UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Study of bacteriophage biology and its potential application in therapy

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniero Biomédico

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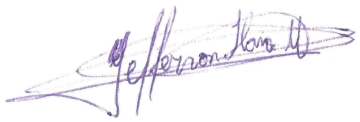

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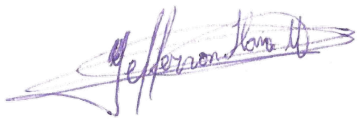

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Abstract

Antibiotic microbial resistance has become a significant threat to global health. The medical community is in an urgent search for an alternative treatment to deal with antimicrobial resistance. In this context, bacteriophages have appeared as a potential option. This study is focused on presenting a general overview of the potential of phage therapy to be used as an alternative treatment for bacteria-resistant infections. This approach starts with an introduction to phage history, biology, and applications. Then addresses the problem of antibiotic resistance and their implications. Finally, there is a description of phage therapy, with a presentation of the advantages and constrains in comparison with current antibiotic therapy. From the previous analysis, phage therapy is seen as a viable treatment choice; however, it is also advised that further studies and research is implemented, with especial attention in the realization of clinical trials to ensure phage therapy efficacy, efficiency and safety.

Keywords: bacteriophage, phage, antibiotics, antibiotic-resistance, therapy

Resumen

La resistencia antibiótica de los microorganismos se ha convertido en una grave amenaza a la salud global. La comunidad médica se encuentra en una búsqueda urgente de un tratamiento alternativo para lidiar con la resistencia antimicrobiana. En este contexto, los bacteriófagos han aparecido como una opción viable. Este estudio se enfoca en presentar una revisión general del potencial de la fago-terapia para ser utilizada como un tratamiento alternativo para infecciones causadas por bacterias resistentes a antibióticos. El enfoque inicia con una introducción de la historia, biología y aplicaciones de los fagos. Luego se aborda la problemática de la resistencia antimicrobiana y sus implicaciones. Finalmente, se describe la fago-terapia presentando sus posibles ventajas y limitaciones comparados con la terapia antibiótica. La evidencia analizada prevé el posible uso de la fago-terapia, sin embargo se reconoce la necesidad de realizar más estudios y sobre todo de ensayos clínicos que puedan sustentar la eficiencia, eficacia y seguridad en su aplicación terapéutica.

Palabras clave: bacteriófagos, fagos, terapia, resistencia antibióticos.

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AMR: antibiotic microbial resistance

ARB: antibiotic resistant bacteria

DNA: deoxyribonucleic acid

ds: double strand/ed

ss: single strand/ed

FDA: Food and Drug Administration (US)

HIC: high income country

ICTV: International Committee on Taxonomy of Viruses

INSPI: Instituto Nacional de Investigación en Salud Pública

LMIC: low-and-middle income countries

MDR: multi-drug resistant (bacteria)

RNA: ribonucleic acid

USSR: Union of Soviet Socialist Republics

US: United States

WHO: World Health Organization

MSP: Ministerio de Salud Pública del Ecuador

1. Introduction

1.1. Bacteriophages

Bacteriophages, the most abundant biological entity on the planet, are small viruses that are capable of infecting and killing bacteria.[1] Bacteriophages play an important role in maintaining microbial balance by acting as natural predators for bacteria. Bacteriophages act as natural drivers of bacterial evolution and virulence. Phages are known as obligate bacterial parasites that use bacterial host machinery to produce their progeny. Studies of phage biology have been conducted for almost 90 years (see 1.2 for detailed historical background) and have led to phages' development as useful tools for several biotechnological applications. Phage research has been vital in developing modern molecular biology, including sequencing and genome engineering, and recent discovery and exploitation of CRISPR-Cas systems, due to the ability of phages to develop proteins inhibitors known as anti-CRISPR proteins [1]. Tough being extensively studied, mainly in the former Soviet Union and eastern Europe, phages have not been present as expected in the clinical treatment of infective diseases caused by bacteria. Mainly because of antibiotic development and proved efficacy, phage therapy was overlooked by the medical community. However, there is a renewed interest in phage research driven by humanity's actual antibiotic crisis. Bacteriophages are now seen as a promissory option to treat infective diseases caused by antibiotic-resistant bacteria (ARB). This does not imply that phages are taken antibiotics away, but they represent an opportunity to look and study a long-forgotten ancient technique with great potential. [2–4].

1.2. A brief history of phages

The first approach to bacteriophages dates back over a century, especially in the pre-antibiotic era. In 1896 the British bacteriologist Ernest Hankin described a phenomenon of waters from the Ganges being able to destroy *Vibrio cholera*-inducing bacteria cultures. However, it was not until the year 1915 that Frederick Twort described a filterable agent capable of breaking down bacteria from a culture into granules; and two years later Felix d'Hérelle described a similar experimental finding devised the term "bacteriophage" meaning bacteria-eater.[2,3]. After bacteriophages being co-discovered by Twort and d'Hérelle, phages were used in the clinical setting for treating infectious diseases (see **Fig 1**. for details).

Until the 1940s, the nature of phages was still in discussion. Some experts were skeptical of bacteriophages 'viral nature and stated that phages were enzymes capable of bacterial lysis. The discovery of electron microscopy (EM) allowed the visualization of phages and proved their viral nature.[2]. Helmut Ruska was the first to describe phages as "sperm-shaped" particles adhering to a bacterial membrane.[3]. The appearance of antibiotics caused phages to be left apart, especially by the western scientific community. Meanwhile, phage therapy continued to be studied and used extensively in France, Germany, Russia, and eastern Europe. [5]

Phage therapy had an enormous development in eastern Europe, especially in two countries: Georgia and Poland. In these countries are located two of the most representative phage research facilities: Hirszfeld Institute (Wroclaw Poland), which was focused on the development of individualized phage therapy, and Eliava Institute (Tbilisi, Georgia) with a focus on the production of phage cocktails and their clinical application. [6]. Both facilities are still operational and are two of the major research centers for phage therapy. It was not until 1980 that phages reappeared in the western world, caused by Smith and Huggins' attention because of their work. Bacteriophages and their clinical application have regained interest in recent years due to the current antibiotic resistance crisis. Nowadays, it is a hot topic in the scientific community, and there is a notable increase in research focused on phages, especially on their bactericidal effect.

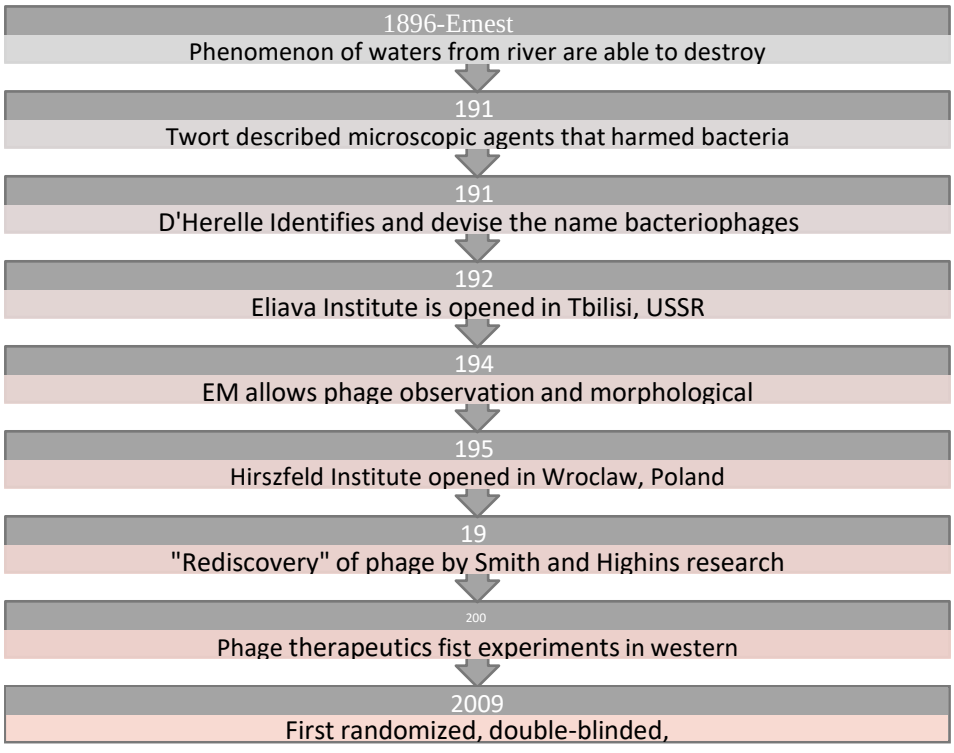


Fig. 1 Timeline showing important bacteriophage milestones. Adapted from 1, 45

1.3. Bacteriophage morphology

Bacteriophages are small viruses that vary in size from 24 to 400nm [7], depending on the species of phage. The phage structure is usually composed of a head(capsid) and additional structures that are responsible for the interaction with the bacterium membrane.[8] [9](see **Fig. 2**). The head or capsid usually has a geometric shape formed structurally by two or more different proteins. The capsid encloses and protects the genetic material, which could be either DNA or RNA [7]. The baseplate contains receptor-binding proteins responsible for bacterial strain recognition and genetic material transfer into the host [7,10]. The study of bacteriophages' morphology is useful beyond a possible phage classification based on certain characteristics (Table 1) because it helps understand specificity mechanisms mediated and depend on certain phage structures [9]. From a morphological standpoint, bacteriophages are a highly diverse group of viruses that display various structural elements, shapes, and proteins [9] (see **Table 1**).

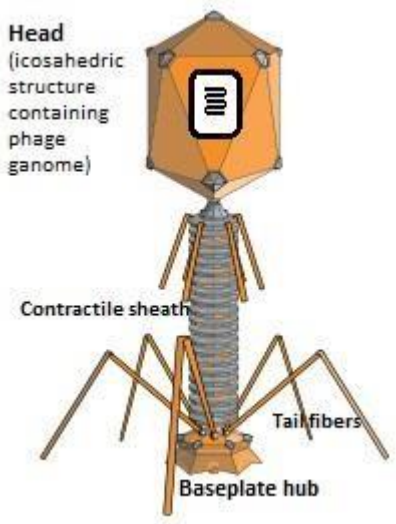


Fig. 2 Schematic representation of phage morphology

Table 1. Most critical structural characteristics of phages. Adapted from data in (1)

Phage characteristic	Description
Genetic material	Ss or ds *ss= single strand, ds= double strand DNA or RNA
Genome size	Simple (3.5kb ss RNA) to complex (500kb ds DNA)
Morphological capsid shape	Tailed Polyhedral Filamentous or pleomorphic
Capsid structure	Lipid or lipoprotein envelopes

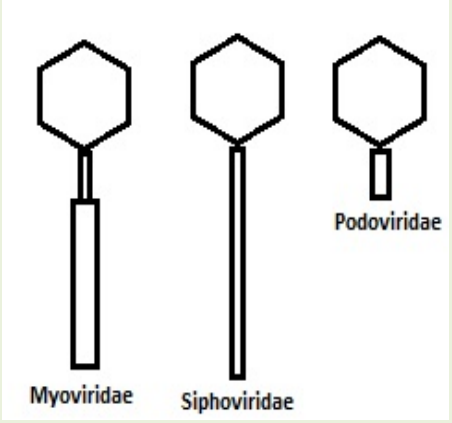
1.4. Bacteriophage classification

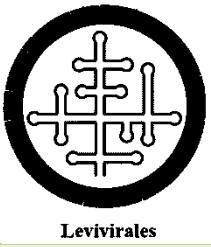
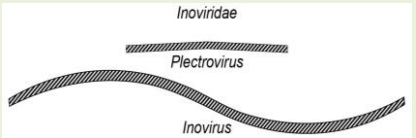
More than 6000 different bacteriophages have been discovered and described, including 6196 bacterial and 88 archaeal viruses [3]. Bacteriophages could be classified according to different characteristics, such as morphology, genetic content, specific host, location, and life cycle.

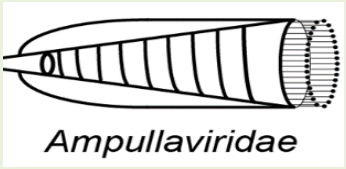
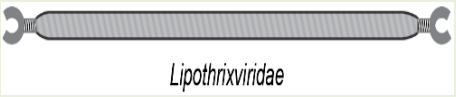
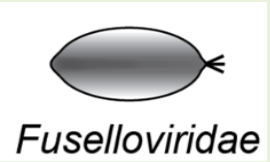
1.4.1. Bacteriophage taxonomical classification

During the early years of bacteriophage discovery, the insights into fundamental phage biology were limited. Until the 1940s, electron microscopy visualization allowed bacteriophages' characterization and further taxonomical classification based on morphology [2]. The taxonomical classification of phages is carried out by the International Committee on Taxonomy of Viruses. In the ICTV report in 2008, phages were grouped into five families, 26 subfamilies, 363 genera, and 1320 species [11]. Several changes have been made since this report. The current phages classification was updated in the year 2019, as described in (Table 2). The vast majority of bacteriophages (96%) are tailed and belong to the Caudovirales order; other types are cubic, filamentous, or pleomorphic [12].

Table 2. Phage classification, according to ICTV 10th report. The structural phage representations were obtained from ICTV website: https://talk.ictvonline.org/ictv-reports/ictv_online_report/introduction/w/introduction-to-the-ictv-online-report/422/hosts-bacteria-and-archaea

Order	Nucleic acid	Family	Example	Structure
Caudovirales	DNA, ds, linear	Ackermanviridae		
9 families		Autographiviridae		
		Chaseviridae		
44 subfamilies		Demerecviridae		
		Drexlerviridae		
		Herelleviridae		
1967 species tailed		Myrioviridae	T4	
		Podoviridae		
		Siphoviridae	T7	

Belfryvirales	DNA, ds, linear	Turriviridae		
Halopanivirales	DNA, ds, linear	Sphaerolipoviridae		
Haloruvirales	DNA, ss, circular DNA, ds, circular DNA, ds, linear	Pleolipoviridae		
Kalamavirales	Linear dsDNA	Tectiviridae		
Levivirales	Linear ssRNA	Leviviridae	MS2, Qβ	
Ligamenvirales	DNA, ds, linear	Lipothrixviridae	Acidianus filamentous virus 1	
		Rudiviridae	Sulfolobus islandicus rod-shaped virus 1	
Mindivirales	Segmented dsRNA	Cystoviridae		
Petitvirales	DNA, ss, circular	Microviridae	ΦX174	
Tubulavirales	DNA, ss, circular	Inoviridae	M13	
Vinavirales	Circular dsDNA	Corticoviridae	PM2	
Unassigned	DNA, ds, linear	Ampullaviridae		

	DNA, ds, circular	Bicaudaviridae		
	DNA, ds, circular	Clavaviridae		
	DNA, ds	Finnlakeviridae	FLiP	
	DNA, ds, circular	Fuselloviridae		
	DNA, ds, linear	Globuloviridae		
	DNA, ds, circular	Guttaviridae		
	DNA, ds, circular	Plasmaviridae		
	DNA, ds, circular	Portogloboviridae		
	DNA, ss, circular	Spiraviridae		
	DNA, ds, linear	Tristromaviridae		

1.5. Remarkable bacteriophages

1.5.1. T4 Phage

Bacteriophage T4 is one of the seven phages capable of infecting *Escherichia coli* (T1-T7). T4 is closely related to other T-even phages (T2 and T6). T4, as a member of the Myoviridae family, has a contractile tail connected to an icosahedral head and a relatively large genome with approximately 170000 bp in a double strand DNA [13]. T4 phage study has served to obtain several advances in molecular biology, such as recognizing the chemical nature of genes, understanding DNA replication mechanisms, discovering protein gene coding, and unraveling how the genetic code is read.

T4 has some unique features: eukaryote-like introns, high-speed DNA replication mechanism, DNA repair mechanisms[14]. T4 bacteriophages have been used in therapeutics; the called coliphages were products developed by the American Eli Lily Company™. The products were offered as preparations for human use against *E. coli* infections [14].

1.5.2. Lambda (λ) Phage

Lambda phage was discovered in 1951 by Esther Lederberg. Its fortuitous discovery turned into lambda phage, becoming a model system for studying fundamental biological processes [15]. Lambda phage is cataloged as temperate because of its lysogenic cycle to infect bacteria. Lambda phage infects *E. coli*; however, other lambdoid phages can infect *Salmonella*, *Shigella*, *Pseudomonas*, and *Burkholderia*. As a member of the Siphoviridae family, its structure is formed by an icosahedral head and a non-contractile tail [13]. Lambda phage was one of the first models to study the nature of DNA and genes [16]. According to Casjens (2015), lambda phage had a significant contribution to the development of DNA cloning technology. Besides, lambda phage is considered as a study model for phage lysogeny. The use of lambda was useful to establish the mechanism of phage genome insertion into the host genome. Due to its lysogenic nature, there is no evidence of Lambda phage applied in any therapeutic approach.

1.6. Bacteriophage lifestyle

Phage's lifestyle classification is based on what strategies phages employ to infect their hosts. Traditionally phages have been grouped into either lytic or lysogenic life cycles. However, recently two additional life cycles have been observed: pseudo lysogenic and chronic infection.

According to the life cycle, it is possible to determine their role in bacterial/archaeal biology. Bacteriophages are classified into two different classes based on their life cycles: temperate and virulent. To know the lytic or lysogenic nature of phage has vital importance to determine if it is suitable for phage therapy. [10,17,18].

1.6.1. Lytic cycle

The phages that undergo a lytic life cycle are known as virulent. During a lytic cycle, bacteriophages infect their target bacterial host, inject its genome, and replicate viral progeny using the bacterial machinery. Once the replication has generated enough copies, the host cells are lysed, and it generally kills the host. The lytic lifestyle can shape bacterial population dynamics, and generalized transduction might assist in their long-term evolution [6,17,18].

In the lytic process, phages use two types of proteins: holins and lysins [6]. Holins function is to define the duration of the infective cycle and the yield of progeny phage. [19]. Holins are accumulated in the membrane until they reach a critical concentration that leads to the membrane's disruption. Due to holins' action, the membrane becomes permeabilized to the fully folded endolysin [20]. Endolysins are muralytic enzymes that degrade the murein or peptidoglycan layer (peptidoglycans are the major structural component of the bacteria cell [21,22]. Given the bacterial killing potential that lytic phages present, it is suggested that they might be more efficient and should be used for bacteriophage therapy [6,23].

1.6.2. Lysogenic cycle

Bacteriophages that undergo a lysogenic cycle are known as temperate. In the lysogenic cycle, the phages infect the bacterial hosts and incorporate their genome into the host. During lysogenic infection, temperate phages are found in a dormant state and are known as prophages. The phage replicates along with the host; this cycle can be stable for several generations.

The phage remains in this cycle until it is exposed to certain environmental stimuli, such as presence of antibiotics or host cell inflammation, that might trigger a lytic cycle [17]. Lysogeny may create a symbiotic phage-bacterium relationship that can be beneficial for the bacteria; due to the phage's ability to encode genes that might enhance the fitness of the bacterial host in a process known as lysogenic conversion. [6,18,24].

1.6.3. Pseudolysogenic cycle

Pseudolysogeny is a less common phage life cycle. It is defined as an unstable situation in which the genetic material of a phage has entered into a bacterial cell but has not integrated stably (as it does in lysogeny) neither replicate and lyses its host (as it does in the lytic cycle). Instead of being incorporated into the host genome in the pseudo -lysogenic cycle, the phage's genetic material remains as free DNA in the cytoplasm. Pseudolysogeny is often observed in nutrient-deprived conditions, in which bacterial cells are not able to support DNA replication or protein synthesis. The phage remains in a carrier state until the nutritional status is restored and enters either lytic or lysogenic life cycle. [17,18,24].

1.6.4. Chronic infection

Chronic infection is usually observed in filamentous phages containing single-stranded DNA. It is similar to the lytic cycle; however, the cell is infected, and phage progeny is constantly released without the host cells' substantial disruption. The phage viral progeny is exported by a variety of mechanisms such as budding or extrusion. [9,17,18,25].

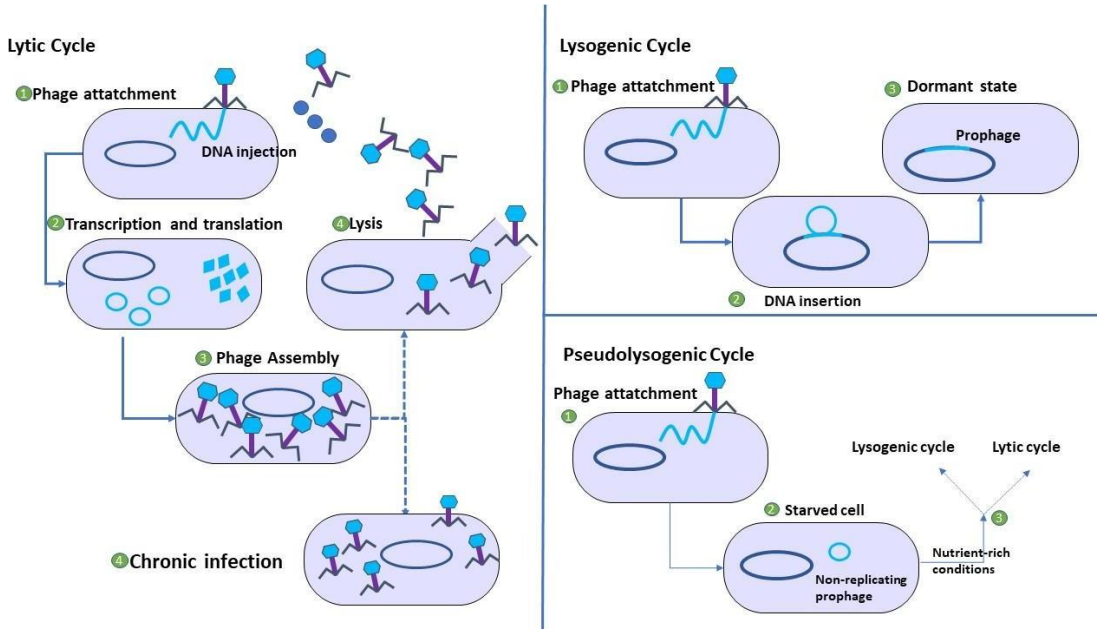


Fig. 3. Schematic representation of the different life cycles of bacteriophages. ***Lytic cycle:*** *1) phage is attached to the bacterial surface through specific binding site, and there is a process of phage DNA injection into the host. 2) Through transcription and transduction processes, phage genetic material and capsid proteins are replicated. 3) Phages are assembled inside the host cell. 4) Phage progeny is released causing the disruption of membrane cell and leading to bacterial death.* ***Chronic infection:*** *Steps 1,2, and 3 are the same as* ***lytic cycle,*** *the main difference is that in step 4) phage progeny is released without any major disruption of the cell membrane.* ***Lysogenic cycle:*** *1) Phage attachment mediated by specific binding sites, and phage DNA is injected into the host. 2) The phage genetic material is inserted into the bacterial genome. 3) The phage remains in a dormant state (prophage) and replicates along with the host.* ***Pseudolysogenic cycle:*** *1) Phage attachment and DNA injection into the host. 2)DNA is not inserted into the host genome nor replicated, it is usually seen in starved bacterial cells. 3)By the time nutrients are available, the phage might enter either a lytic or a lysogenic cycle.*

1.7. Phage non-therapeutical applications

Even though the western world overlooked bacteriophage research, bacteriophage studies contributed to several advances and developments of the current molecular biology. Besides, phages have been used for food industry applications, agriculture, diagnosis, and as biocontrol agents. The use of bacteriophages out of the clinical field presents the advantage of a significantly reduced regulatory burden, meaning that companies might have lower expenses in development and products reach the market faster [26].

1.7.1. Food biocontrol and safety

Food used for human consumption is not always free of pathogens, usually containing germs responsible for severe diseases in humans [27], and might represent a serious health problem, as well as cause economic losses. As a result, bacteriophages have been introduced into the production chain of food, emphasizing food safety. Phages are used as natural food additives and accepted due to high phage levels in the human digestive tract and the environment [28]. Combined with characteristics as specificity, auto replications, keeping on the sensory properties, and previously mentioned environmental safety makes bacteriophage an exciting option to control foodborne bacterial pathogens[29]. However, it is necessary to ensure phage lytic activity and performance under certain conditions (pH, temperature, and storage conditions) before using either additive or a surface decontaminant [28,29].

There already exist phage products in the market, which have the GRAS (Generally Recognized as Safe) status approved by the US FDA. Some of the approved products are ListShield™, EcoShield™, and SalmoFresh™. Those products target *E. coli*, *Salmonella*, *Listeria monocytogenes*, which are some of the bacteria responsible for major foodborne outbreaks [26].

1.7.2. Vaccine carriers

Another application of bacteriophages is to be used as vehicles for vaccine delivery. There are animal model studies (mice, rabbits, and sheep) that have demonstrated a long-lasting and more significant antibody response produced as a result of the phage delivery of DNA vaccines. Compared to those after vaccination with naked DNA, and even comparable to results obtained with recombinant protein vaccination [30].

There are two main approaches for phage vaccine carriers: 1) phage display vaccines 2) phage DNA vaccines. Those approaches are often used to obtain phage particles that bear foreign genes and display a protein or peptide on their surface [10]. The first phage vaccination approach is phage display vaccines; this method aims to produce immunogenic bacteriophage particles. The other approach is phage display for vaccine delivery (see Phage display technology).

1.7.3. Phage display technology

First described in 1985, phage display is a technique used to create phage libraries. Phages are genetically engineered by fusing DNA that encodes a polypeptide into the phage coat genome. As a result, the protein is displayed on the phage's surface—the proteins displayed on the phage surface act as recognition peptides with different biological targets. The recognition and attachment mechanisms are similar to the antigen-antibody interaction. Phages employed in phage display are M13, lambda, and T7. [31]. The generated phage libraries can contain an almost infinite number of recognition sites that could be used to test pathogenic agents. Phage display has several applications: epitope identification, antigen delivery, vaccine design, bioimaging, and biosensing [32].

2. Problem Statement and Objectives

2.1. Problem Statement

The development of bacterial resistance rises as a challenge to the global health system. In the case of Ecuador, the problem seems more critical. Ecuador is a developing country and is listed in the Low-and-Medium Income countries. According to WHO and Vikesland (2019) [33], those countries might experience an aggravated crisis caused by AMR. The current antibiotic resistance crisis has led to the search for viable solutions to overcome AMR and treat infections caused by those. Unhappily in Ecuador, there is no current development of any alternative treatment, other than plans for controlling antibiotic consumption to delay the spread of resistant strains. Specifically, there are very little data regarding the potential use of bacteriophages as alternative "antibiotics."

2.2. Objectives

2.2.1. General Objective

To introduce the possibility and urgent need to focus on new alternatives to overcome the current antibiotic crisis.

2.2.2. Specific Objective

- To review bibliographical sources to develop a better understanding of bacteriophages.
- To analyze the potential advantages and constraints of phage therapy.
- To raise awareness of the urgent need to find alternatives or complementary therapiesto deal with resistant-bacteria.

3. The Antibiotic Resistance Problem

3.1. Antibiotic Resistance

Resistance to antibiotics is ancient; it was recorded even before the first clinical use of penicillin in the early 1940s [34]. Antibiotic Resistance is a natural evolutionary process; it results from bacteria's interaction with their environment. The development of antibiotic resistance cannot be stopped; however, it has been accelerated due to the intense selective pressure that bacterium have been exposed to [34]. Antibiotics themselves act as the source of the evolutionary pressure that accelerates the development of bacterial resistance. Resistance genes are located on mobile genetic elements such as plasmids, carrying one or more resistance genes. [34] Once resistance genes are successfully integrated in gene-transmission elements, antibiotics resistance can persist and spread in the absence of antibiotics. [35] Due to the transmission of resistance genes, it is possible to find bacteria resistant to multiple antibiotics. Those are known as multi-drug resistant bacteria (MDR). Antibiotic Resistance can be acquired by different mechanisms (Table 3).

Table 3. Description of antibiotic mechanisms developed by bacteria, adapted from data obtained in Wright 2010.

Mechanisms for antibiotic resistance	Effect
1._ Target modification	Mutation of the target itself
2._ Efflux	Large family of proteins that eject antibiotics from inside the cell.
3._ Immunity	Antibiotics or targets are bound by proteins that prevent the antibiotics binding site.
4._ Enzyme-catalyzed destruction	The most specific and evolved mechanism, enzymes can recognize antibiotics and modify them in such a way as to eliminate the functional characteristics.

World Health Organization (WHO) defines antibiotic resistance as one of the biggest threats to global health, food security, and development [36]. WHO has estimated approximately 50 million deaths due to bacterial infections by the year 2050. [37]. Besides the difficulty of treating infectious diseases, the WHO also concerns about other pathologies in which antibiotic prophylaxis is required to avoid associated infections. [35]. In the WHO Model List of Essential Medicines [38], antibiotics were classified according to the urgency of bacterial resistance against them. The categorization is based on three categories: Access, Watch, and Reserve. In the case an antibiotic is listed under access, it is potentially safer to use and is available as a treatment for a wide range of common infections. Antibiotics listed in the Watch group are recommended as the first or second choice for a small number of infections. Finally, antibiotics listed in the Reserve group should be used only when first-choice antibiotics were used without success (Table 4)

Table 4. List of Priority antibiotics listed in the Essential Medicines of the WHO

Priority Group	Antibiotics
Access	• Amoxicillin • Amoxicillin + clavulanic acid • Ampicillin • Benzathine benzylpenicillin • Cefalexin • Metronidazole • Clindamycin • Cefazolin • Sulfamethoxazole+trimethoprim • Gentamicin
Watch	• Quinolones/ Fluoroquinolones • 3rd gen. Cephalosporins • Macrolides • Glycopeptides • Antipseudomonal

	• Penicillin + β -lactamase inhibitor • Carbapenems • Penems
Reserve	• Aztreonam • Fosfomycin • 4th gen. Cephalosporins • Oxazolidinones • 5th gen. Cephalosporins • Tigecycline • Polymyxins • Daptomycin

3.2. The gap between antibiotic development and bacteria and therapeutical implications

The introduction of new antibiotics into therapeutics has been accompanied by the rapid appearance of resistant strains in most parts of the world [23]. The development of new antibiotics has experienced a paucity over the last years. [34]. WHO Report on Surveillance of Antibiotic Consumption states that since the 1980s, only a few new antibiotics classes have been placed into the market, and their target mostly Gram-positive bacteria. [23]. The prevalence and transmission of resistance genes have reached a critical level, and the new developed antibiotics cannot keep pace with microbial evolution. [34]. Another reason for slow antibiotic development is related to the increased expenses that new drug development represents. Failures between phase 2 and submission cause the major increases due to toxicity and Efficacy. [37]. Pharmaceutical companies do not find economic interest in developing new antibiotics; due to the short time window of use before resistance arises and the expectation of affordable pricing. The international consortium DRIVE-AB estimated the cost of developing a new antibiotic at $2.7 billion. [39].

It is necessary to establish a difference between in vivo and in vitro bacterial resistance. The in vivo and clinical response is not well defined given the many factors that might lead to a therapeutic failure. Pharmacodynamics and pharmacokinetics factors could cause an undesired response to antibiotic treatment. Besides, research shows a discrepancy between the in vitro and in vivo activity of resistant bacteria. [40].

Infections caused by multidrug-resistant (MDR) organisms present increased mortality compared to infections caused by susceptible bacteria. Treatments for several bacterial infections, including urinary tract, tuberculosis, sepsis, gonorrhea, and foodborne diseases, have become less effective [23]. To sum up, infections caused by resistant strains are associated with higher mortality, longer hospital stances, morbidity, and higher expenses if compared to infectionscaused by susceptible strains [40].

3.3. Antibiotic pollution

As stated before, antibiotic resistance is a natural process; however, this process has been accelerated by using antibiotics in humans and animals. Antibiotic treatment without medical prescription or surveillance has worsened the emergence and spread of resistance. The considerable misuse of antibiotics has diminished their effectiveness and released many antibiotics in the environment. High concentrations of antibiotics can be found in water and soils, and it is usually related to areas of human activity. Even though antibiotics can be degraded by several processes, such as photodegradation, chemical degradation and biodegradation, there are environments where antibiotic waste is continually being released. Settings like hospitals, houses and farms are expected to present high levels of bacteria that might carry antibiotic resistance determinants [35].

Another factor that contributes to bacterial resistance is globalization. Human and animal transit has allowed AMR organisms to move between different ecosystems and facilitate the spread antibiotic resistance genes. As a result, it is possible to find resistant bacteria in places where antibiotic pollution has not been an issue. According to Martinez (2009) [35], finding resistance genes in a particular environment does not directly imply antibiotic pollution. To determine antibiotics are a pollutant is necessary to analyze the average levels of resistance genes prevalence in a singular environment. In case an antibiotic pollutant is present, those values are expected to increase.

3.4. Economic burden effect of AMR

The World Economic Forum identified antibiotic resistance as a global risk that cannot be managed or mitigated by any organization or nation alone [41]. The current rise of bacterial resistance affects the ability to treat bacterial infection-related diseases and has economic effects. According to the Global Action Plan issued by the WHO[41], AMR is a drain on the global economy. The presence of resistant bacteria leads to an increase in healthcare costs of patients with resistant infections.

Compared to non-resistant infections, resistant ones are more expensive because of the longer duration of illness, additional tests, and more expensive medicines [23]. As reported by Weinbauer (2004) [9], the cost of treating a patient from an antibiotic-resistant infection can be found in the range of $18,588 to USD 29 069. In countries like Ecuador, where health is recognized as a free human right and the government affords therapy, it might represent a considerable increase in the health system budget.

3.5. Antibiotic Resistance in Ecuador

With antibiotic resistance being a global health problem, finding antibiotic-resistant bacteria in Ecuador is not surprising. In the Report on Surveillance of Antibiotic Consumption, WHO states that there is evidence to suggest the association of antimicrobial use and the emergence of resistance. The global consumption of antibiotics has increased over the last two decades. AMR resistance rates in low and middle-income countries (LMICs) are generally higher than in high-income countries (HICs) [33]. Vikesland (2019)[33] suggests that several factors, such as: environmental, social, and economical, can impact dissemination and magnitude of AMR dissemination. These factors combined with inappropriate use patterns (e.g., Antibiotics used for treating not-bacterial conditions, wrong dosage or administration, the wrong type of antibiotic) are significant drivers of bacterial resistance spread. [23].

The Pan American Health Organization (2016) [42] has reported an increase in the resistance prevalence percentage in Latin American countries, including Ecuador. The report shows data from Latin American centers for antibiotic Resistance control in each country from the years 2014, 2015, and 2016. In Ecuador's case, the report was submitted by the Instituto Nacional de Investigación en Salud Pública (INSPI). The report presented data on:

- *Klebsiella pneumoniae:* Seven countries exhibited an increase in the resistance percentages. Ecuador reported an increasing trend in non-susceptible strains. The data showed an increase from 20% to 24% of resistant strains from a total of 1382 tested isolates.

- *Escherichia coli*: The average nonsusceptibility ranges from 21% to 60%. Ecuador data showed a small decrease from 42% to 38% from a total of 3840 tested isolates.
- *Acinetobacter baumannii*: The report showed mixed data, with some countries increasing resistance trends while others decreased. The data from Ecuador showed an average 59% nonsusceptibility from a total of 212 analyzed isolates.
- *Staphylococcus aureus*: Three countries showed a significant increase, Ecuador being one of those and increasing 5% to reach a 48% resistance.
- *Pseudomonas aeruginosa:* From the data, it was observed that the resistance values were maintained at 33% average non-susceptible strains from 825 isolates.

A report from 2018 on Antimicrobial Resistance issued by the Ecuadorian Health Ministry [43] showed resistant strains in several hospitals that are part of the Public Health System. The report shows that resistant strains of *E. coli* are the most predominant, exhibiting resistance in more than 50% of the tested samples. The other major bacterial resistant strains are *Klebsiella pneumoniae* (20%), *Staphylococcus aureus* (12%), and *Pseudomonas aeruginosa* (10%). Some of these bacteria are listed in the global priority list of antibiotic-resistant bacteria released by the WHO [44](See Table 5).

Table 5. The global priority list of antibiotic-resistant bacteria. Extracted from WHO

Priority	Bacteria	Antibiotic
Critical	*Acinetobacter baumannii*	Carbapenem
	Pseudomonas aeruginosa	Carbapenem
	Enterobacteriaceae	Carbapenem, 3rd generation cephalosporin
High	*Enterococcus faecium*	Vancomycin
	Staphylococcus aureus	Methicillin, vancomycin-intermediate and resistant
	Helicobacter pylori	Clarithromycin
	Campylobacter	Fluoroquinolone
	Salmonella	Fluoroquinolone
	Neisseria gonorrhoeae	3rd generation cephalosporin, fluoroquinolone
Medium	*Streptococcus pneumoniae*	Penicillin-non susceptible
	Haemophilus influenzae	Ampicillin resistant
	Shigella	Fluoroquinolone

4. Bacteriophages: The "natural antibiotics."

There is a resurgent interest in the use of bacteriophage therapy, mostly caused by the current challenge of multi-drug resistant bacteria and its effect on human health. Historically, especially before the "antibiotics boom," phage therapy was used to control and treat human infections [45]. Bacteriophages being able to kill bacteria, combined with several properties that will be further discussed in the chapter that follows, presented them as an alternative or supplement to antimicrobial therapy [5]. There is a steady increase in the number of research in Europe and the US, which has led to the development of several phage's applications, especially for healthcare, veterinary, and agriculture [28]. Bacteriophages have also been used as biological control agents; their use has been described in several different fields, such as food safety, industry, and clinical diagnostics. Bacteriophage therapy presents a novel, non-antibiotic approach to treat bacterial pathogens [3].

4.1. Bacteriophages in Ecuador:

To obtain data about bacteriophage research in Ecuador, it was conducted a bibliographic search. As a result, three scientific articles were found that contained bacteriophages as their primary study focus. From the three, only one was relevant to phage clinical application. The article titled: "*Bacteriófagos como alternativa para eliminar cepas de Acinetobacter baumannii resistente a antibióticos presentes en tres hospitales del Ecuador*" [46] presented the process for isolating and testing bacteriophages capable of infecting *Acinetobacter baumannii*. The other studies [47,48] aimed at using bacteriophages in agriculture, especially in bacterial biocontrol.

4.2. Phage therapy

4.2.1. Phage therapy mechanisms

4.2.1.1. Phage choice and isolation

Phage choice and isolation are critical processes for any successful phage therapy development. For the choice of phages, it is crucial to take into account if the disease or condition to be treated is caused by a single bacterial strain or by multiple. That is why phage choice can be applied following two different methods: i) phage cocktails and ii) on-demand isolated phage. The first one involves the use of a cocktail containing multiple phages. If phage choice is a phage cocktail, it may target a more comprehensive array of bacteria and virtually prevent any resistance from developing in the short term. This approach seems suitable for commercial production in the western world [49].

For the second one, it is necessary to isolate the pathogenic bacteria and test it against previously isolated phages [49]. Bacteriophages for therapeutic purposes can be isolated from any environmental source in which the target bacteria are likely to be contained. For clinically significant pathogens in the hospital setting, wastewater and sewage connected to the hospital environment are suggested as the primary source of phage isolation [6]. Phage isolation can be performed relatively quickly; however, it varies depending on the bacteria host. It usually is more challenging to isolate lytic phages for some hosts than others [6]. The process of isolation is carried out through a process known as enrichment [49,51]. Phage enrichment consists of removing endogenous bacteria from a sample and adding it to bacterial culture media and a growing culture of your host and incubating it. This allows that even if a single phage capable of infecting the target bacteria is present in the sample, it will replicate [52]. Enrichment presents two useful functions for phage therapy: 1) Isolation is biased and prioritizes those phages with a greater antibacterial virulence, meaning that they are able to propagate by targeting the bacteria under specific conditions within which enrichment takes place. 2) Phage isolation is biased towards phages that are freely propagated in the laboratory.

After the process of isolation, it is necessary to submit the phages isolates to purification processes. Purification can be done only as a clarification of lysed cultures via either centrifugation or filtration or more rigorous purification involving ultracentrifugation, a series of filtration, or several forms chromatography [49].

4.2.1.2. Host range and susceptibility testing

The determination of the range of bacteria targeted is the most critical approach towards phage characterization for phage therapy. It is desired that phage can infect the targeting bacteria and display reasonable specificity [51]. After isolation and purification, a phage has to be characterized and genetically sequenced to be available for therapeutic use [6]. Host range characterization has vital importance in developing efficient phage cocktails, given that multiples phages form those and that should present synergistic characteristics, especially in terms of host range [51]. However, it should be considered that the host range is not a fixed property and might evolve. It could be used to favor the treatment, given that modifying the host range of a therapeutic phage might have a desirable impact on its efficacy [6].

Gill (2010) [53]claims that a term usually associated with the phage host range is virulence. Virulence is defined as the potential of a phage strain to drive a particular bacterial culture to extinction. Virulence is an important concept to consider in the clinical application of phages. Phage must present a minimum level of virulence. A sufficient virulent phage might ensure treatment success before being removed by the immune system [53].

As reported by Cui (2019) [54], phage-susceptible bacterial testing is a precondition for the success of therapy. The most favorable standard method is the two-layer agar plate, considered a simple and straightforward method.

4.3. Potential advantages of phages over antibiotics

4.3.1. Specificity and host range

There is clear evidence proving that not all bacteria are infected by all phages [55]. However, it is estimated that approximately ten bacteriophages are capable of infecting every bacterial cell [56]. Bacteriophages are specific to their hosts, and indeed phages can only infect a subset of bacterial species [55]. The phage's specificity is driven by phages' ability to recognize and attach to binding sites in the bacterial cell [57].

Proteins or sugar moieties are usually the receptors in binding sites, and in case a bacterium does not have any receptor for a specific bacteriophage, there is no interaction between those two [56]. Bacteriophage specificity reduces significantly the chances of a secondary infection caused by the phage; given that phages only interact with bacterial cells and not with human cells [3,58]. Due to their host specificity, phages might only minimally impact health-protecting normal microbiota [59]. Tough the phage specificity is beneficial for treating monomicrobial diseases; it may become a significant limitation in polymicrobial infections. For these cases, it is suggested to administer a phage cocktail or phage combined with a suitable antibiotic [60]. Those phages that are highly specific for a single host are known as monophagous, while phages with a broader host range are called polyphages [56]. See **Table 6** for details of potential advantages and limitations related to specificity.

Table 6. Comparison of potential pros and cons of phage specificity

Bacteriophage specificity	
Potential advantages	**Potential limitations**
Due to the host cell specificity, there are not reports indicating phage interacting with different microorganisms rather than the specific target.	It was not covering the diversity of pathogenic bacterial strains that may be encountered in the clinical environment.
Bacteriophages' specificity is what differentiates antibiotic treatment from phage therapy.	In the case of infection caused by intracellular bacteria, it could become inaccessible for phage therapy.
In the case of phage-resistant bacteria, it is possible to use genome engineering to make up for some disadvantages.	Phages are selecting for resistant bacteria that may retain their virulence and evade phage infection.
Bacteriophages are capable of co-evolution. Meaning that as bacteria develops phage-resistance, phages might also evolve to avoid bacteria resistance.	Companies might not be willing to risk their inversion in phage therapy due to a narrow range of pathogenic bacterial species to be targeted by a single phage.

4.3.2. Safety

The main advantage regarding phage safety in comparison to antibiotics is phage specificity. Phages used during clinical treatment are likely to only infect and destroy bacterial cells. The assurance of phages safety is still under development. Studies regarding phage therapy in the former Soviet Union and Eastern Europe showed phages were applied through several administration routes, including: orally, rectally, topically, and intravenously. Reporting no serious complications associated with phage use, and there is evidence to support that phages appear to be innocuous from a clinical standpoint [61]. However, those studies do not meet current rigorous standards for clinical trials, therefore it is necessary to conduct new studies that follow modern regulations.

Humans have developed tolerance towards bacteriophages due to constant exposition to large numbers of phages present in their environment, as well as phages routinely consumed with our food [62]. According to Furfaro (2018), the exposure to bacteriophages is not evidence of their safety per se, as it differs from the clinical context. Skurnik (2007) and Furfaro (2018) suggest the need of processes such as purification, and phage preparation prior the clinical application.

Therefore, it would be essential to carry out more clinical trials regarding phage therapy in order to ensure the safety. Meaning that it is necessary to test that phages do not possess gene sequences that carry antibiotic resistance genes, phage encoded toxins, or genes for other bacterial virulence factors [61].

4.3.3. Abundance, low dosage, and self-replication

As stated before, bacteriophages are the most abundant viruses on the planet. Besides, phages are distributed to any ecosystem where a bacterial host can be found. Tough, given that bacteriophages act as obligate parasites, their distribution and abundance are controlled by their hosts. There is evidence that phage capable of infecting bacteria exists in a ratio of 10:1 compared to bacteria, and there is plenty of phage options to be selected for phage therapy [56]. A remarkable advantage of phages is related to their capacity to increase their density at the infection site. This could mean that therapy costs could be reduced, provided that lower doses (either in number or amount) are needed to achieve an effective treatment.

Besides, the application of phages in low doses may improve their safety, provided that the required number of phages might not raise any essential immune reaction. Even there is a potential for bacteriophage therapy to be applied in a single dose; however, it needs to be further studied because currently, a single dose is expected to not be enough to achieve therapeutic efficacy [59].

4.3.4. Avoidance of antibiotics pollution

Bacteriophages are natural components of the environment and can be found in any environment where host bacteria exist [7,63]. In the case of therapeutic use and later discard of phages, due to their narrow host cells, it is expected to have no impact in the environmental bacteria given that phages only interact with host bacteria [59]. Meanwhile, large amounts of antibiotics have been released in the environment due to non-essential uses: animal or plant diseases increased animal food growth rate, or inefficient use for human diseases. Consequently, the environmental bacterial population is exposed to antibiotics and might develop resistance mechanisms, leading to a decrease in susceptible microbiota population and the enrichment of resistant microorganisms [35,59].

4.3.5. Overcome MDR

The current existence of multi-drug-resistant bacteria has affected the Efficacy of antibiotic treatments. A single antibiotic is usually used to treat different bacterial infections; however, some bacterial species have developed resistance to multiple antibiotics [7]. Given that bacteriophages' infection and lysis mechanisms are different from those of antibiotics, the specific antibiotic resistance mechanisms do not translate into phage resistance mechanisms [59]. Bacteriophages are active against either Gram-positive or Gram-negative bacteria and even against MDR pathogens [3].

4.3.6. Biofilm clearance

Most bacterial species live in complex communities that consist of an extracellular matrix surrounding bacterial cells. Bacterial communities found in biofilms tend to be more resistant to antibiotics. Additionally, the extracellular matrix acts as a physical barrier that potentiate bacterial resistance, limiting antibiotics' access to the target bacteria's interaction [10,21,64]. The use of bacteriophages to combat bacterial biofilms has enormous potential for phage therapy.

Phages can be used to disrupt biofilms found on intracorporeal medical devices like catheters, which tend to be important sites for bacterial colonization and biofilm formation. Besides, in case of an infection caused by AMR, phages have been studied as a viable option to be applied either by themselves or in combination with antibiotics like vancomycin.

In contrast to antibiotics, phages have an impressive ability to break down biofilms [65]. Phages have different mechanisms to disrupt bacterial biofilms. One of the mechanisms is done through bacterial killing, meaning that phages can lyse bacteria at the outer layer of the film and keep going to penetrate their way into the biofilm actively. Another mechanism displayed by phages is their ability to degrade the extracellular matrix's polymeric materials that protect the bacteria inside the biofilm. This activity is mediated by depolymerizes capable of disassembling the surface polysaccharides of bacteria [51,59,64].

4.4. Potential constraints

4.4.1. Development of phage resistance

Bacteria can develop phage resistance; some of the phage-resistant bacteria's mechanisms are blockage of phage adsorption, inhibition of phage's genome injection in the host, restriction-modification systems, and abortive infection systems [66]. Due to the relatively narrow host range of most phages, the number of bacterial types selected with specific phage-resistance mechanisms is limited [59]. Phage-resistance is not as problematic as antibiotic resistance; however, it is still one of the major limitations of phage therapy. It is possible to observe the emergence of phage-resistance bacteria during therapy, which might cause the whole treatment to fail. Bacteriophage resistance, as well as antibiotic resistance, is inevitable. [54,65].

Table 7. Description of mechanisms developed by bacteria against bacteriophages

Resistance mechanisms	
Blockage of phage adsorption	Loss of specific cell surface receptors mediated via mutation. There is a structural modification and, or masking of the receptor that might further prevent phage adsorption. [3]
Inhibiting the injection of phage genomes	Through transcription and transduction methods, phage DNA is prevented from entering the host.
Restriction-modification systems	Prevention of phage DNA integration. [3]
Abortive infection system	Resulting in blocking of phage replication, transcription, translation, or virions assembly. The abortive infection process is a programmed cell death to prevent the spread to surrounding bacteria [3,67]

To counter phage-resistant bacteria, some techniques and strategies have been proposed: phage therapy combined with another antimicrobial, cycling through different phage mixtures, and phage engineering. To sum up, phage resistance is not a sustainable problem due to the several mechanisms through which it can be overcame. Moreover, new phages can be selected from natural samples and used to treat phage-resistant bacteria [68]. Phage-resistant bacterial mutants may be susceptible to infection by phages with a similar target range. Moreover, selecting these new phages can be performed "easily and quickly" within days or weeks instead of long-time development of new antibiotics. [63].

4.4.2. Immunological response

The possibility of giving rise to immunological response may lead to the removal of phages by the patient's immune system. It might become a significant hurdle for phage therapy; challenging immune responses do not compromise phage safety; they may result in a drastic reduction of treatment efficacy [27,56]. Immunological reactions triggered by phages depend on the site of infection and administration route [69]. Studies about the immunogenicity of phages have evidenced that phages being removed by immune response are higher when phage administration is systemic. This has conditioned research, resulting in several studies focusing only on treating non-systemic diseases [27,66]. In most studies, phages are administered topically or orally, which have not significantly affected [69].

In contrast, systemic administration presents many extra barriers that limit extensive use [27]. For this reason, in the case of systemic administration, it is suggested to analyze the phage's potential to cause any immunological response before application. Furthermore, it is proposed to carry a process of high purification of the phage preparation to ensure that toxic or allergenic substances (such as endotoxins) are not delivered to the patient's bloodstream [59,69]. Immunogenicity of phages is not limited only to the route of administration, because there is a possibility that phages' lytic activity might result in the release of bacterial toxins and possible allergens. Leading to the development of an inflammatory cascade on the host [3,49,69].

4.5. Phages vs. MDR bacteria

The use of bacteriophages has been limited to being used as biological tools. However, there is an increased interest in the use of phage as therapeutic antimicrobials. One of the main advantages that bacteriophage promises are treating bacteria that have shown resistance to antibiotics. The analysis is focused on the finding of supportive evidence of phages used to combat infective diseases. [43].

Pseudomonas aeruginosa (*P. aeruginosa*) has been widely studied. It is an opportunistic pathogen, usually related to nosocomial infections, cystic fibrosis, burn infections, and urinary tract infections. *P. aeruginosa* has developed antibiotic resistance and an enhanced ability to produce high biofilm levels. The antibiotic resistance added to the biofilm formation makes the antibiotic treatment of *P. aeruginosa*-related diseases more challenging. Bacteriophages were used to treat P. aeruginosa infections more than 50 years ago.[67,70]Evidence of "successful" studies that used phages to combat *P. aeruginosa* infections was conducted in eastern Europe. Sulakvelidze (2001) [61] has listed some of those studies to treat infections, such as: suppurative skin, postoperative wound, and gastrointestinal.

Escherichia coli is a Gram-negative enterobacterium and is frequently found in the human digestive tract. Capable of causing intestinal infections, extraintestinal infections, and nosocomial infections [42]. The use of therapeutic phages against *E. coli* has been one of the most studied bacteria, especially after discovering T-phages and their potential applications. As reported by Hobbs (2016), phage products against *E. coli* were the first to be commercialized just a few years later to phage discovery in 1931. Several studies to test *E. coli* to treat bacterial infection were conducted in Poland and the former Soviet Union. Some of the infections listed by Sulakvelidze (2001), such as: intestinal dysbacteriosis, inflammatory urologic disease, bacterial dysentery, and recurrent

subphrenic abscess; were analyzed in clinical studies for human therapy application. However, those studies' regulations are considered insufficient to demonstrate the efficacy and safety of phage therapy.

Klebsiella pneumoniae has become one of the most challenging bacteria to combat. According to Tagliaferri (2019), it is based on the increase of resistant strains and the ability to form biofilms. *K. pneumoniae* is responsible for infections such as pneumonia, urinary tract, bacteremia, and sepsis. Its ability to survive on inert surfaces for long periods might cause the spread of resistant strains in hospital settings [42]. Human therapy studies were applied to treat several infections caused by *K. penumoniae*. As reported by Sulakvelidze (2001) [61], some of the conditions treated with phages were: suppurative skin, head, and neck infections.

4.6. Phages in the present

4.6.1. Recent human trials

Due to the long period in which bacteriophage as human therapy was omitted in the western world, one of the challenges for bacteriophage therapy to enter into the clinic is the lack of validated and adequately controlled clinical trials [60]. Although there is good experience from studies conducted in eastern Europe and some recent studies due to renewed interest, there is a shortage of adequately controlled, double-blind clinical trials [6].

To obtain a view of the current state of clinic related to bacteriophages, data were collected from https://clinicaltrials.gov/ [71] and https://globalclinicaltrialdata.com/[72]

. As a result of searching the term "bacteriophage therapy," the web sites displayed 18 studies (Table 8). From the obtained results, eight of the studies are aimed to treat or prevent bacterial infections. Six are dedicated to the study of phage relationship with a singular bacterium and safety evaluation. There are two that are related to cancer treatment. In the remaining two, phages are not an essential part of the primary treatment, are only employed in supplementary activities. The trials are mostly under phase I or phase II of clinical essays. Only three studies have at least completed one phase and are pending to proceed with the next phase or the publication of results.

Table 8. Selection of the most relevant clinical trials

Reference	Condition	Pathogen	Phase	Summary
NCT04287478[73]	Urinary Tract Infection Bacterial	*E. coli and K. pneumoniae*	Phase I, Phase II	Evaluation of safety and Efficacy of bacteriophage in patients with urinary tract infection. Two administration routes: intravenous and intravesical.
NCT02664740[74]	Diabetic Foot Staphylococcal Infections	*S. aureus*	Phase I, Phase II	Application of a topical anti-staphylococcal phage cocktail to analyze its efficiency and safety. It was used to treat MRSA. The evaluation would be based on the ability to reduce wound surface area.
NCT02116010[75]	Wound Infection in Burns	• *E. coli* • *P. aeruginosa*	*Phase I, Phase II*	A double-blind, randomized, and controlled trial to assess tolerance and Efficacy of local bacteriophage treatment of wound infections in burned patients.
NCT04323475[76]	Wound Infection	• *E. coli* • *P. aeruginosa* • *K. pneumoniae*	Phase I	A randomized, open-label, controlled trial evaluates the safety and tolerability of phage cocktail SPK as therapy for second-degree burn wound in adult patients.
NCT01818206[77]	Cystic Fibrosis	*Pseudomonas aeruginosa*	Completed	The study aims to evaluate the Efficacy of bacteriophages to infect *P. aeruginosa* strains. The treatment is the application of a 10-bacteriophage cocktail applied to sputum samples
NCT03140085[78]	Urinary Tract Infection Bacterial	• *Enterococcus spp* • *E. coli* • *Proteus mirabilis* • *Staphylococcus spp* • *Pseudomonas aeruginosa*	Completed	Aimed to Investigate the Efficacy of intravesical bacteriophage treatment. There is an application of a controlled drug(antibiotic), a placebo, and the test drug (bacteriophages).
NCT04596319[79]	Cystic Fibrosis, Lung infection	*Pseudomonas aeruginosa*	Phase I, Phase II	To evaluate the safety, tolerability, and phage recovery of AP-PA02 multi-bacteriophage therapeutic administered by inhalation.
NCT00663091[80]	Venous leg ulcers	*Pseudomonas aeruginosa, S. aureus, E. coli*	Phase I	The determination of safety of WPP-201 a pH neutral, polyvalent phage preparation containing 8 bacteriophage components.
NCT02828774[81]	Chronic Lymphocytic Leukemia (CLL)	Not applicable	Observational, in vitro, and animal models	Isolation of phage internalized by B-CLL cells from recently diagnosed and untreated patients. Therapy based on the utilization of phage display technique
NCT04191148[82]	Urinary Tract Infection Bacterial	*E. coli*	Phase I	Randomized, double-blinded study to evaluate the pharmacodynamics of the phage cocktail LBP-EC01.
NCT00089180[83]	Nonmelanoma skin cancer	Not applicable	Completed	Randomized trial to determine T4N5 Efficacy in preventing recurrence of nonmelanoma skin cancer in kidney transplant patients.

From the completed clinical trials, two articles were found. The article with the published results for phage trial NCT02116010[75], shows the methods and result obtained in the study of Efficacy and tolerability of phage cocktail to treat burn wounds infected by *P. aeruginosa*. The study was conducted in 25 patients that were aged 18 years old or older and had a burn infected by *P. aeruginosa*. The study consisted of testing PP1131 phage cocktail versus standard treatment. Patients were divided into two groups; one was administered PP1131 (n=12), and standard care (n=13). The study was stopped due to insufficient Efficacy of PP1131. The time to obtain a sustained reduction in bacterial burden Patients treated with PP1131 phage took longer (144h) than standard antibiotic treatment (48h). The authors concluded that PP1131 was slower to reduce the bacterial burden and that further studies are required. [75]

The clinical trial article NCT0314008574 presents the result obtained from the study of treating patients with urinary tract infection (UTI) during transurethral resection of the prostate with Pyo bacteriophage. The study was conducted on 474 male older than 18 years of age. Patients were divided into three groups; one group was administered Pyo phages, the second was given a placebo, and the other was administered antibiotics. The results data showed that bacteriophage therapy was not inferior to standard antibiotic treatment. According to the authors, despite bacteriophage therapy not being a recognized or approved treatment, their study provides a new insight to optimize the design of future large-scale clinical studies. [78]

The results obtained from those studies depict the current state of phages clinical trial. Bacteriophage therapy is still under development and requires further research to get a better perspective. [60]

4.6.2. Phages in COVID-19 treatment

The current pandemic coronavirus disease, caused by the SARS-CoV-2 virus, constitutes a significant health crisis. Worldwide has affected millions of people and caused over 500,000 deaths. The lack of a proven effective therapy and the possibility of secondary infections might constitute risk factors that would increase the severity and mortality rates of COVID-19. The immune system of patients infected with SARS-CoV-2 can be weakened by the viral infection, which might predispose the patients to develop viral-bacterial co-infection [84,85].

Several clinical and epidemiological studies suggest that secondary infections and bacterial co-infection are critical factors that might increase the severity and mortality rates of COVID-19. The application of antibiotics is the usual practice for COVID-19 patients, with over 70% of hospitalized patients getting antibiotics as part of their treatment. However, there is the possibility of facing infections caused by antibiotic-resistant bacteria. In this context, Wojewodzic (2020) suggests that bacteriophages could play an essential role in preventing secondary infections and decreasing patients' mortality rates.

The application of phage therapy might result in the prevention of bacterial growth and prevent a major inflammatory response of the patient. The initial viral infection causes the trigger of the innate immune response with SARS-CoV-2. The role of bacteriophages might be relevant in the reduction of bacteria found in the lung of patients. In case that bacterial infection is not treated, it might provoke an inflammatory process and the accumulation of inflammatory material (fluid and cells)[84]. Since COVID-19 requires the adaptive immune response, which takes longer than the innate response, the bactericidal effect of phages might help the patient reach that point without severe complications.

Controlling bacterial growth is vital in treating patients; however, the viral infection causes an inflammatory response. The adaptive response to COVID-19 takes much longer than the innate response because it is a new pathogen. To scale down the inflammatory response is necessary to find an alternative to decrease viral load. Another approach proposed by Wojewodzic (2020) is using phage display technology to produce antibodies to target SARS-CoV-2. There is evidence obtained from treating MERS-CoV with phage-display engineered phages. Several studies show the isolation and development of antibodies with high neutralizing activity against MERS-CoV[86]. The proposal is to engineer phages capable of blocking ACE2 (functional receptor of SARS-CoV-2) to prevent further expansion of the virus and reduce viral load.[84]

5. Discussion

The use of bacteriophages has been documented for almost a century, and despite those studies not being carried under current regulations, they are a convenient source of information for future research. There is an undoubtedly need for further studies before bacteriophages being widely accepted as safe therapeutics. However, the current data present promising results that might encourage continuing the efforts and promoting Phage Research development. Devoting resources to phage investigation may be beneficial in terms of developing an alternative treatment. Besides, the development of a safe phage

therapy might not demand as many resources or time as those required to establish new antibiotics.

Although phage therapy is getting attention, it remains practically unnoticed in the western medical community [87]. This same medical community is now facing the challenging incapacity of treating patients that have been infected by AMR. Doctors are in the urgent search for an alternative to treat their patients and, in many cases, to save their lives [88]. There is an opportunity to generate an openness of the western medical community towards the use of phage therapy as a possible valuable tool to combat AMR by publishing clinical trials of Phage therapy in major medical journals. Another essential member, if not the most important, is the patient. There have been cases in which patients have heard about bacteriophages and decided to travel to eastern Europe countries for treatment, leading to an increment in medical tourism. Many countries have approved regulations to allow the doctor to apply experimental treatments. To prevent patients from being forced to travel abroad seeking treatment or applying bacteriophages only in exceptional cases, it is advised to develop regulation policies. Some phage products were listed as natural additive to foods and pointed as safe by the FDA. That approval is insufficient for phages to be used as therapy. Therefore, it is vital that regulatory agencies issue policies, based on clinical documented results, to regulate phage therapy application in humans.

The pharmaceutical industry has not picked the phage therapy approach, and phage applications have been relegated to agriculture, food safety, and biocontrol. Surprisingly, a therapy promoted as the solution for antibiotic resistance is not attractive to pharma. At this point, it is valid to analyze a viable approach by which pharmaceuticals might be attracted to invest in phage therapy. First, it would be essential to maintain a phage biology investigation, focusing on their interaction with the bacterial host in natural habitats. It might result in phage therapy (PT) researchers learning how to achieve adequate bioavailability and *in vivo* phage lysis [88]. Another approach is to increase the number of clinical trials that are currently conducted. With an increase in the experimental data obtained from carefully documented controlled clinical trials, it would be easier to demonstrate PT efficacy fully. And finally, despite phages being intended to combat the most urgent antibiotic resistant pathogens, this approach might not be the most advantageous.

Given that the currently number of patients infected with resistant-bacteria is still relatively small. Concentrating phage therapy solely in resistant bacteria could contradictory be unhelpful for phage therapy settlement in the pharmaceutical industry. The Pharma industry might not select those phages because of the small market they would represent [88]. Bacteriophages present a remarkable potential for therapeutical applications. As mentioned before, phages show several characteristics that might place them as a viable alternative to antibiotics in AMR treatment. Bacteriophage study is an almost centenary technique and has regained interest in the last decade. However, the current attraction of phage therapy is driven by the appearance of AMR. It is improbable that phage therapy would have been considered for treating infectious diseases if not because of antibiotic resistance escalation.

In comparison, the relative ease of use and a well-defined regulatory path of antibiotics is far from being matched by phage development. It is expected that phages might not fully replace antibiotics. Because of the antibiotic crisis degree, a multiple approach strategy would be needed, and phage therapy can be part of the solution [22].

6. Concluding Remarks

The current antibiotic crisis presents the urgent need for a correct approach to get around antibiotic resistance. Organizations as WHO expect that antibiotics will no longer be efficient in treating the most common and currently non-lethal infections at some point. In this context, bacteriophages appear like a feasible option to treat bacteria-related infections, especially those caused by antibiotic-resistant bacteria. Driven by the concern of AMR, there is a revitalized attraction towards bacteriophages. With the renewed interest in bacteriophages, it's expected that their reintroduction into the clinical setting would be accelerated.

There is an obvious need for further research and the realization of clinical trials to provide the required data to promote phage therapy. One of the main limitations of phage therapy was found to be the lack of supportive evidence. The collected evidence of phage clinical application, mostly from the research in eastern Europe, is insufficient to determine certainty about phage therapy safety and efficacy. It is required to conduct more clinical trials that fulfill the current regulations. The accomplishment of several clinical trials through the required clinical phases may provide the supportive data needed to promote pharmaceuticals to invest in phage therapy.

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