 184	Clear	-	-	-	-	-	-
	I640	Clear	-	-	-	-	-	Turbid
	I975	Turbid	-	-	-	-	-	Turbid
	I018	-	-	-	-	-	-	-
	I435	Clear	-	Turbid	Turbid	Turbid	Turbid	-
	I986	Turbid	Turbid	Clear	Clear	Turbid	Turbid	Turbid
	I837	Turbid	-	-	-	-	-	-
	I581	Clear	-	-	-	-	-	-
	I429	Turbid	Turbid	-	Turbid	Turbid	-	-

Clear: clear halo; Turbid: turbid halo; - no halo

Phage 99-1 inhibited eight of the *E. faecalis* strains tested, causing clear halos in five and turbid haloes in three strains. Using another phage, 99-2, clear halos in six strains and turbid halos in two strains were observed. The lytic effect of phage 00-1 I was observed in six strains, with two of them presenting turbid haloes. A similar effect was observed using phage 00-1 II, with the difference of presenting a turbid halo on the strain I986. *E. faecalis* phage 80-1 showed lysis in four analyzed strains

and turbid halos in three other strains. Moreover, phage 80-2, inhibited eight strains. Using the same criterion as described in *S. aureus* the selected phage for further studies was the 09-2, being able to infect 15 strains out of the 16 strains tested (Table 4.1-3).

iv. *E. faecium* phages

E. faecium is a Gram-positive spherical cell. Its normal habitat includes the gastrointestinal tract, oral cavity and vaginal tract of animals[175,177]. This bacterium is a human pathogen that causes nosocomial bacteremia, surgical wound infection, endocarditis, and urinary tract infections[176].

An effluent of Frossos, Braga was enriched with 18 *E. faecium* strains and one phage was isolated. The same panel of 18 strains was used to determine the phage host range (Table 4.1-4).

Table 4.1-4 – Lytic spectrum of one isolated phage against *E. faecium* strains

	Phage	C410
Strain	C803	-
	C410	Clear
	I006	Clear
	I406	Turbid
	I999	-
	I769	Turbid
	I405	Turbid
	I459	Turbid
	C759	Turbid
	I738	Turbid
	I951	-
	I567	Turbid
	I647	Turbid
	C637	Turbid
	VR II36	Clear
	I677	Turbid
	I266	Turbid
	I577	-

Clear: clear halo; Turbid: turbid halo; - No halo

After lytic spectrum assay, phage C410 showed a broad host range, being able to lyse 14 out of 18 strains tested (78 %). For that reason, this phage was included in the further studies.

After evaluating the host range of all isolated phages, *S. aureus* phage I366 B, *E. faecalis* phage 09-2 and *E. faecium* phage C410 were selected for *in vitro* studies for showing the broad lytic spectra on the tested strains.

4.2 Single-species biofilms

4.2.1 Microtiter Plate Model

In vitro models are used to simulate real conditions. Numerous *in vitro* model systems have been developed to evaluate the effect of antimicrobial agents on biofilms, over the years[178].

For the characterization of isolated phages by *in vitro* studies, MTP model was used. This model is often used due to its high reproducibility, simplicity, flexibility, relatively inexpensive (only small volumes of reagents are need) and due to the possibility of performing several tests simultaneously[179]. The MTP model allows the simultaneous study of multiple parameters, for example, composition of growth media, incubation temperatures and pH[180]. Moreover, in this system, biofilms are grown on the bottom and the on walls of the plate[181,182].

Biofilm quantification

In vitro assays started with the evaluation of the biofilm formation ability of the selected phage host strains. Biofilm formation ability was assessed by CFU counting, which counts only viable bacteria using serial dilutions[183].

In Figure 4.2-1 are presented the biofilm CFU counts of the three phage hosts after 48 h of biofilm formation on 96-well microtiter plates.

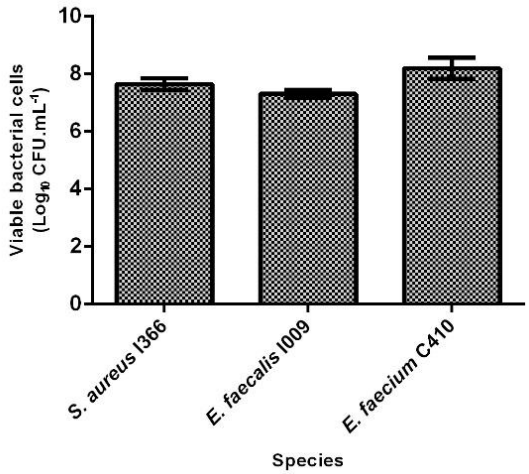


Figure 4.2-1 - Biofilm quantification at 48 h for each species by CFU counting for 96-well microtiter plates using the MTP model. Error bars represent standard deviations from three independent experiments performed in duplicate.

All strains tested in this study produced biofilm in microtiter plates wells with the levels of biofilm formation varying between 7 and 8 orders-of-magnitude (Figure 4.2-1). In other studies, a concentration about 1 x 10^9 CFU/mL was observed in a 24 h biofilm of *S. aureus*[111]. For *E. faecalis*, a biofilm formed in 96-well plates for two weeks showed about 10^8 CFU/mL of concentration[184], and in another case biofilms with a concentration between 2.25 x 10^8 and 3.35 x 10^8 CFU/biofilm were observed for two different *E. faecalis* strains. These latter biofilms were grown in 24-well plates for 48 h[185]. These results allowed to conclude that the strains used are high biofilm producers and the model seems adequate to form biofilms of these species.

Efficacy of phages in biofilms

Phage infection was performed in biofilms formed on the conditions described above, using a MOI of 1. Phage suspensions were applied in SM buffer or in TSB, depending on the experiment. In the first case, phage efficacy was analyzed at 3, 6, 8 and 24 h post infection. On the tests with phages applied in TSB, only the time points of 6 and 24 h were evaluated.

i. *S. aureus* phages

According to the infection results, when *S. aureus* biofilms were treated with phage I366 B, prepared in both SM buffer (Figure 4.2-2A) and in TSB (Figure 4.2-2B) there was no decrease in the number of viable biofilm cells in any of the time points analyzed.

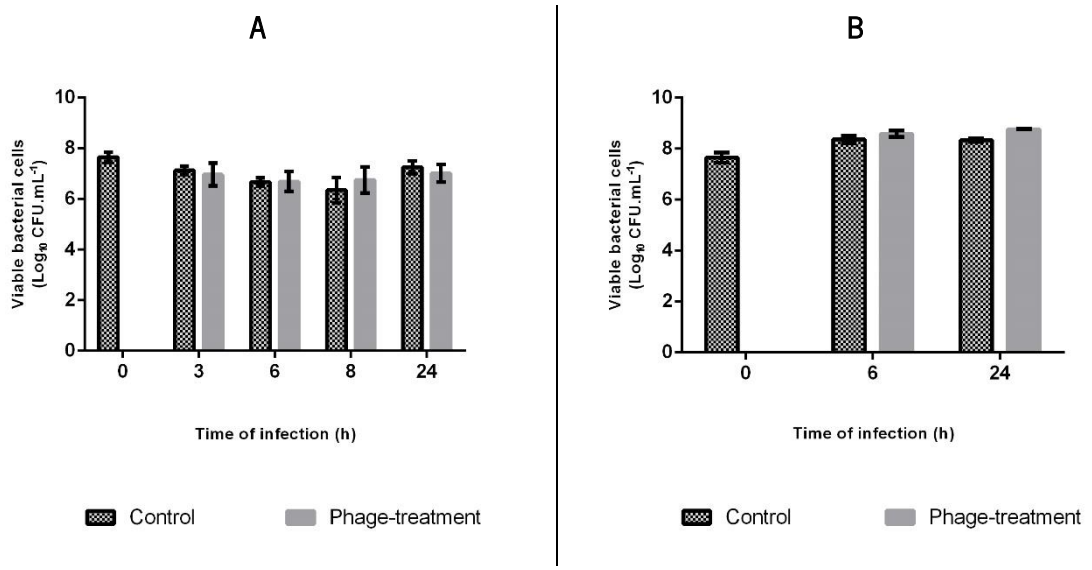


Figure 4.2-2 – Viable bacterial cells of *S. aureus* I366 biofilms formed in 96-well plates using the MTP model, before (0 h) and after phage I366 B infection in SM buffer (A) and in TSB (B). Error bars represent standard deviations from three independent experiments performed in duplicate.

These results were not expected, as the phage broad lytic spectrum and the formation of clear plaques were indicative of a promising phage. Moreover, the efficacy of *S. aureus* phages against biofilms were demonstrated in other *in vitro* studies. For example, in a study with the aim to characterize the antimicrobial activity of *S. aureus* phages, a *S. aureus* phage cocktail at MOI 1 was tested against 48 h biofilms[186]. In the referred study, it was shown that the phage cocktail was able to reduce significantly the biofilm load on the polystyrene surface, decreasing more than 50 % of the total biofilm biomass, after 24 h of phage treatment[186]. Drilling *et al.*[187], also investigated, *in vitro*, the ability of a phage cocktail to reduce biofilms of several *S. aureus* strains. Biofilms were treated for 48 h, and the results, showed that treatment with phage cocktail yielded significant reductions in biofilm biomass for 5/6 of the strains tested[187].

Although there are studies demonstrating the efficacy of phages against *S. aureus*, the comparison between results might not be correct, because biofilm formation models vary between studies[188]and the interaction between phages and host cells is different in all cases, particularly in biofilms[189].

Due to the referred results, several attempts to produce the other three *S. aureus* isolated phages were performed with the aim of selecting other phage with biofilm-reducing ability. However, it was not possible to obtain phages at a concentration that could be tested against biofilms at a MOI of 1. Difficulties to propagate a phage of a bacterium of the *Staphylococcus* genus have also been reported[145].

The formation of small plaques in their host was also a problem in the enumeration of *S. aureus* phage I366 B (Figure 4.2-3). Difficulties in the detection and enumeration of *S. aureus* phages plaques

are not new, because they have already been demonstrated in a study with the phage of host *S. aureus* ATCC 43300 (MRSA)[190].

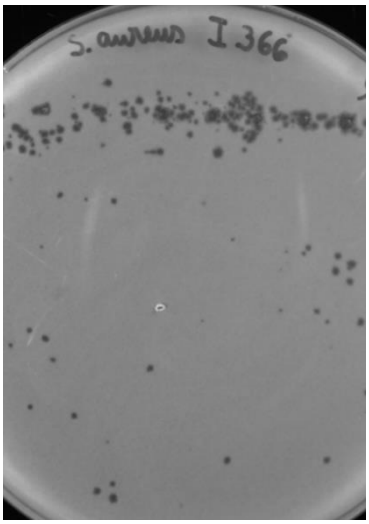


Figure 4.2-3 - Phage plaques of *S. aureus* phage I366B.

As the phage I366B not showed reduction of biofilm cells of their host, and the production of the other isolated phages was not possible, phages of *S. aureus* were not included in the following experiences.

ii. *E. faecalis* phages

Regarding *E. faecalis* phage 09-2, it reduced about 1 order-of-magnitude ($p<0.05$) the biofilm population, after 8 h of infection, when applied in SM buffer (Figure 4.2-4A). No statistical significant reduction was observed, at the other time points. On the other hand, when the biofilm was challenged by the phage applied in TSB, a significant decreased of almost 3 orders-of-magnitude ($p<0.05$) of the cellular concentration of *E. faecalis* I009 biofilm was observed after 6 h of treatment. Moreover, a statistically significant ($p<0.05$) decrease (≈ 1 order-of-magnitude) was also observed after 24 h of infection (Figure 4.2-4B).

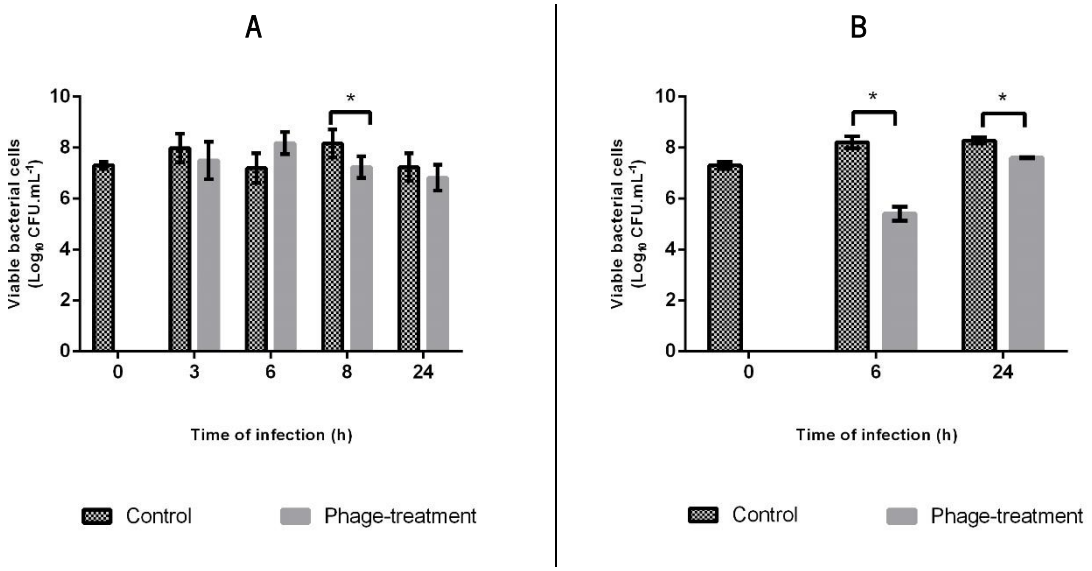


Figure 4.2-4 - Viable bacterial cells of *E. faecalis I009* biofilms formed in 96-well plates using the MTP model, before (0 h) and after phage 09-2 infection in SM buffer (A) and in TSB (B). Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

One possible explanation for this difference on the results obtained, might be related with the fact that phages need metabolically active bacterial cells to replicate successfully[105]. As the concentration of certain nutrients in culture medium is an important factor for bacterial growth[191], when phages are applied in SM buffer the available nutrients for the bacteria are smaller than those available when the dilution is performed in TSB. For this reason, it might occur an increase on bacterial growth with a consequent increase on the release of new phage particles.

The last event during phage lytic cycle is the lysis of bacterial host, and it is dependent on the composition of culture medium[192]. A study characterized the influence of well-defined physiological conditions on *E. coli* T4 phage growth and development[192]. The authors concluded that culture medium can affect phage lysis activity, with the maximum growth and lysis of phage being obtained in LB culture medium supplemented with nutrients. Moreover, in that study it was shown that with the use of minimal culture medium the rate of growth and lysis activity of phage was lower than in another rich culture media, which corroborates our SM buffer results. In another study, it was showed that the activity of lytic phages λ, P1 and T4 was severely inhibited by nutrient limitations of *E. coli* host cells[193]. More recently, in a study with T4 phage, the authors showed that the infection of stationary *E. coli* cells, by this phage, at low MOIs only occurred when nutrients were added to the culture[194]. The authors of that study, hypothesized that phage T4 infects the *E. coli* cells in starvation state, but in a mode that authors call "hibernation", in which T4 initiates protein synthesis but arrests further phage development[194].

Although the *E. faecalis* phage 09-2 showed good activity in reducing biofilm cells, when applied in TSB culture medium, its antibiofilm activity was very poor when applied in SM buffer. The wound environment is rich of nutrients and therefore *in vivo* the phage could have a better performance. Nevertheless, because the phage preparation should be made in a buffer and not in a rich nutrient media, the activity of the other isolated phages was tested in SM buffer, aiming at selecting phages with high antibiofilm activity even in a low nutrient medium.

In this study, almost all tested *E. faecalis* phages reduced the number of viable cells of each respective host, after 6 h of infection (Figure 4.2-5).

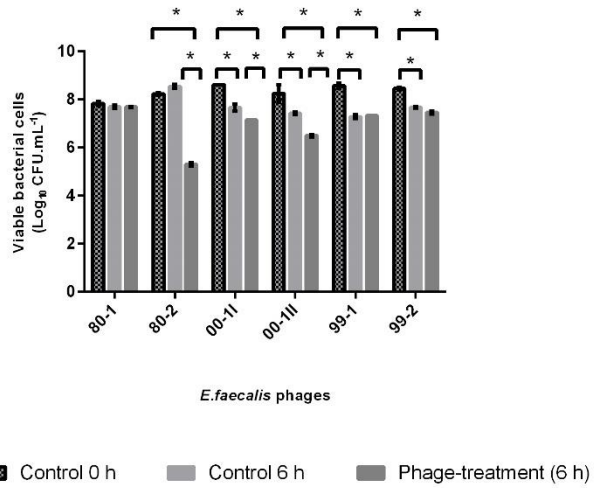


Figure 4.2-5 - Viable bacterial cells from *E. faecalis* host biofilms formed in 96-well plates using the MTP model, before (0 h) and after (6 h) *E. faecalis* isolated phages infection in SM buffer Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

With the exception of the phage 80-1, all phages showed significant reductions ($p<0.05$) in biofilm cells after 6 h of infection as compared to the control at 0 h (Figure 4.2-5). The phages 80-2, 00-1I, 00-1II, also decreased the cells in host-biofilm ($p<0.05$), at 6 h when compared to the control at the same time. In conclusion, *E. faecalis* phage 80-2 presented the best results in biofilm reduction, at 6 h, among the isolated phages of *E. faecalis* (Figure 4.2-5). This phage decreased almost 3 orders-of-magnitude the cellular concentration of *E. faecalis* I980 biofilms.

Unexpected results, with the exception for the host of phages 80-1 and 80-2, occurred in the number of cells between controls. A statistically significant ($p<0.05$) loss on the number of viable cells in host of 00-1I, 00-1II, 99-1, 99-2 was observed after 6h. In the latter stage of the biofilm lifecycle cell detachment occurs, probably because, the cells are limited by nutrient and/or oxygen depletion. Moreover, there is an accumulation of waste products with consequent pH modifications[195]. During the experiment, the

biofilms were subjected to a washing procedure that might have removed less adhered cells, which can justify the loss on the number of viable cells between control experiments.

In order to compare the results obtained with phage 80-2, with the ones obtained using phage 09-2, an experience with *E. faecalis* phage 80-2 applied in TSB was also performed (Figure 4.2-6).

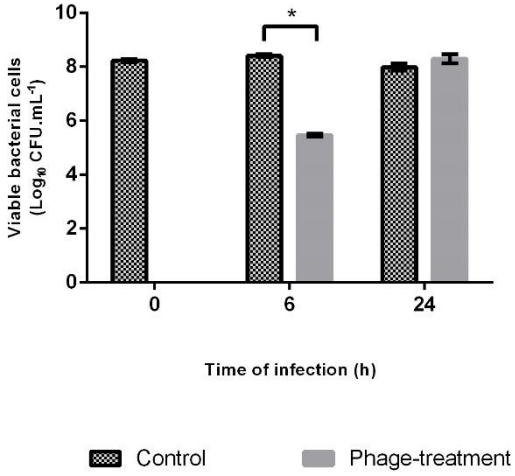


Figure 4.2-6 - Viable bacterial cells of *E. faecalis* I980 biofilms formed in 96-well plates using the MTP model, before (0 h) and after (6 h) phage 80-2 infection in TSB. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

As it happened when phage 80-2 was applied in SM buffer, when the phage was applied in TSB it also decreased almost 3 orders-of-magnitude ($p<0.05$) the cellular concentration in biofilms of *E. faecalis* I980, at 6 h. At 24 h no significant reductions were observed.

This phage was the only one that presented similar results in *in vitro* assays when applied in SM buffer and TSB culture medium. These results might be related with the host ability to grow in SM buffer. As can be seen in Figure 4.2-5, the host bacterium of phage 80-2 showed in the 6 h control a growth of about 0.5 orders-of-magnitude compared to the control at 0 h. This was not observed in any other strain. On Figure 4.2-5 it is also visible that this strain reached cell concentration of about 8.5 orders-of-magnitude, at 6 h (54 h of biofilm growth), while the remaining strains obtained concentrations $\leq$ 8 orders-of- magnitude. Thus, as this strain can grow in SM buffer it could be expected that the phage 80- 2 might infect and replicate in this host. Other possible explanation for these results might be related with phage 80-2 ability to infect stationary-phase cells. Although it is a rare feature, several phages were shown to infect these cells with low metabolic activity, namely in *E. coli*[194], *Pseudomonas fluorescens*[196] and

S. epidermidis[162]. To understand which phenomenon cause this results, more detailed studies are required.

Although this phage did not present the highest lytic spectrum, it lysed 50 % of the strains tested (8 out of 16 strains, Figure 4.1-3), it was decided to include it on the further studies.

The efficacy of *E. faecalis* phages in *in vitro* biofilms has already been described in literature. Static *E. faecalis* biofilms were grown for two weeks in 96-well plates. After this time, phage was added (10^7 PFU/well). CFU counts, showed a 5 orders-of-magnitude reduction after seven days of exposure to the phage, when compared with untreated biofilm[184]. In another study, the ability of a genetically modified *E. faecalis* phage to disrupt biofilms of two *E. faecalis* strains was study. A static biofilm with 48 h was used, and a solution of phages at 5.8 x10^9 PFU/mL was added for 1 h. A reduction between 1 and 2 orders-of-magnitude in both treated-biofilms, was observed[185].

Regarding to the phage plaques morphology, 09-2 showed large phage plaques (Figure 4.2-7A) and 80-2 displayed large and halo-generation phage plaques (Figure 4.2-7B).

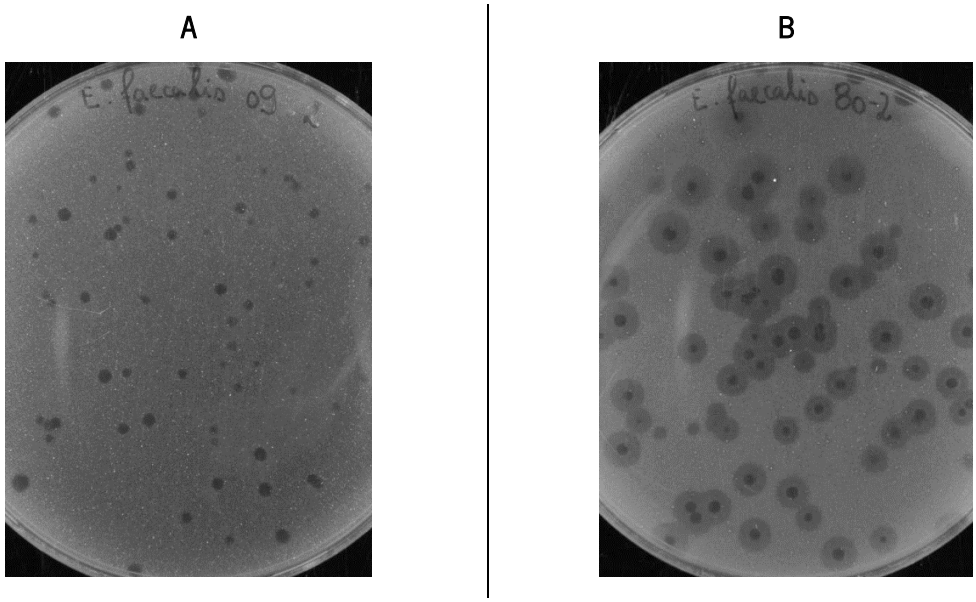


Figure 4.2-7 - Phage plaques of *E. faecalis* phage 09-2 (A) and *E. faecalis* phage 80-2 (B).

Analyzing all the results, *E. faecalis* phages 09-2 and 80-2 were selected for the following tests due to their broad lytic spectra and their promising *in vitro* results against 48 h biofilms.

iii. *E. faecium* phages

Regarding *E. faecium*, it was only possible to isolate one phage. After showing a broad lytic spectrum, this phage was tested against biofilms applied in SM Buffer and TSB (Figure 4.2-8).

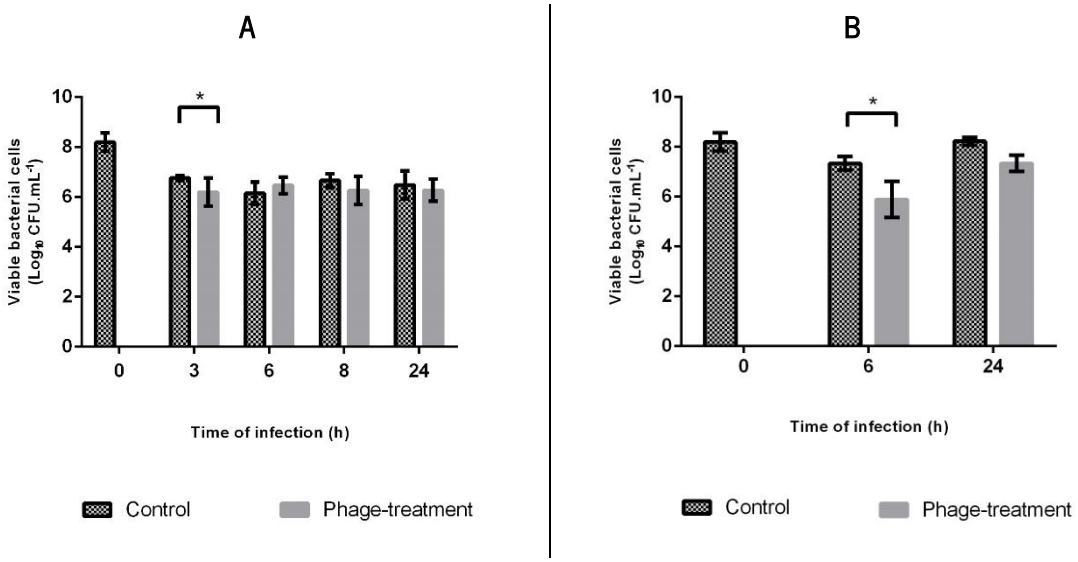


Figure 4.2-8 - Viable bacterial cells of *E. faecium* C410 biofilms formed in 96-well plates using the MTP model, before (0 h) and after phage C410 infection in SM buffer (A) and in TSB (B). Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

When *E. faecium* phage C410 was applied in SM buffer, it showed a slight reduction ($p<0.05$) of about 0.6 orders-of-magnitude at 3 h on the number of viable cells. However, there was no observed effect at the other time points analyzed (Figure 4.2-8A).

Similarly, to *E. faecalis* phage 09-2, an increased biofilm reduction was obtained when TSB was used to apply *E. faecium* phage C410, where at 6 h of infection biofilms had almost 1.5 orders-of-magnitude ($p<0.05$) the cellular concentration lower than untreated biofilms (Figure 4.2-8B). These results emphasize phages requirement of active bacteria in order to complete phage lifecycle.

No studies were found in the literature on the efficacy of *E. faecium* phages *in vitro*. However, an *in vivo* study with mice is reported[197]. In that study, phages at 3×10^8 PFU were injected after 45 min of an injection with *E. faecium* bacteria at 3×10^9 CFU. In 48 h, all non-treated mice were dead, while in phage-treated groups, 100 % of animals survived[197].

Relative to plaque morphology, phage C410 showed small and non-halo-generating phage plaques (Figure 4.2-9).

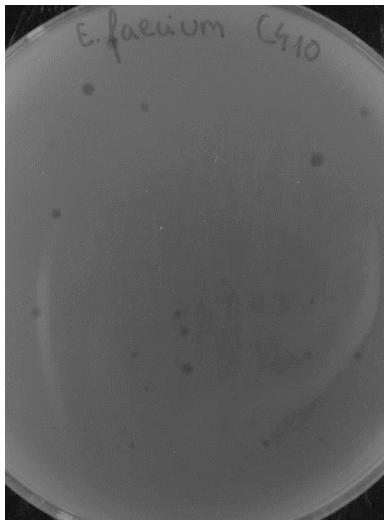


Figure 4.2-9 - Phage plaques of *E. faecium* phage C410.

At this stage, *E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410 showed to be efficient *in vitro* against their host biofilms, mainly when the assays were performed in the presence of culture media. Since the main purpose of this study is to develop a phage-based product to control biofilms in chronic wounds, it is important to study the effect of these phages in a model that better mimics wounds environment, for that the CWM was used.

4.2.2 Collagen Wound Model

Although MTP model is a common method to study biofilms, mainly due to being simple, cheap and allows multi-parameter analysis it has the problem of not reflecting the environmental conditions present in the wound bed[144]. For this reason, several researchers have made adaptations to *in vitro* models (Table 4.8-1 – Supplementary material).

In 2010, Werthén *et al.*[144] developed an *in vitro* model, CWM, to simulate wound injury. In this model, a SWF with 50 % FBS and 50 % physiological saline in 0.1 % peptone is used, and bacterial cells do not attach to well-defined solid surfaces, but a matrix of collagen. In the referred study, it was shown that the biofilms formed in this model are structurally similar to biofilms observed in *in vivo* conditions[144]. For this reason, CWM was already used for the study of antimicrobial activity of antibiotics and silver containing wound dressings[198,199] and was, also, selected for the use in this study.

Biofilm quantification

In the same way of the previous *in vitro* studies, the ability of the bacteria to form biofilms was evaluated by CFU determination (Figure 4.2-10). Biofilms were formed in collagen pre-coated 96 and

24- well plates, for 48 h (with medium renewal at 24 h) at 37 °C, with agitation. The culture medium used was composed by a mix of 50 % (v/v) TSB and 50 % SWF (v/v).

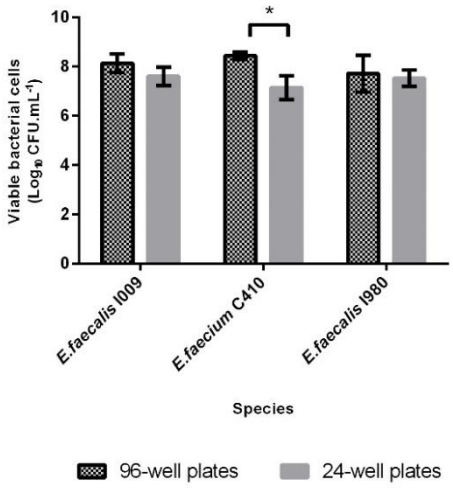


Figure 4.2-10 - Biofilm quantification at 48 h for each species by CFU counting for 96-well and 24-well microtiter plates using the CWM. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between the conditions tested.

As it can be seen in Figure 4.2-10 there were no significant differences on the total biofilm viable cells present in biofilms formed in the two plates and in *E. faecalis* strains. Only *E. faecium* strain C410 showed a slight increase ($p<0.05$) of about 1 order-of-magnitude in biofilm concentration in 96-well plates when compared to 24-well plates.

When comparing biofilm formation in this model with the MTP biofilm (without collagen and SWF), no differences were observed in the amount of biofilm cells. This fact was not observed in a study of Werthén *et al.*[144]. In that study, it was observed that the presence of collagen and serum proteins decreased the biofilm cells in both bacteria analyzed (*S. aureus* and *P. aeruginosa*), when compared with the cells in uncoated wells and without SWF[144]. However, in the referred study, only SWF was used as culture medium. As, SWF is a nutrient-limited medium and in this work it was used a mixture of TSB and SWF, the differences obtained might be justified by these differences.

Efficacy of phages in biofilms

After biofilm quantification, the efficacy of selected phages against biofilms was tested in this model. Phage infection of *E. faecalis* I009, *E. faecium* C410 and *E. faecalis* I980 biofilms was performed in both 96 and 24-well plates (Figure 4.2-11 and Figure 4.2-12 respectively). In both cases, to obtain a MOI of

1, phages were applied in TSB/SWF (1:1 v/v). The use of culture medium to apply phages instead of SM buffer, was based on the better results obtained in the previous *in vitro* characterizations (4.2.1).

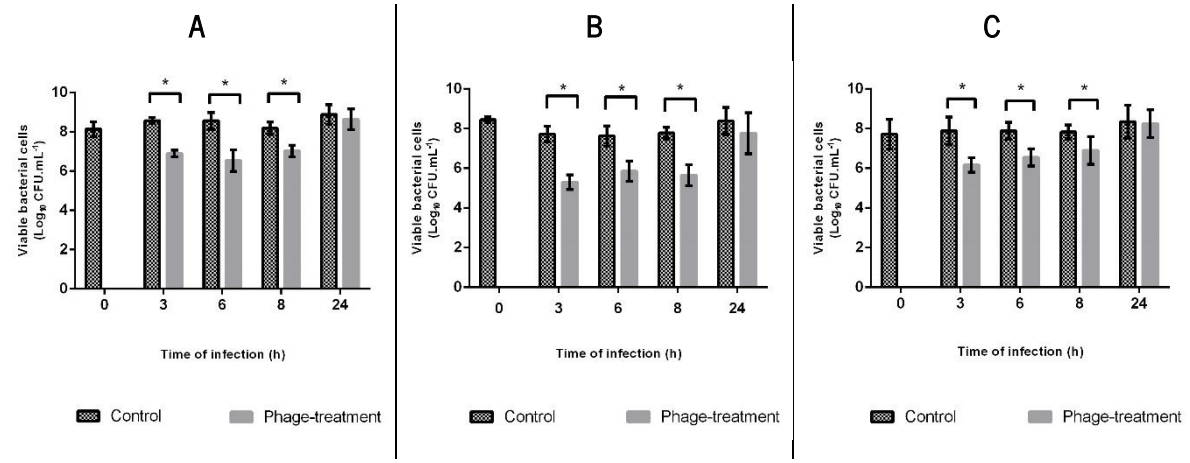


Figure 4.2-11 - Viable bacterial cells of biofilms formed in 96-well plates using the CWM before (0 h) and after (3, 6, 8, and 24 h) phage infection in TSB/SWF. (A) phage 09-2 against *E. faecalis* I009 biofilm; **(B)** phage C410 against *E. faecium* biofilm C410; **(C)** phage 80-2 against *E. faecalis* I980 biofilm. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

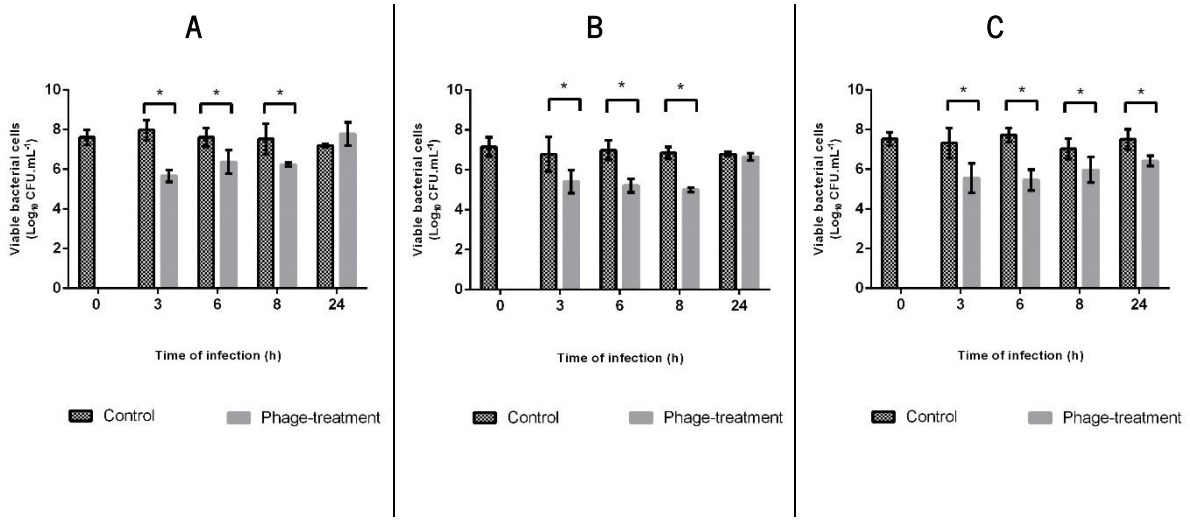


Figure 4.2-12 - Viable bacterial cells of biofilms formed in 24-well plates using the CWM before (0 h) and after (3, 6, 8, and 24 h) phage infection in TSB/SWF. (A) phage 09-2 against *E. faecalis* I009 biofilm; **(B)** phage C410 against *E. faecium* biofilm C410; **(C)** phage 80-2 against *E. faecalis* I980 biofilm. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

Briefly, using the three different phages it was observed a similar decrease on the viable cells in biofilms, in both conditions tested (96 and 24-well plates). *E. faecalis* phage 09-2 reduced significantly soon after 3 h, about 2 orders-of-magnitude the number of viable cells ($p<0.05$), comparing with the control, in 96 and 24-well plates (Figure 4.2-11A and Figure 4.2-12A). Reductions between 1.5 and 2 orders-of-magnitude were maintained after 6 h, however at 8 h, the reduction decreased to 1 order-of-magnitude, in the two conditions tested. Concerning *E. faecium* phage C410, this caused a reduction of

approximately 1.5 orders-of-magnitude between at 3 and 6 h of infection (Figure 4.2-11B and Figure 4.2-12B). Curiously, a reduction of biofilm cells for about 2 orders-of-magnitude was observed, at 8 h, in the two conditions tested. All reductions are statistically significant ($p<0.05$). Finally, phage 80-2, caused a statistically significant reduction ($p<0.05$) between 1.5 and 2 orders-of-magnitude in viable cells for 6 h of infection (Figure 4.2-11C and Figure 4.2-12C). However, at 8 h this reduction, decreased for 1 order-of-magnitude in the two tested conditions.

At 24 h, in almost all cases, was observed a similar number of biofilm cells between control and phage-treated biofilms. One possible explanation for these results might be related with the emergence of phage resistance. Phage resistance occurred in a study with *P. aeruginosa* biofilms, where the proliferation of phage-resistant variants was observed in the time-points after 6 h of single-phage treatment[200]. In the same study phages revealed to be the less effective 12 h after biofilm infection, and at 48 h post-infection, no statistical differences in the number of biofilm cells between control and phage-treated biofilms, were observed[200]. Interestingly, this is not observed in the case of *E. faecalis* phage 80-2, in 24-well plates (Figure 4.2-12C), which after 24 h of infection it still shows a reduction of approximately 1 order-of-magnitude of viable cells in biofilm.

In general, the tested phages presented ability to infect and kill biofilm cells in the presence of medium simulating wound injuries. The presence of nutrients in the TSB/SWF mixture might justify the better efficacy of the phages in comparison with their application in SM buffer (Figure 4.2-4A, Figure 4.2-5 and Figure 4.2-8A). It is also important to emphasize that the reductions obtained using the CWM model were similar to the obtained using TSB (with the double of the nutrients) in the MTP model (Figure 4.2-4B, Figure 4.2-6 and 4.2-8B).

In fact, CWM possess limitations as it is a closed system, without the presence of host immune cells and physiological factors (e.g. hypoxia, presence/absence of exudate and drainage of the wound) and therefore, as occurred with antibiotics[201], the phage can also accumulate in plates. Nevertheless, this is a model that better mimic wounds, suggesting that these phages may be promising in combating biofilm in wounds *in vivo*.

4.3 Dual-species biofilms

The majority of the studies about chronic wounds detected more than one bacterial species on the injuries[46,48]. *Enterococcus* species is one of the most common isolated from diabetic foot ulcers[202], venous

leg ulcers[203] and pressure ulcers[204]. Consequently, in order to approximate to the real biofilms, phage efficiency in mixed-*Enterococcus* strains biofilms was analyzed.

Biofilm quantification

Biofilm assays of dual-species were only performed using CWM on 24-well plates, due to the fairly similar amounts of viable cells present in 96-well and 24-well plates in single-species biofilm assays (as described above, Figure 4.2-10). Biofilm quantification was performed on the conditions described in 4.2.2.

Two different consortia were studied: *E. faecalis* I009 + *E. faecium* C410 and *E. faecalis* I980 + *E. faecium* C410 (Figure 4.3-1). Biofilms were characterized in terms of CFU counts under selective conditions, as bacteria were diluted in the presence of the phage acting against the opponent species.

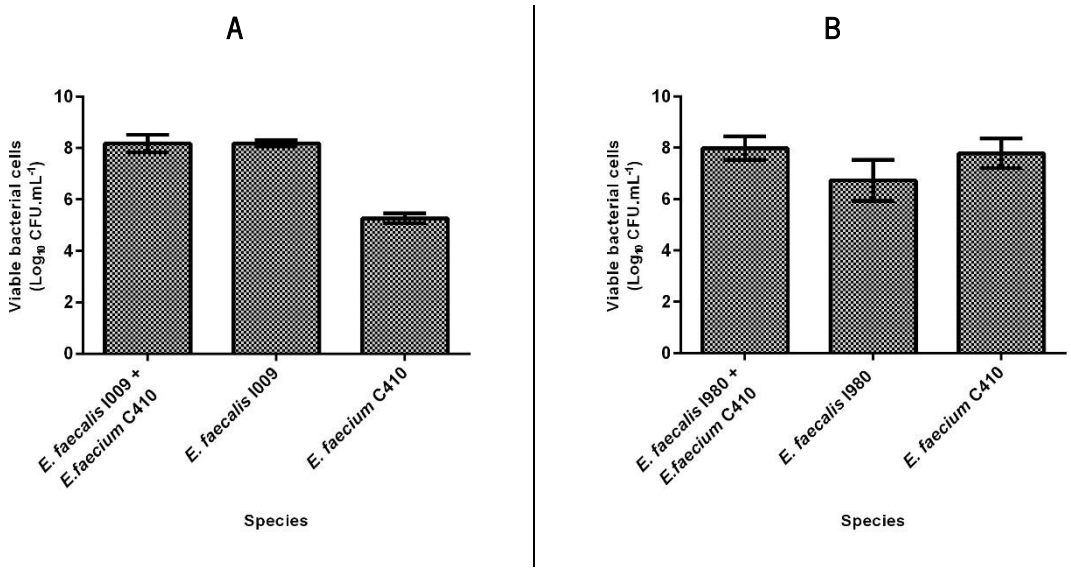


Figure 4.3-1 - Biofilm quantification for dual-species biofilms at 48 h by CFU counting for 24-well plates using the CWM. (A) *E. faecalis* I009 + *E. faecium C410* and **(B)** *E. faecalis* I980 + *E. faecium C410.* Error bars represent standard deviations from three independent experiments performed in duplicate.

Competition between *E. faecalis* and *E. faecium*, were observed in both combinations tested. In both cases the concentration of biofilm cells in mixed biofilms was about 8 orders-of-magnitude. In the first case, an adverse effect of the dual-species culture was observed on the *E. faecium* C410 population. The mixed biofilm was composed by *E. faecalis* I009 cells in about 8 order-of-magnitude, a value significantly greater in 3 orders-of-magnitude than the number of *E. faecium* C410 cells. In the second combination, the difference in biofilm formation was not so sharp, however the number of *E. faecium* C410 cells was

1 order-of-magnitude higher than *E. faecalis* I980 cells. The results show that the total cells of a multi-species biofilm are not necessarily the sum of the cells of each single species.

When comparing the total biofilm cells of strains in mono- and dual-species biofilms, some differences were observed. Although *E. faecalis* I009 kept the number of cells constant at approximately 8 orders-of-magnitude under both conditions (Figure 4.2-10), *E. faecalis* I980 and *E. faecium* C410 was significantly affected by mono- or multi-species culture conditions. In the first case, incubation of dual-species cultures resulted in biofilms with the lower counts than on mono-species biofilm (Figure 4.2-10), suggesting that *E. faecium* C410 might inhibit the growth of *E. faecalis* I980 biofilm cells. On the other hand, for *E. faecium* strain, had different behaviors depending on the combination tested. When combined with *E. faecalis* I009, this strain showed lower counts than in mono-species biofilm (Figure 4.2-10), while when combined with *E. faecalis* I980 it presented higher counts than in mono-species biofilm (Figure 4.2-10).

Although several researchers noticed that some specific strains of enterococci strains can inhibit the growth of other pathogenic bacteria such as *Listeria monocytogenes*, *Clostridium tyrobutyricum* and *S. aureus*, due to the production of bacteriocins[205,206], no study have described the interactions between two *Enterococcus* strains. Other factors, namely problems in the culture growth before biofilm formation, competition for nutrients and even the production and accumulation of toxic metabolites during biofilm formation may have contributed to these results[207].

Efficacy of phage cocktail

An advantage of the use of phages is that they can be mixed as cocktails to broaden collectively their antibacterial spectrum of activity[208]. On the other hand, the use of phage cocktails can be the solution to tackle the problem of phage insensitive mutants (BIM). BIM is associated with point mutations in genes encoding receptors on the bacterial cell surface and it is a major drawback on phage[109,208].

In this work, phage infection of dual-species biofilm was performed with cocktail composed by two phages: 09-2/C410 and 80-2/C410 for each mixed biofilm composed by heir host strains (Figure 4.3-2 and Figure 4.3-3).

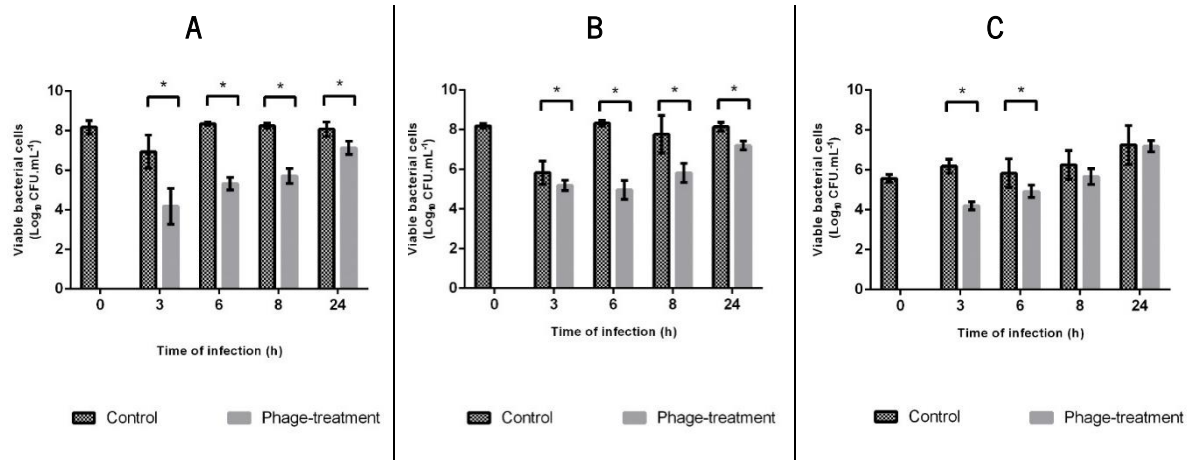


Figure 4.3-2 - Viable bacterial cells of dual-species biofilms formed in 24-well plates using the CWM before (0 h) and after (3, 6, 8 and 24 h) phage cocktail 09-2/C410 infection in TSB/SWF. (A) Viable bacterial cells of *E. faecalis* I009+*E. faecium* C410; **(B)** Viable bacterial cells of *E. faecalis* I009 cells in mixed biofilm; **(C)** Viable bacterial cells of *E. faecium* C410 cells in mixed biofilm. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

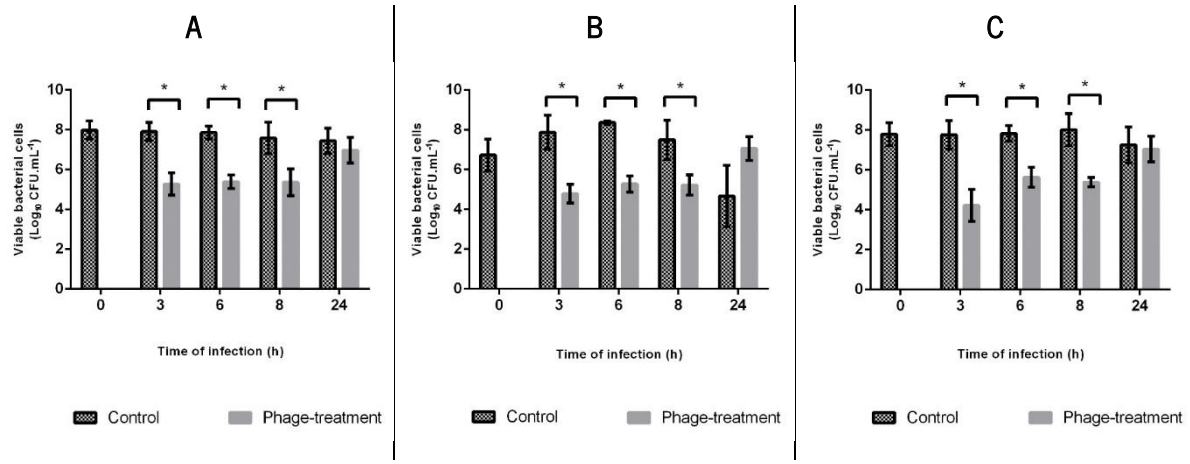


Figure 4.3-3 - Viable bacterial cells of dual-species biofilms formed in 24-well plates using the CWM before (0 h) and after (3, 6, 8 and 24 h) phage cocktail 80-2/C410 infection in TSB/SWF. (A) Viable bacterial cells of *E. faecalis* I980+*E. faecium* C410; **(B)** Viable bacterial cells of *E. faecalis* I980 cells in mixed biofilm; **(C)** Viable bacterial cells of *E. faecium* C410 cells in mixed biofilm. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

In both cases a statistically significant reduction ($p<0.05$) on cells concentration at 3, 6 and 8 h, on treated biofilms with phage cocktail comparing to the controls was observed (Figure 4.3-2A and Figure 4.3-3A).

Phage cocktail 09-2/C410 was particularly efficient in reducing 48 h old biofilm, where the cell concentration was reduced by approximately 2.5 orders-of-magnitude after 3 h of infection. The reduction remained after 6 and 8 h. However, after prolonged treatments, cells might have acquired resistance to the phages, as previously explained in chapter 4.2.2 and consequently, after 24 h of phage infection the reduction was only of 1 order-of-magnitude ($p<0.05$) (Figure 4.3-2A).

Analyzing each strain, it is possible to observe that phage cocktail showed greater reductions in dominant strain (*E. faecalis* I009, Figure 4.3-2B) than in *E. faecium* C410 (Figure 4.3-2C). As the concentration of each phage in the cocktail was fixed at 10^8 PFU/mL, the MOI applied at *E. faecium* C410 strain was 100. However, larger MOIs do not always mean larger reductions[209]. In a study with a phage against *E. coli* it was showed that infection with the phage at an MOI of 10, 3 or 1 caused similar reduction in cell concentration within the first 2 h of treatment. In addition, after 3 h of infection and until 24 h, the cell concentration of the four phage-infected cultures at the initial MOI of 10, 3, 1 and 0.3 remained similar[209].

In the other biofilm combination tested (I980 + C410) soon after 3 h, there was a statistically significant reduction, of approximately 2.5 orders-of-magnitude in viable cells, which remained after 6 and 8 h of biofilm infection (Figure 4.3-3A). After 24 h of phage infection, the cocktail showed a slight reduction of 0.5 orders-of-magnitude in viable biofilm cells, but this was not statistically significant (Figure 4.3-3A). In the same way, as in the previous mixed-biofilm, concentration of each phage in the cocktail was fixed at 10^8 PFU/mL, therefore, in this case, the MOI applied at strain *E. faecalis* I980 was 10. Analyzing the cell reduction of each species was possible to observe that despite the cell reductions being very similar, a slight improvement in the reduction of *E. faecalis* I980 cells was observed, probably due to the effect of a higher MOI (Figure 4.3-3B).The positive effect of high MOIs has also been demonstrated[120]. In adult BALB / c mice, burns infected with *K. pneumoniae* were treated with a single injection of phage at various MOI (0.001, 0.01, 0.1, 1, 10, 100, and 200). After 24h, in higher phage doses (MOI>1), a 96.66 % survival rate was observed, while with MOI<1, the animals became critically ill with 0 - 53.33 % survival[120].

Compared with any single phage, the two phage cocktails were more efficient in reducing biofilm cells in the CWM (Figure 4.2-12)). In other studies it was shown that phage cocktails were more effective in restricting the bacterial growth for longer periods[112,208]. A study with the aim to examine the therapeutic potential of a phage cocktail (with three phages of *K. pneumoniae*) in a murine model of infection reported that when a single phage administration was performed, mice survival rates were between 0 and 40 %. On the other hand the phage cocktail administration presented a survival rate about 100 %[208]. In another murine model, the therapeutic efficacy of *K. pneumoniae* phages was evaluated in comparison to the phage cocktail in the treatment of wound infection. In that study, mice receiving phage treatment showed a decrease of 6 orders-of-magnitude in bacterial load as compared to control, while for single phage treatment a decrease of about 4.5 orders-of-magnitude was observed[112]. In an *in vitro* study, it was possible to observe that 8 h post-treatment, the phage cocktail was more efficient than other treatments, namely

with a single phage administration in eliminating *P. aeruginosa* biofilm cells. Moreover, 24 and 48 h after phage cocktail significant reductions were still observed[200].

4.4 Transmission electron microscopy

To better characterize the phages with the best anti-biofilm activity, the morphology of phages particles, (*E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410), was observed by TEM.

This analysis revealed that all phages are from families within the *Caudovirales* order. According to the morphological evaluation[210], *E. faecalis* phage 09-2 belongs to the *Siphoviridae* family of phages as indicated by the presence of a long tail and the absence of a contractile sheath (Figure 4.4-1A). This phage has a tail with approximately 220 nm in length and 12 nm in width. The capsid size is 58 nm in diameter (Table 4.4-1). *E. faecium* phage C410 has a capsid with 46 nm in diameter and a short, noncontractile tail with 19 nm of length (Table 4.4-1), and consequently belonging to the *Podoviridae* family (Figure 4.4-1B). Finally, *E. faecalis* phage 80-2 also belongs to the *Siphoviridae* family, having a noncontractile tail with a tail with 238 nm in length and 13 nm in width and a capsid with 56 nm in diameter (Figure 4.4-1C; Table 4.4-1).

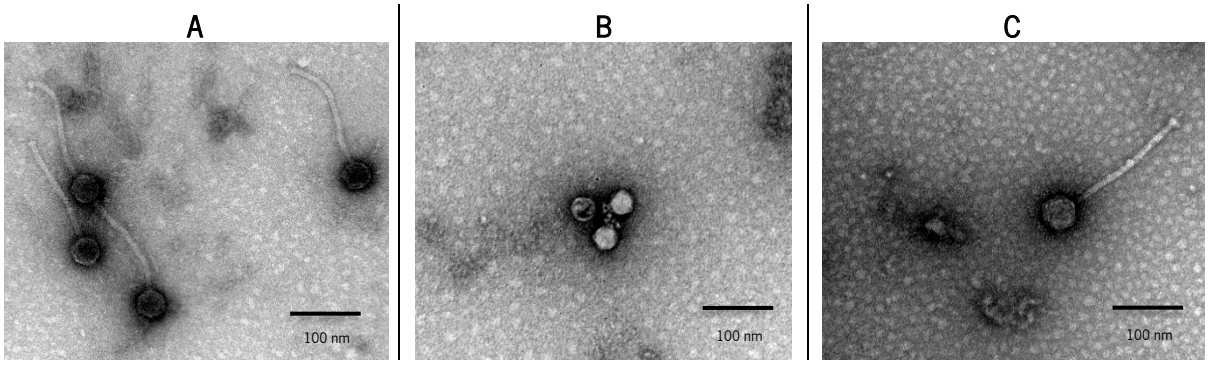


Figure 4.4-1 - **TEM images (A)** *E. faecalis* phage 09- 2, **(B)** *E. faecium* phage C410 and **(C)** *E. faecalis* phage 80-2. Scale bars represent to 100 nm.

Table 4.4-1 – Taxonomy and morphology of the isolated phages

Phage	Family	Capsid size (nm)	Tail length (length x width) (nm)
E. faecalis phage 09-2	*Siphoviridae*	58	220 x 12
E. faecium phage C410	*Podoviridae*	46	19
E. faecalis phage 80-2	*Siphoviridae*	56	238 x 13

4.5 Genome Analysis

Genome analysis revealed that the three phages are virulent, because no gene associated of temperate phages was detected, namely integrases, recombinases and transposases genes[211]. This observation together with the fact that *in silico* analysis no protein with toxic potential was found in *E. faecalis* phage 80-2 and *E. faecium* phage C410, allow to conclude that these two phages may have potential for therapeutic purposes[115]. Concerning *E. faecalis* phage 09-2 its genome encodes a protein of hemolysin XhlA family, which is a potential toxic protein and therefore this phage can be potentially toxic. This protein in *Bacillus subtilis* is a cell-surface associated hemolysin that lyses granulocytes and plasmatocytes in insects and mammalian erythrocytes *in vitro*[212]. In another study, the presence of this gene on a phage genome allowed the authors to conclude that the referred phage was not suitable for to control *E. faecium* infections[213]. However, in the case of this work, a more detailed analysis about the effect of the XhlA gene on the *E. faecalis* phage 09-2 is needed. As no conclusions were taken regarding the safety of this phage, it was defined to proceed with its use.

E. faecalis phage 09-2 has a linear dsDNA genome consisting by 40 975 bp with a G+C content of 34.7 %. Phage 09-2 encodes 65 putative CDSs, of which only 28 of the predicted proteins have an assigned function. The 63 of the CDSs possess ATG (methionine) as start codon, while only 3 % possess GTG (valine). Concerning *E. faecium* phage C410, this has a linear dsDNA genome consisting by 18 742 bp. The G+C content represents 35 % of the genome. Phage C410 encodes 22 putative CDSs, of which 10 of the predicted proteins have an assigned function; one of the CDSs is unique as no hits were found. All CDSs possess methionine as start codon. Finally, *E. faecalis* phage 80-2 has a linear dsDNA genome consisting by 39 742 bp with a G+C content of 35 %. This phage encodes 62 putative CDSs, of which 26 of the predicted proteins have an assigned function; two of the CDSs are unique as no hits were found. The 97 % of the CDSs possess ATG as start codon, while only 3 % possess GTG.
DNA homology by BLASTN[150] revealed that, for the three phages, no hit with other sequenced genomes was found. Therefore, a more intensive genome analysis will be needed in the future to understand the behavior of *E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410.

4.6 pH and temperature stability

The use of phages in pharmaceutical applications requires the knowledge of their stability in harsh conditions, for example, low and high temperatures or pH values[214].

Stability tests were performed exposing the phages to different temperatures and pH values for 24 h at room temperature (Figure 4.6-1 and Figure 4.6-2 respectively).

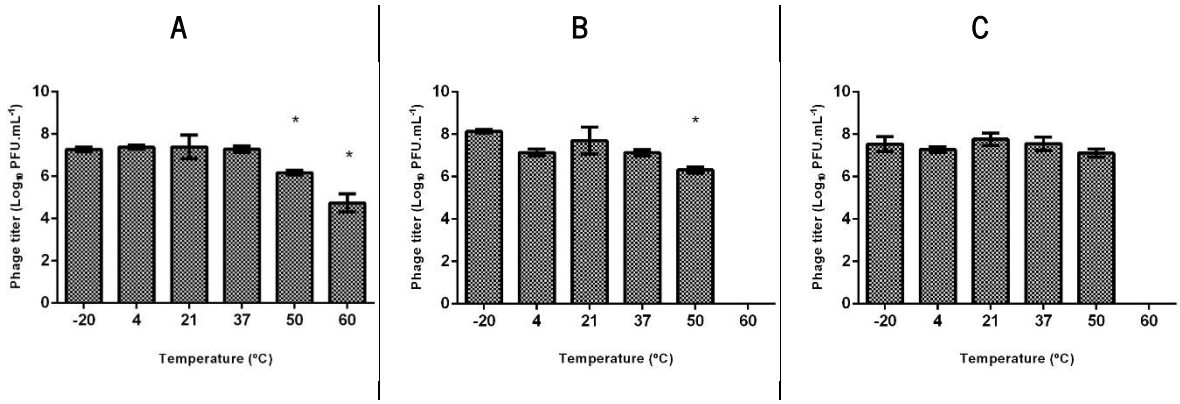


Figure 4.6-1 - Thermal stability test of phages (A) *E. faecalis* phage 09-2; **(B)** *E. faecium* phage C410; **(C)** *E. faecalis* phage 80-2. The experiences were performed for 24 h at room temperature (21 °C). Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control (4 °C) and test assays.

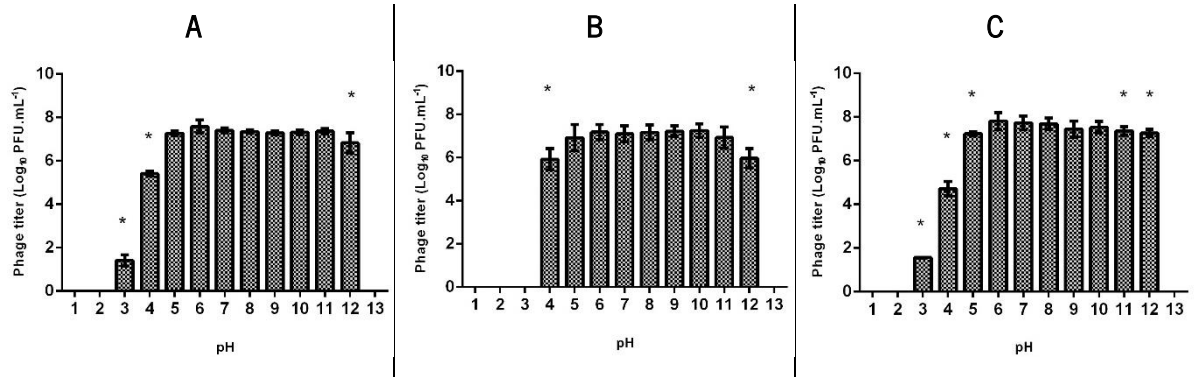


Figure 4.6-2 - pH stability test of phages (A) *E. faecalis* phage 09-2; **(B)** *E. faecium* phage C410; **(C)** *E. faecalis* phage 80-2. The experiences were performed for 24 h at room temperature (21 °C). Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control (pH 7) and test assays.

Temperature plays a fundamental role in phage attachment, genetic material ejection and phage multiplication[215]. The thermal stability test was carried out to determine the heat resistance of isolated phages at pH 7.0. All phages were stable after 24 h, at −20°C, 4°C, 21°C and 37°C, showing a concentration about 7 orders-of-magnitude (Figure 4.6-1). At 50 °C, phages 09-2 and C410 lost titer ($p<0.05$) between 0.5 and 1 order-of-magnitude (Figure 4.6-1A and Figure 4.6-1B). *E. faecalis* phage 80-2 and *E. faecium* phage C410 were completely inactivated at 60 °C (Figure 4.6-1B and Figure 4.6-1C)

and at the same temperature *E. faecalis* phage 09-2 concentration decreased about 2 orders-of-magnitude ($p<0.05$), in comparison with the control (4 °C) (Figure 4.6-1A).

For *E. faecalis* phages, these results are similar to those reported by Lee *et al.*[216]. In their study, *E. faecalis* phages belonging to the *Siphoviridae* family, showed heat resistance at temperatures below 60 °C[216]. In general, members of *Siphoviridae* family are considered to be able at to large temperature fluctuations[217], but in the case of this work only *E. faecalis* phage 09-2 showed similar results, being active at -20 and 60 °C.

For *E. faecium* phage C410 the results are similar to observed for a phage *Podoviridae* from *Citrobacter freundii*[218]. This phage was incubated for 1 h, at respective temperature, and it showed maximum stability at 37 °C and gradually decreased the concentration as the temperature was increased up to 65 °C. At 70 °C no PFU counts were observed[218].

Acidity and alkalinity of the environment are other important factors influencing phage stability. Optimal pH value was determined by testing the stability of phages at different pH after 24 h of incubation at room temperature (21 °C) (Figure 4.6-2). All phages were completely inactivated at extreme pH values of 1.0, 2.0 and 13.0. *E. faecalis* phage 09-2 seems to be extremely stable in the pH range 5.0–11.0, however PFU counts decreased ($p<0.05$) by 6 and 2 orders-of-magnitude at pH 3.0 and 4.0, respectively (Figure 4.6-2A). Concerning *E. faecium* phage C410, it showed to be very stable in the pH range 5.0–11.0. Viable phage counts were reduced in 1 order-of-magnitude at pH 4.0 and 12.0 ($p<0.05$) and phage particles were completely inactivated at pH values of 1.0, 2.0, 3.0 and 13.0 (Figure 4.6-2B). Regarding *E. faecalis* phage 80-2 it was stable in the pH ranges of 5.0 to 12.0, showing a slight decrease (about 0.5 orders-of-magnitude) at pH 5, 11 and 12 ($p<0.05$), comparing with control (pH 7, Figure 4.6-2C). Viable phage particles count reduced 6 and 3 orders-of-magnitude respectively at pH 3.0 and 4.0 ($p<0.05$) and particles were completely inactivated at pH of 1.0, 2.0 and 13.0 (Figure 4.6-2C).

Recently, Lee *et al.*[216] reported that two *E. faecalis* phages, belonging to *Siphoviridae* family, were stable in a pH range of 5.0-9.0, and coagulation of the phages at pH 2.0 was observed[216]. Similarly, to temperature stability results, *Siphoviridae* phages are described as the most resistant to adverse pH conditions[217]. *E. faecalis* phages 09-2 and 80-2 classified as members of this family showed to have some activity at pH 3.0, unlike the *E. faecium* phage *C410* (*Podoviridae*), which was completely inactivated at that pH value. For *Podoviridae* phages the loss of titer at pH 3 was also reported in a study with a phage of *Citrobacter freundii*. In that, the phage was stable in a pH range of 4 to 9[218].

In conclusion, the phages studied in this work may be promising in combating biofilms in *in vivo* wounds, as they present stability in a pH range from 5-11, usually found in wounds. A pH value of about

4.7 is characteristic of the skin surface of adults and healthy children[219]. However, a study proved that wounds that develop an infection appear to increase the pH value. The researchers analyzed 26 cases of second degree burns, and in cases of local infection the pH ranged from 6.5 to 9.0[220]. Moreover, concerning temperature, the skin surface temperature is about 33.2 °C[221], but when there is an infection, the temperature in wound can increase due to immune response, inflammatory cytokine-induced vasodilation and increased tissue metabolism[222]. Dini *et al.*[223] analyzed the skin temperature in 18 patients affected by venous insufficiency and lower leg ulcers. In final, they proved that wound injury temperature range was between 31 °C and 35 °C, and the perilesional skin temperature range was between 31 °C and 34 °C[223].

The properties demonstrated by the isolated phages in this work, namely lytic spectrum, stability at different temperatures and pH ranges, genome and efficacy in *in vitro* biofilms, suggest its therapeutic use. However, *E. faecalis* phage 09-2 needs a more detailed genomic analysis due to on one putative toxic protein -hemolysin XhlA family. Finally, the high efficacy of the phage cocktail suggests its formulation for therapeutic application.

4.7 Purification of phages

It is expected that for the application of phages for biological and medical purposes, phages solutions should be free of contamination[166].

Although PEG precipitation was used as purification methodology throughout this work, in this chapter the concept of phage purification using chromatographic methods was tested.

It was previously reported that chromatography can be a promising alternative for phage, purification, as it is associated with high resolving power, high capacity, simplicity and controllability[143].

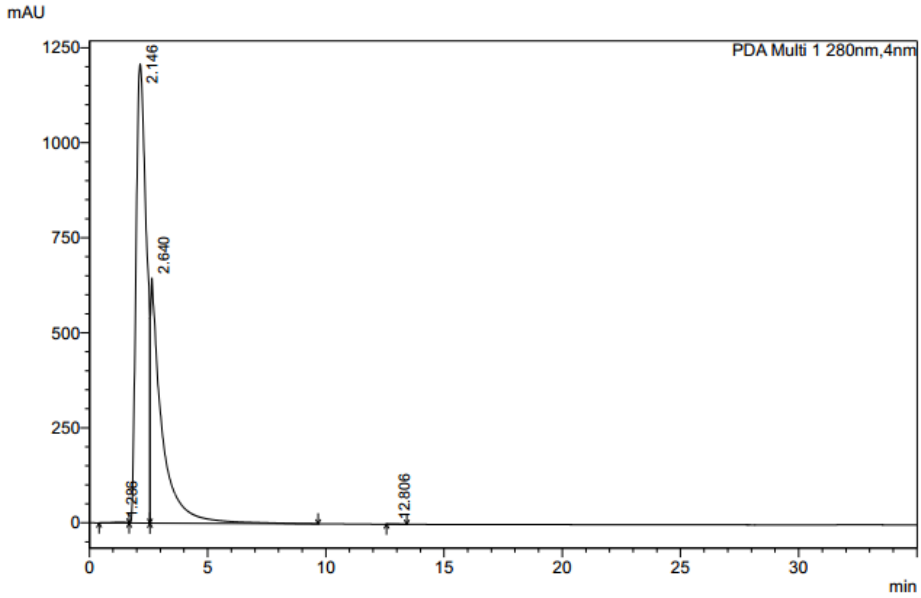
4.7.1 Chromatography – CIMmultus® columns

The crude lysates from the three phages used in the mixed-biofilm experiments (*E. faecalis* phage 09-2, *E. faecium* phage C410 and *E. faecalis* phage 80-2, see chapter 4.3), were tested on the laboratory scale anion-exchange columns, CIMmultus® QA and DEAE. The purification of phages with variants of these columns was already successfully reported by several researchers[141–143]. In the referred studies, different equipment was used to connect the columns, namely the FPLC system[143] and the HPLC

equipment[141,142]. In this work, the first experiments were performed on an Ultra-High Performance Liquid Chromatography (UHPLC) system.

As each purification protocol needs to be optimized for each individual phage[143], several buffers are described as possible[142,224,225]. Herein, Tris-buffer (20 mM Tris-HCl, pH 7.5) was used as loading buffer because it is one of the components of the solution used in the production of phages (SM buffer). For elution, a gradient of 0 to 2 M of NaCl was added to the loading buffer. In ion exchange chromatography there is an electrostatic attraction between the protein solution and the column matrix[226]. As the structure of *Caudovirales* phages is mainly composed by proteins[95], it can possible to consider that phage binding to the stationary phase of the column occurs through charges present on the surface of the phage. The elution step is performed by gradually increasing the ionic strength of the buffer through the salt gradient. Addition of salt increases the number of ions competing with the bound-particles for functional groups in the stationary phase[226]. A linear elution gradient and a flow rate of 1 mL/min were used. The assay was performed at 4 °C and the injection volume was fixed at 5 mL, after several conditions have been tested (data not shown).

Chemically, CIMmultus® QA is characterized as a strong column anion exchanger. Chromatograms from each phage were performed with a small volume injection (50 µL) because the UV-detector of the UHPLC system does not support higher sample volumes. Phages concentration were determined with plaque assays, but to facilitate comparisons between the amount of phage recovered in each fraction, concentration values were multiplied by the volume of each fraction recovered. Thus, Figure 4.7-1 represents the chromatogram obtained and the titer of each fraction recovered for *E. faecium* phage C410.



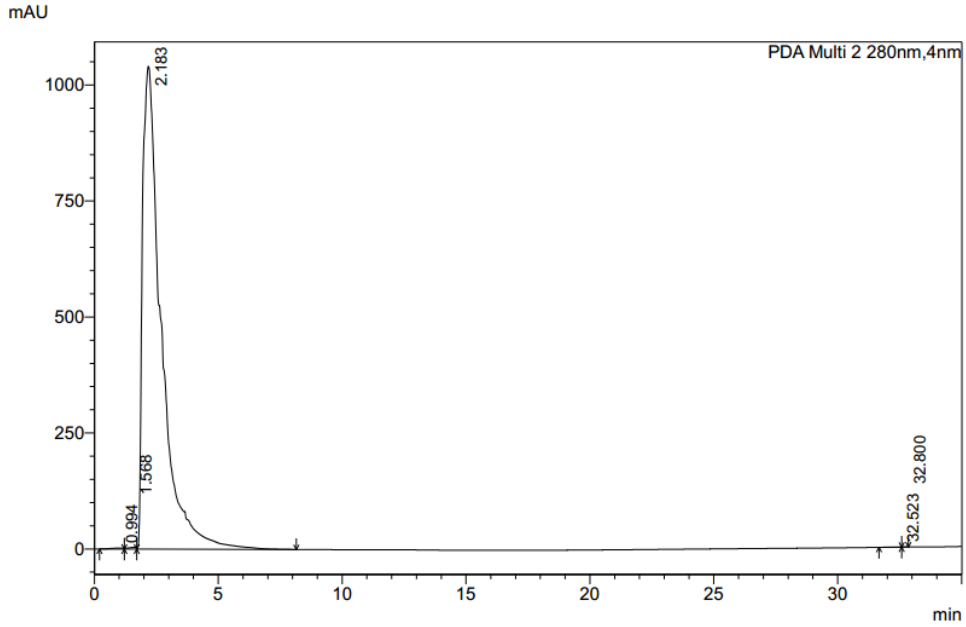
Samples / Assay	Load	Peak 1 (1.8 min-7.5 min)	Final (11 min-13 min)
1	1.30E+10	1.94E+07	4.40E+08
2	1.30E+10	2.39E+09	7.60E+08
3	1.30E+10	2.17E+09	6.60E+07

Figure 4.7-1 - Purification of *E. faecium* phage C410 in linear gradient mode. Conditions: CIMmultus® QA column was loaded with 50 µL of bacterial lysate containing *E. faecium* phage C410 and eluted using a linear gradient of NaCl (0 - 2M); Flow rate:1 mL/min; UV detection at 280 nm; Titer of each fraction in Total PFU.

Despite of testing the three phages, it was decided to include only *E. faecium* phage C410 data, due to the similarity of the behavior of all phages. For all phages, the chromatogram showed, for QA column, a single peak at approximately 0.08 M NaCl when a linear gradient was used (0–2 M NaCl). In other phage purification experiments, phage recovery is described to commonly occur between 0.6-0.8 M NaCl[141,143,227]. Moreover, unexpected results were obtained, with the existence of high phage concentration recovered out of the peak, namely at the end of the run (11-13 mins). As different phage concentrations were detected throughout the run, the purest phage fraction could not be determined.

In parallel, CIMmultus® DEAE column was also tested under same conditions and with the same phages samples. Chemically, DEAE is characterized as a weak anion exchanger column. However, again, for all phages, unexpected results occurred with the existence of high phage concentration out of the peak. Although the three phages were tested, it was decided to include only data from *E. faecium* phage C410 (Figure 4.7-2), due to the similarity of the behavior of all phages. A smearing effect, mainly in

E. faecalis phage 80-2 chromatogram was also observed, probably because the lower affinity between phages and stationary phase of this column (data not shown).



Assay \ Samples	Load	Peak 1 (1.8 min-7.5 min)	Final (11 min-13 min)
1	1.30E+10	0.00E+00	2.00E+05
2	1.30E+10	1.37E+08	4.00E+05
3	1.30E+10	8.36E+07	5.20E+06

Figure 4.7-2 - Purification of *E. faecium* phage C410 in linear gradient mode. Conditions: CIMmultus® DEAE column was loaded with 50 µL of bacterial lysate containing *E. faecium* phage C410 and eluted using a linear gradient of NaCl (0 - 2M); Flow rate:1 mL/min; UV detection at 280 nm; Titer of each fraction in Total PFU.

The chromatographic method for purifying phages is a stepwise process that begins with the phage binding on the column, followed by the elution step and recovery of phage. Optimizing these steps is the key to a high yield purification[143]. The results obtained in this work showed that there were problems with the use of the method, particularly in the binding step. In a work on phage purification, the authors stated that before the start of the purification process, the specific binding conditions for each phage must be determined. For this, a phage suspension was passed through the column and the flow fraction (FT) was recovered, with the aim to have no phage in this fraction and to eliminate most of the contaminants[143].

Thus, after failed results in HPLC system and mainly due to the detectable sample limit in this equipment, a test in an FPLC system was performed. CIMmultus® QA monolithic column and the same mobile phases from the previous experiments were used. Chromatography of *E. faecium* phage C410 is

shown in Figure 4.7-3. The linear gradient of NaCl and collected fractions of 2 mL analyzed by plaque assay can be observed in Figure 4.7-4.

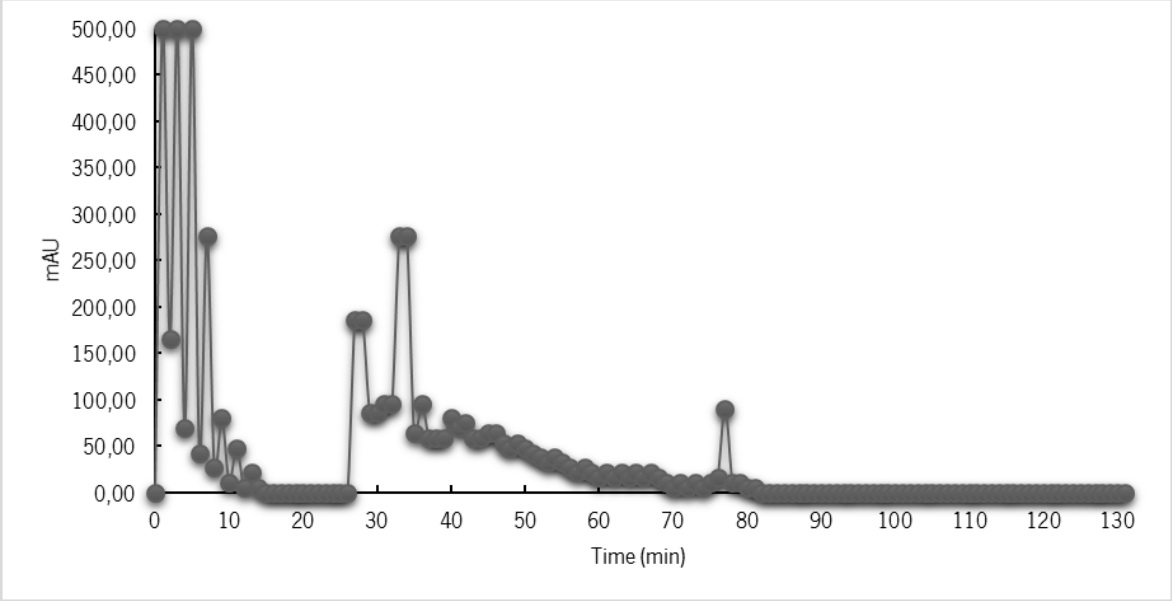


Figure 4.7-3 - Chromatography of *E. faecium* phage C410 on the FPLC system. Conditions: CIMmultus® QA column was loaded with 5 mL of bacterial lysate containing *E. faecium* phage C410 and eluted using a linear gradient of NaCl (0 - 2M); Flow rate:1 mL/min; UV detection at 280 nm.

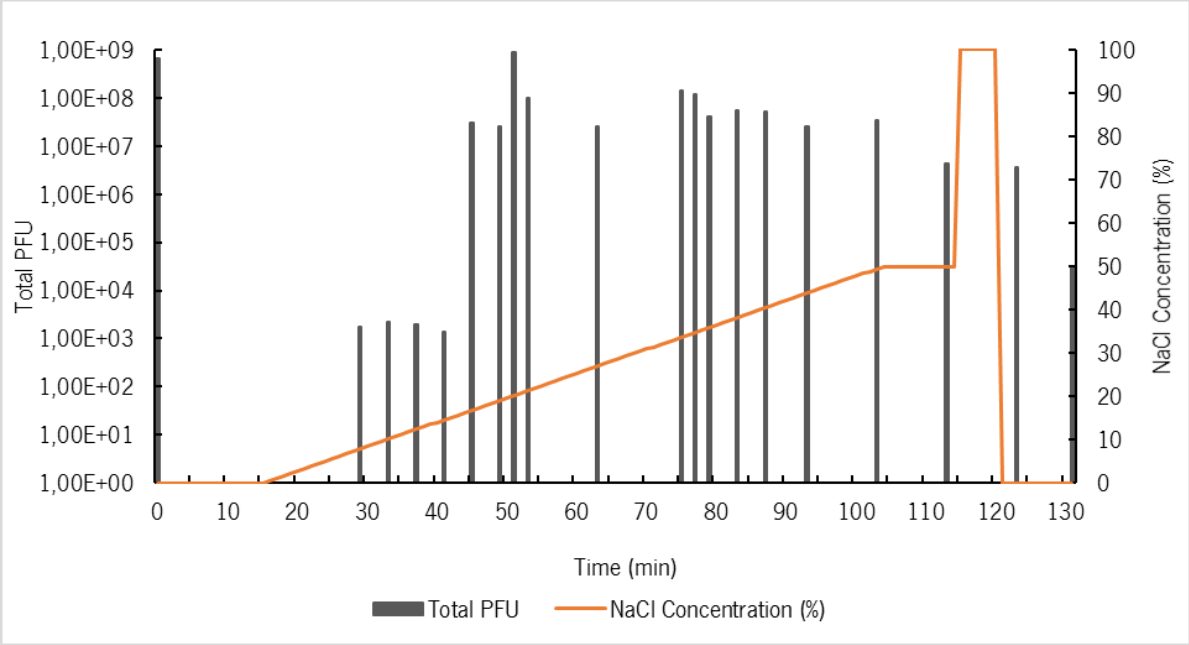


Figure 4.7-4 - NaCl gradient and titer of samples collected during phage purification of *E. faecium* phage C410.

Five milliliters of *E. faecium* phage C410 lysate were passed through the column, with a flow rate fixed at 0.1 mm/min, and then 10 mL of loading buffer were also passed (flow rate at 1 mL/min) in order to remove most of the impurities, as described in several studies[142,143]. Despite the high UV absorbance detected in the first 10 min of the purification run (Figure 4.7-3), no phage was detected with plaque assay (Figure 4.7-4). Therefore, as expected the UV signal might represent non-bound impurities[142].

After starting the NaCl gradient, three distinct peaks at 28, 34 and 74 min were observed in the chromatogram. However, unexpectedly phage was detected in several samples collected between 29 and 131 min. From 83 to 131 min there was phage detection, between 8 and 4 orders-of-magnitude (Figure 4.7-4), even when the signal detected in the chromatogram was zero (Figure 4.7-3).

This fact might happen due to the loading solution, as phages solution should be diluted in loading buffer, before passing the column, to reduce ionic strength and promote binding of the phage particles in the column matrix[143]. This dilution might also reduce the amount of proteins (consequent of bacterial lysis) that can also bind to the matrix[143]. In these experiments, there was no equilibrium between buffer and phage samples. Phage lysate was diluted in SM buffer and an elution buffer with Tris-HCl and NaCl was used.

In all experiments (HPLC and FPLC) it was observed that phage recovery occurred during several fractions, i.e. during various concentrations of NaCl and not at a specific value. When larger amount of highly concentrated phage is required, the stepwise gradient can be used, because in this, narrower peaks are achieved and fraction collection is easier[142]. Consequently, a stepwise gradient of NaCl would be a valuable alternative to purify these three phages on the tested columns.

Overall, due to the sample volume limitations presented by the UHPLC system, FPLC system seemed to be more efficient than the UHPLC system. On the other hand, the QA column presented better results, since no smearing effect was observed in any of the samples tested. The selection of the QA column to optimize the phage purification method has been reported in a study with phage VDX-10 of *S. aureus*[141]. Another study with phage T4 of *E. coli* also selected the QA column for purification assays[142].

In conclusion, the CIMmultus® columns offer a valid alternative for traditional purification methods, as was already proved in another studies[140,143]. Experiences have also demonstrated that each phage needs an optimized protocol[143]. In this work, it was possible to conclude that FPLC system have shown better results than HPLC, because this last, presents limitations in the volume sample. This work also allowed to conclude that QA column presents better results than DEAE, because in this last a smearing effect, in chromatogram was observed, probably because there is a lower affinity between phages and stationary phase of this column.

4.8 Studies in 3T3 mammalian cells

The use of phages against a pathogen is an exciting prospect, but for application in humans, particularly for wound infections, their cytotoxicity and efficacy needs to be evaluated. The use of models with cell lines allows to study cytotoxicity, apoptosis assays, biochemical effects and enzymatic reactions[228]. Furthermore, these models allow to study the administration time of a drug and the different dosages that may cause cell death[130].

3T3 mouse embryonic fibroblast cells are an example of a cell line that can be used for this type of studies. They were initiated from primary mouse embryonic fibroblast cells isolated in 1962 at the New York University School of Medicine Department of Pathology[229].

4.8.1 Cytotoxicity assays -Neutral Red Uptake

There are many assays available to measure cell cytotoxicity, however there is not described a standard assay for determining the cytotoxic effect of phages[230].

The NR is a weak cationic dye used to identify viable cells in culture. This assay consists on the uptake of NR into lysosomes and its subsequent accumulation on living cells, indicating quantitative viability. If cells are damaged or dead, NR is no longer retained within the vacuole of cells, so it quantifies the number of viable, uninjured cells after their exposure with test agents, through reduction in the absorbance[229]. NRU cytotoxicity test is described in Annex A of ISO 10993- 5:2009.

Cytotoxicity assays were performed using *E. faecalis* phage 09-2, *E. faecium* phage C410 and *E. faecalis* phage 80-2. These phages were tested in two different concentrations (10^8 and 10^7 PFU/mL) and in three different conditions (lysate, after PEG purification, and after chromatography purification).

3T3 cells were incubated with phages for 24 h prior to NRU test. SLS at four different concentrations (0.05, 0.10, 0.15, 0.20 mg/mL), was used as a positive (assay) control. Figure 4.8-1 shows the viability values for each condition tested.

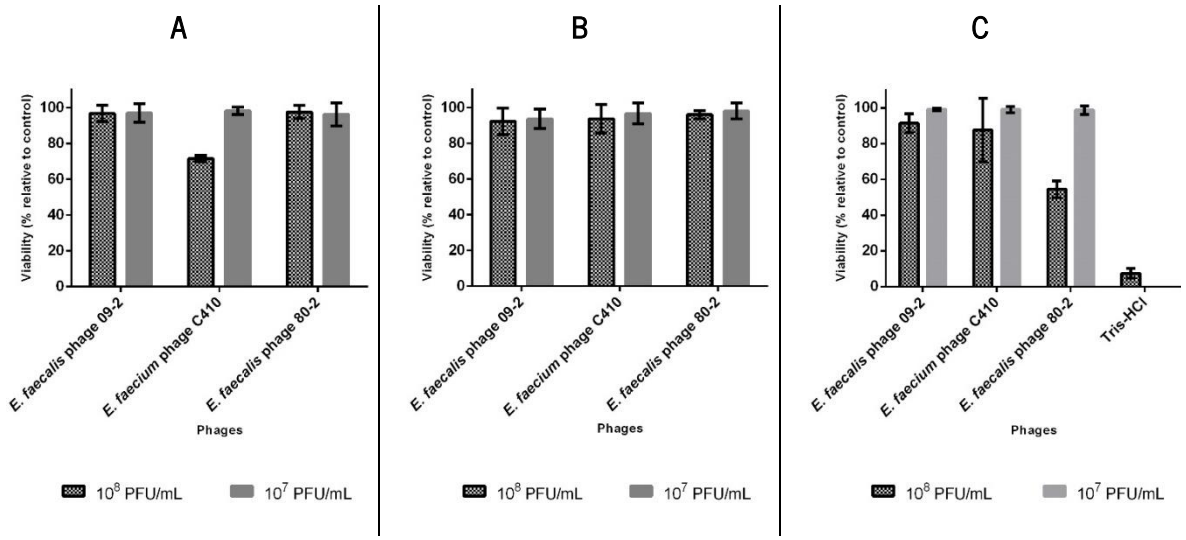


Figure 4.8-1 – Viability values (%) of 3T3 cells exposed to *E. faecalis* phage 09-2, *E. faecalis* phage 80-2 and *E. faecium* phages C410 at different concentrations and three different conditions for 24 h (A) Phage lysate **(B)** Phages purified with PEG **(C)** Phages purified with chromatography. Viability was calculated as percentage of negative control (3T3 cells without phages). Error bars represent standard deviations from three independent experiments performed in duplicate.

In general *E. faecalis* phage 09-2 was not toxic to 3T3 cells in all conditions tested (viability of cells was about 100 %). *E. faecalis* phage 80-2 shows toxicity to mammalian cells, with loss of about 50 % of cells, for a concentration of 10^8 PFU/mL when chromatography purification was used (Figure 4.8-1C). Finally, *E. faecium* phage C410 was nontoxic to animal cells when their concentration was 10^7 PFU/mL, in all conditions tested. When it was purified with PEG, it also showed no toxicity at 10^8 PFU/mL (Figure 4.8-1B). The lysate of phage C410 at a concentration of 10^8 PFU/mL showed some toxicity, causing a loss of about 30 % of cells (Figure 4.8-1A). When the C410 phage solution was purified by chromatography and tested at 10^8 PFU/mL, there were losses of about 15 % of cells (Figure 4.8-1C). However, since the standard deviation is large, it is not possible to conclude with certainty whether this solution could be toxic to 3T3 cells. Concerning the results of the chromatography, it is possible to observe that this method showed more losses in cellular viability than the other conditions tested. This fact may be due to the solution in which the phages are eluted (Tris-HCl). As can be seen in Figure 4.8-1C, Tris-HCl showed high toxicity to animal cells. However, for phage 09-2 and for low concentrations of phages 80-2 and C410 the viability is about 100 %. So, in this case the justification for the results obtained might be due to the lack of culture medium for animal cells. As high phage concentrations were not obtained when chromatography purification was used, for the dilutions in the cytotoxicity assay, the phage solution (with Tris-HCl) ratio was greater than DMEM medium.

Studies on phage cytotoxicity in mammalian cells have shown that they have no effect on cell viability. For example, Merabishvili *et al.*[231] concluded that a phage cocktail active against *P. aeruginosa* and

S. aureus strains was not cytotoxic against human neonatal foreskin keratinocytes[231]. Another study with an *A. baumannii* phage also showed that this was not cytotoxic to the 3T3 mouse fibroblast cell line[230]. Although several *in vivo* studies reported the safety of phages[89,131,197,232], similar studies should be performed to every new isolated phage that could be used for therapeutic purposes.

After obtaining these results it was considered important to understand the behavior of phages against bacterial infections in animal cells by investigating the mechanisms by which bacteria and phages interact with these cells.

4.8.2 Bacterial adhesion of 3T3 cells

Bacterial adhesion assay was performed in 3T3 cells. *E. faecalis* I009 and I980, and *E. faecium* C410 strains were used. Time-points at 2 and 24 h were analyzed for bacterial adhesion assay (Figure 4.8-2A). Cell viability evaluation was also performed in this experience by counting 3T3 cells under the microscope, at the time points analyzed (Figure 4.8-2B).

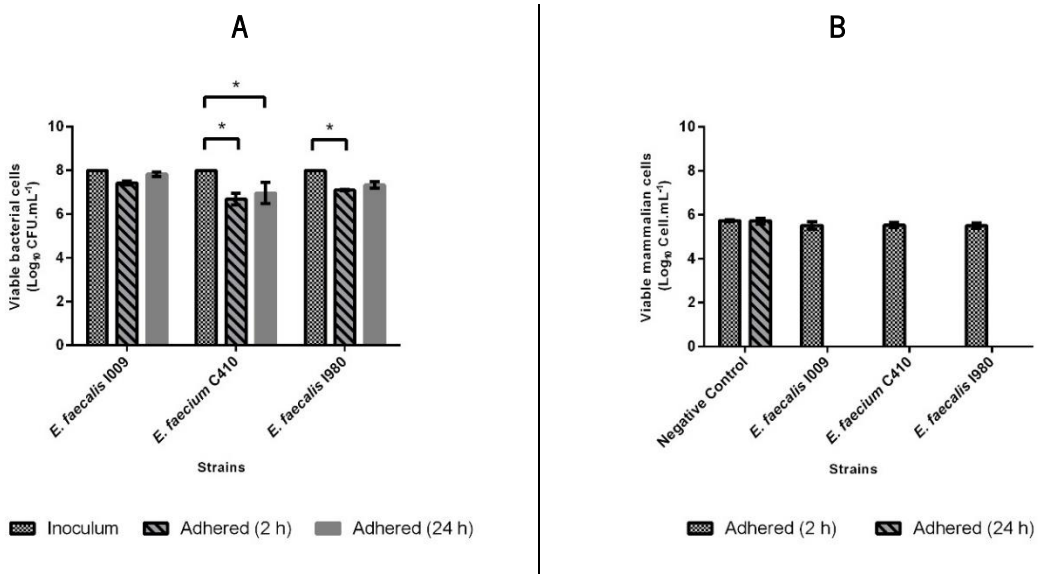


Figure 4.8-2 – Assessment of the bacterial adhesion to 3T3 cells. (A) Concentration of bacteria adhered in CFU/mL and **(B)** Viability of 3T3 cells (Cell/mL) at 2 and 24 h of post-contact. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

As it can be seen on Figure 4.8-2A, all strains tested adhered to 3T3 cells after 2 h of contact, in a concentration of about 7 orders-of-magnitude. The number increases slightly (between 0.2 and 0.4 orders-of-magnitude) when assays were prolonged for 24 h. Regarding the number of mammalian cells, no cell death was observed in the first 2 h of assay, but after 24 h of contact, cells were completely eliminated (Figure 4.8-2B).

The total death of mammalian cells in the presence of bacteria is a prominent strategy of many bacterial pathogens. Several bacteria, especially those capable of invading and multiplying within cells, secrete toxins and activate virulence factors to kill the host cells[233,234]. Cell death allows bacterial pathogens to escape efficiently from the host cell, spread to neighboring cells, and/or gain nutrients[234,233].

Several *Enterococcus* adhesion factors, that confer ability to bind to epithelial surfaces and facilitate host colonization, were already identified[235]. The aggregation substance (AS) is one of the potential virulence factors of *Enterococcus* strains[236]. This virulence factor promotes adhesion and effective colonization of bacteria in endothelial cells[237]. In a study aiming to prove the importance of AS in adhesion of bacteria to epithelium, the authors showed that this substance is involved in the attachment of different *E. faecalis* strains to intestinal epithelial cell lines originating from the colon (HT 29 and T84), ileum (HCT-8), and duodenum (Hutu 80)[236]. In a similar study, human umbilical vein endothelial cells (HUVEC) primary cultures were infected with an *E. faecalis* clinical isolate. In that case, after 4 h of infection it was observed that the bacteria also adhered to HUVEC[238]. In another study using three *E. faecalis* strains and a monolayer of colonic epithelial cells (Caco-2) it was concluded that after 2 h of contact, two strains adhered to epithelial cells with a concentration of 1 x 10^5 CFU/mL and another strain with a concentration about 5 x 10^4 CFU/mL, when a inoculum of 1 x10^8 CFU/mL was used[239]. The concentration of adhered cells in this study was much lower than observed in the referred work. Moreover, for *E. faecium*, adhesion to animal cells has been reported. In a study with *E. faecium* strains isolated from the oral cavity, the ability of these bacteria to adhere to human epidermoid cancer (Hep-2) and human lung adenocarcinoma epithelial cell lines (A549) was tested and the researchers concluded that all strains tested adhered to at least one of the cell lines tested[240].

Bacterial adhesion activates the signal transduction cascades of the host and it is an important step for the internalization of bacteria[238,241]. Although the *Enterococcus* strains are considered extracellular pathogens, it has already been proven that they can invade eukaryotic cells[238,242].

4.8.3 Bacterial internalization of 3T3 cells

Bacterial internalization assay was also performed in 3T3 cells. Internalization of *E. faecalis* I009 and I980 and *E. faecium* C410 strains was studied after 2 h of contact with 3T3 cells (Figure 4.8-3). For internalization assay 150 μg/mL Vancomycin was selected, among other antibiotics tested (data not shown), to kill the viable extracellular bacteria.

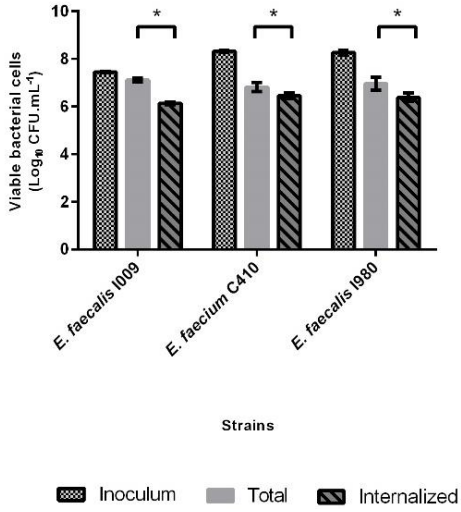


Figure 4.8-3 - Internalization of *E. faecalis* I009, *E. faecium* C410 and *E. faecalis* I980 on 3T3 cells. Internalization experiments was performed for 2 h. Inoculum, Total and Internalized, represent respectively, the initial total bacteria, total bacteria in cells without antibiotic treatment, and concentration of bacteria in cells treated with antibiotic. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

For *E. faecalis* I009 the number of internalized bacteria cells, is 1 order-of-magnitude lower than the number of total cells ($p<0.05$), but it can be concluded that the majority of the bacterial cells are internalizing the 3T3 cells (Figure 4.8-3). In the other tested strains (*E. faecium* C410 and *E. faecalis* I980), the number of internalized bacteria is about 6.5 orders-of-magnitude, very similar to the total number of bacteria counted (only differences between 0.3 and 0.5 orders-of-magnitude), suggesting that all bacteria were internalized after 2 h of contact (Figure 4.8-3).

The internalization of *Enterococcus* strains has already been reported in other studies. The *in vitro* ability of *E. faecalis* clinical isolates to adhere, and to invade, human epithelial cells was already studied[242]. In that study, it was observed that *E. faecalis* strains internalized HeLa cells in two distinct modes of interaction after 2 h of contact[242]. The direct absorption of the bacterial cell was one of the possible modes of interaction observed. In the other, the internalization of the bacteria possibly occurred through the formation of cellular pseudopodal[242]. In a recent study, it was possible to observe intracellular *Enterococcus* cells 4 h after infection on HUVEC cultures[238].

Since the studied bacteria can internalize animal cells, it was considered important to perform phage internalization experiments to determine if the phages would have the ability to infect and kill internalized bacteria.

4.8.4 Phages internalization

3T3 cells were incubated with the three studied phages for phages internalization assay. To remove extracellular-bound phages 10 mM FAS was used. After 2 h of assay, the number of intracellular phages was determined by PFU assay (Figure 4.8-4).

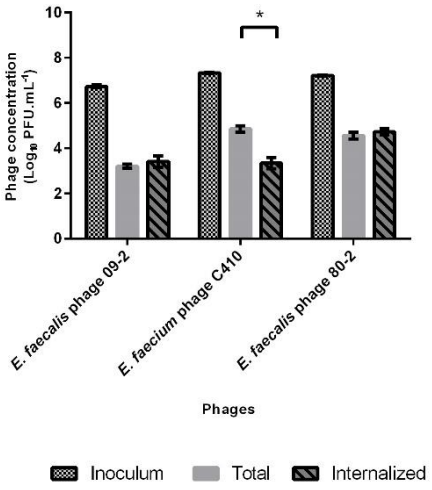


Figure 4.8-4 – Internalization of *E. faecalis* phage 09-2, *E. faecium* phage C410 and *E. faecalis* phage 80-2 on 3T3 cells. Internalization experiments was performed for 2 h. Inoculum, Total and Internalized, represent respectively, the initial total phages, total phages in cells without FAS treatment, and concentration of phages in cells treated with FAS. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

On Figure 4.8-4 it is shown that all phages are able to interact with the 3T3 cells, in 3 to 5 orders-of-magnitude. Results suggest that all phages could internalize 3T3 cells, after 2 h of contact. *E. faecalis* phages 09-2 and 80-2 internalization was about 3 and 5 orders-of-magnitude, respectively. *E. faecium* phage C410 internalization was about 3 orders-of-magnitude.

In the literature, there is no information about the internalization of phages in animal cells. However, the structure of *Caudovirales* phages is mainly composed by proteins, and in this case, some studies on peptide internalization are reported. Some synthesized peptides were put in contact with NIH-3T3 fibroblasts and with Jurkat E6 human leukemia cells. Using confocal laser scanning microscopy (CLSM) and flow cytometry, the authors concluded that the cellular uptake of peptides was very efficient for both culture cells[243]. In another study, the internalization of other synthesized peptides was studied against cancer cells (HeLa, HCT116, B16 / F10) and normal cells (HaCat, BJ and NIH 3T3). The results were

observed by CLSM and it was concluded that there was internalization of peptides in cell lines, but more in cancer cells than in normal cells[244].

At the end of these experiments, it was possible to conclude that both bacteria and phages interact with animal cells by adhering and/or internalizing them. If bacteria kill the cells by internalizing them, it is crucial to determine if phages could control internalized bacteria.

4.8.5 Efficacy of phages in bacteria colonizing 3T3 cells

3T3 cells were infected with 10^8 CFU/mL of each bacterium (*E. faecalis* I009, *E. faecium* C410 and *E. faecalis* I980) and treated at 2 h post-infection by 10^7 PFU/mL from respective phage. The concentration of viable bacterial cells and the number of 3T3 cells were quantified at 6 and 24 h post-treatment (Figure 4.8-5).

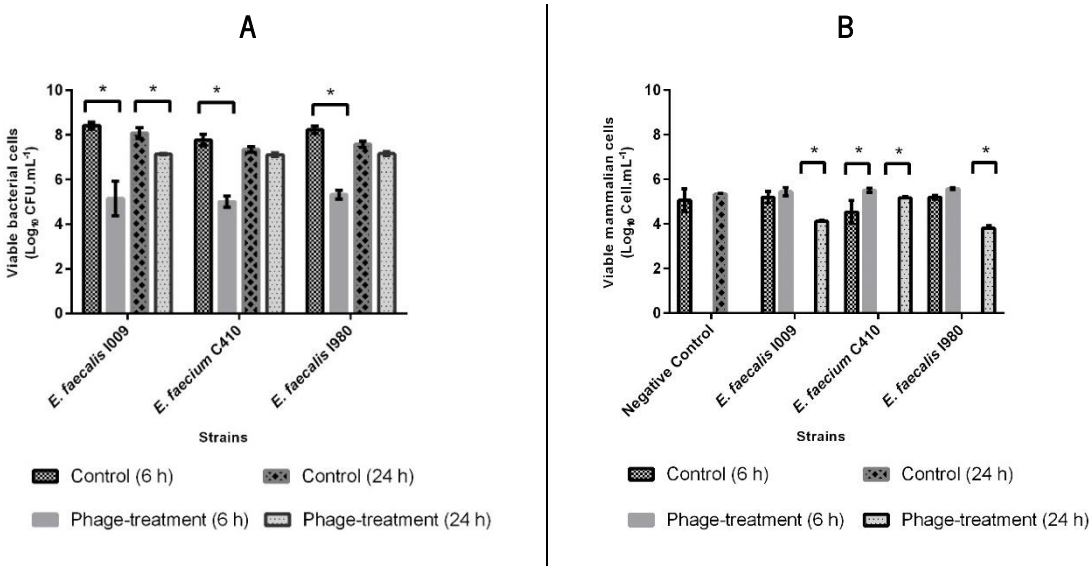


Figure 4.8-5 - Efficacy of phages against bacteria adhered/internalized to 3T3 cells (A) Concentration of viable bacterial cells and **(B)** Number of 3T3 cells in control (without treatment) and after 6 and 24 h of phage treatment. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

Concerning the reduction of infection, the three tested phages, showed a decrease in viable bacterial host cells ($p<0.05$) after 6 h of treatment (Figure 4.8-5A). The reduction was about 3 orders-of-magnitude, which was more than the reduction previously obtained on the CWM for 24-well plates (Figure 4.2-12). In 3T3 cells, 6 h after the application of *E. faecalis* phage C410, it was possible to observe a greater concentration (1 order-of-magnitude, $p<0.05$) of viable 3T3 cells than in the untreated control cells (Figure 4.8-5B). In the other cases, the concentration of 3T3 cells was about 5.5 orders-of-magnitude, very similar between treated and untreated cells. Twenty-four hours after phage treatment, it was possible to observe

that only phage-treated cells presented viability (Figure 4.8-5B), although at this time point, phages efficacy in the reduction of bacterial cells, was only between 0.2 and 1 order-of-magnitude (Figure 4.8-5A).

To prove the efficacy of phages in killing internalized bacteria an experience that used Vancomycin to kill external bacteria, was performed. Phages were administrated 1 h after the antibiotic and their efficacy was analyzed 6 h post-treatment.

Concentration of viable bacterial cells after 6 h of treatment can be observed in Figure 4.8-6.

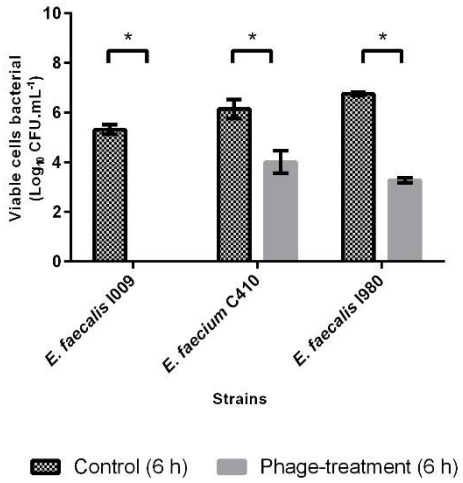


Figure 4.8-6 - Efficacy of phages against internalized bacteria in 3T3 cells. Concentration of viable bacterial cells in control (without treatment) and after 6 h of phage treatment. Error bars represent standard deviations from three independent experiments performed in duplicate. *Statistically significant ($p<0.05$) conditions between control and test assays.

Statistically significant reductions ($p<0.05$) were obtained in all species after phage-treatment (Figure 4.8-6). After 6 h of treatment, *E. faecium* phage C410 reduced by 2 orders-of-magnitude the number of host viable cells, comparing with the control. *E. faecalis* I980 strain treated with *E. faecalis* phage 80-2 showed a decrease in bacterial cells of 3 orders-of-magnitude. In the other *E. faecalis* strain tested (I009), when was used *E. faecalis* phage 09-2 no bacterial cells were observed in treated mammalian cells.

The efficacy of phages in the presence of cell lines was observed by Mirzaei *et al.*[132], when they studied the interaction between immortalized cell lines (HT-29 and Caco-2 intestinal epithelial cells) and four *E. coli* phages. In that study, phages showed a significant reduction between 1.5 and 4.5 orders-of-magnitude of the bacterial content over a period of 8 h[132].

In the literature, the information about the effects of direct addition of phages against bacteria in immortalized cell lines is scarce. However, phages efficacy on *in vivo* models suggests that this approach

might be a valuable therapy. Duerkop *et al.*[245] tested the ability of phage φVPE25 to kill *E. faecalis* growing in the intestinal tract of a mouse model. Bacteria and phages were orally administered with a difference of 6 h. After 24 h of phage treatment a reduction in the number of *E. faecalis* cells was observed compared with phage–untreated animals[245]. Another study, used BALB/c mice as *in vivo* model, with an intraperitoneal administration of *E. faecalis*. An *E. faecalis* phage isolated from a sewage sample was administrated after 30 min and 4 h, saving 60 and 40 % of the animals, respectively[246].

In general, all phages were not toxic to 3T3 cells when a concentration of 10^7 PFU/mL was tested. Concerning the interactions between bacterial cells and mammalian cells, it was possible to observe in all bacteria tested, that they adhered and internalized the 3T3 cells. Phages also showed the ability to internalize the tested animal cells. All phages decreased bacterial growth after 6 h of treatment as compared to the control medium. This work also showed that the three phages can infect internalized bacteria. All these results suggest that the *E. faecalis* phages 09-2 and 80-2 and *E. faecium* phage C410 may be promising in therapy.

5 CONCLUSIONS AND FUTURE WORK

The increase of chronic wound cases, the limitations of the current therapies and mainly the increasing bacterial multidrug resistance, led the WHO to report the urgency on the search and development of novel therapeutic approaches. Accordingly, the main purpose of this study was to develop a phage-based product to be incorporated in a wound dressing to control biofilms in chronic wounds.

Indeed, phages are seen as a possible novel treatment modality against antibiotic-resistant infections, namely caused by biofilms. Therefore, the first objective of this work was to isolate phages that could be successful killing bacterial species in chronic wounds. Five *E. coli* phages, four *S. aureus* phages, one *E. faecium* phage and seven *E. faecalis* phages were isolated. After this step, phages were subjected to a process of characterization. The first parameter evaluated was its host range. *E. coli* phages were not included on the studies, because they presented a narrow spectrum of activity towards the tested strains. *S. aureus* phage I366 B, *E. faecalis* phage 09-2 and *E. faecium* phage C410 infected respectively 58, 94 and 78 % of the tested strains and consequently were further characterized.

During *in vitro* characterizations, *S. aureus* phage I366 B was discarded of the following assays, because it did not show ability to decrease biofilm cells. Moreover, in these studies *E. faecalis* phage 80-2 was included, due to unexpected results observed when the phages were applied in SM buffer. As future work, it will be important to isolate and characterize more phages of other bacteria predominant in chronic wounds, for example phages against *E. coli*, *P. aeruginosa* and *S. aureus*.

TEM analysis have shown that *E. faecalis* phages 09-2 and 80-2 belong to the *Siphoviridae* family, and *E. faecium* phage C410 belongs to the *Podoviridae* family. Genome analysis revealed that the phages are virulent (no integrases detected) and generally safe (no virulence determined). *E. faecalis* phage 09-2 encodes a putative toxic protein -hemolysin XhlA family, which will require a more detailed genomic analysis. All phages were stable after 24 h, between −20 and 37°C, and in the pH range 5.0–11.0, which allows to conclude that these phages may be promising against biofilms in *in vivo* wound environments, which are basic and have temperatures between 31 and 35 °C.

The three phages were capable of infecting and killing biofilms, however phages 09-2 and C410 showed a low antibiofilm activity when applied in SM buffer, suggesting that they need the presence of active metabolic host to replicate. In almost all conditions a biofilm regrowth was observed at 24 h post-treatment probably due to the development of resistant bacterial variants. Nevertheless, the use of the phage cocktail increased the efficiency of phage killing about 2.5 orders-of-magnitude ($p<0.05$) at 3, 6

and 8 h, and of about 1 order-of-magnitude ($p<0.05$) at 24 h. It was only possible to study two biofilm consortia composed by different *Enterococcus* strains. Although, it was possible to count viable bacteria in each consortium there were some difficulties. In the future, plating methods for strain selection will need to be optimized, using for example other selective marker. Furthermore, it will be important to include other bacteria, such as *S. aureus*, *E. coli* and *P. aeruginosa* for the study of multi-species biofilms.

In the second part of this work the concept of chromatography to purify phages was tested. For all phages, in a HPLC system, the chromatogram showed, for QA and DEAE columns, a single peak at approximately 0.08 M NaCl and, high phage concentration out of the peak was detected by plaque assay. This fact did not allow to determine the purer phage fraction, and therefore in alternative, an assay in a FPLC system was performed. In this, although, several peaks were observed in the chromatogram, non-bound impurities were removed in first minutes of run. As future work, the optimization of this process should include the equilibrium between the mobile phase and the phage solutions, and the test of a stepwise gradient of NaCl instead of a linear gradient.

Concerning the study of phages behavior in the 3T3 cell line, in general, phages were not toxic to cells when a concentration of 10^7 PFU/mL was tested. In addition, all phages and their host bacteria have been shown to adhere and internalize animal cells. Moreover, bacterial reductions between 2 and 3 orders of magnitude ($p<0.05$) were observed in phage-treated cells and it was concluded that the phages infect internalized bacteria, which is a very promising result. However, CLSM observations are required to corroborate these results.

In conclusion, the work developed in this thesis provided good indicators for the formulation of a phage-based bioactive product for biofilm control. This product can be applied in a dressing to control bacterial infections in chronic wounds, namely caused by enterococci. However, there is still some work to be developed, namely, the study of phage efficacy in *ex vivo* biofilms and analysis of the local immune response when phages are present, using, for example, synthetic skin. In parallel studies regarding the selection of the material with the best properties should be performed. After developing the phage-based wound dressing, the *in vivo* validation of the results will also be required, namely using pig wound models.

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SUPPLEMENTARY MATERIAL I – *IN VITRO* CHRONIC WOUND MODELS

Table 4.8-1 - Different *in vitro* chronic wound models. Adapted from[247]

Designation	Surface	Medium	Inoculum	Incubation temp
Poloxamer model	Poloxamer gel	MH-agar	10^5- 10^6 CFU	25-35 °C
Lubbock chronic wound biofilm model (LBCW)	Plastic tip, silicone disk or host derived matrix	Bolton Broth, 50 % (v/v) bovine plasma, 5 % (v/v) freeze-thawed lacked horse-blood	10^4 CFU	37 °C
Collagen wound model (CWM)	Collagen matrix	SWF: 50 % (v/v) FBS and 50 % (v/v) physiological NaCl in 0.1 % (w/v) Peptone or a (1:1 v/v) TSB-SWF solution	10^4-10^5 CFU	35-37 °C
Minimal biofilm eradication concentration (MBEC) wound model	Serum coated pegs	TSB	10^7 CFU/mL	37 °C
Cellulose agar model	Cellulose disks	LB-agar	10^2-10^4 CFU	37 °C
Artificial wound bed model	Plastic	Bolton Broth, 1 % (w/v) gelatin, 50 % (v/v) porcine plasma, 5 % (v/v) freeze-thawed porcine erythrocytes or Bolton broth + 1 % (w/v) gelatin +1.2 % (w/v) agar	10^4 CFU	37 °C