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Review Article

REVIEW ARTICLE

The clinical path to deliver encapsulated phages and lysins

Ana Mafalda Pinto[1,2,†], Maria Daniela Silva[1,2,†], Lorenzo M. Pastrana[2], Manuel Bañobre-López[2] and Sanna Sillankorva[2,*,‡]

[1]Centre of Biological Engineering, LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira, Campus de Gualtar, 4719-057 Braga, Portugal and [2]INL - International Iberian Nanotechnology Laboratory, Avenida Mestre José Veiga, 4715-330 Braga, Portugal

*Corresponding author: INL - International Iberian Nanotechnology Laboratory, Avenida Mestre José Veiga, Braga 4715-330, Portugal. Tel: +351253140112; E-mail: sanna.sillankorva@inl.int

One sentence summary: Applied research with free and encapsulated phages and endolysins has progressed significantly, reassuring their imminent use in clinically significant respiratory, gastrointestinal and integumentary bacterial infections.

†Authors contributed equally

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‡Sanna Sillankorva, http://orcid.org/0000-0002-8408-8550

ABSTRACT

The global emergence of multidrug-resistant pathogens is shaping the current dogma regarding the use of antibiotherapy. Many bacteria have evolved to become resistant to conventional antibiotherapy, representing a health and economic burden for those afflicted. The search for alternative and complementary therapeutic approaches has intensified and revived phage therapy. In recent decades, the exogenous use of lysins, encoded in phage genomes, has shown encouraging effectiveness. These two antimicrobial agents reduce bacterial populations; however, many barriers challenge their prompt delivery at the infection site. Encapsulation in delivery vehicles provides targeted therapy with a controlled compound delivery, surpassing chemical, physical and immunological barriers that can inactivate and eliminate them. This review explores phages and lysins' current use to resolve bacterial infections in the respiratory, digestive and integumentary systems. We also highlight the different challenges they face in each of the three systems and discuss the advances towards a more expansive use of delivery vehicles.

Keywords: bacteriophages; lysins; encapsulation; clinical pathogens; infections; delivery

INTRODUCTION

The benefits of using antibiotics usually outweigh their side effects, which can be severe. According to the Centers for Disease Control and Prevention (CDC), one in five medication-related emergency visits is caused by an adverse reaction to antibiotics (CDC 2019). The systemic administration frequently leads to ototoxicity and nephrotoxicity, low bioavailability in the infected region and accumulation in sites without infection (Swai *et al.* 2009; Ho *et al.* 2019). The two later disadvantages actively contribute to the emergence of antibiotic resistance.

The public health concerns regarding bacterial resistance to antibiotics have driven research groups to develop novel or adopt old strategies to overcome the issue giving a new opportunity to phages and their derived enzymes. Phages are bacterial viruses specific for a given species or strain, fairly rapidly isolated and selected, produced at low cost, considered safe and above all, able to kill antibiotic-resistant bacteria. The renewed interest has augmented their isolation, characterization and *in vivo* efficacy demonstration (animal models, compassionate treatment, case reports, and clinical trials in humans) (Gordillo Altamirano and Barr 2019; Kortright *et al.* 2019). Lysins

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are enzymes produced by phages during their replication cycle (Gondil, Harjai and Chhibber 2020a). While phages are naturally isolated and produced quickly, lysins have to be recombinantly produced in bacterial or yeast expression systems. Nonetheless, today's upscaling techniques grant easy access to large quantities of pure enzymes for large-scale experiments, including clinical trials.

The clinical use of both phages and lysins has seen significant advances, and their use is allowed by the World Medical Association through their Helsinki Declaration (article §37) (Pinto *et al*. 2020). Furthermore, some countries have issued temporary regulations for the use of non-conventional therapeutics in life-threatening situations and when all conventional therapeutic approaches have failed (FDA 2020).

Despite their approved use and success, both free phages and lysins face barriers to their administration. To overcome these challenges, one of the current hot-topics in phage and endolysin research is developing novel delivery systems based on nano- and microencapsulation techniques to protect, preserve, and improve their activity at the target infection sites.

This review focuses on current phage and lysin works, including their encapsulation, specifically addressing bacterial infections in three different human systems – the respiratory (RS), the digestive (DS) and the integumentary (IGS). All the potential problems that defy the successful therapeutic of encapsulated phages and lysins are thoroughly discussed herein.

PHAGES AND LYSINS

Phages are viruses that infect and replicate within bacteria. They are considered the most abundant entities globally, found wherever bacterial development is observed, and exceeding bacteria in number by tenfold (Feiner *et al*. 2015). Their discovery is attributed to Frederick Twort in 1915 (Twort 1915) and independently to Felix d'Hérelle in 1917 (D'Herelle 1917). In the early 1930s, rather promising results were obtained (Bruynoghe and Maisin 1921; Rice 1930; MacNeal and Frisbee 1936), and the model of 'phage therapy' for the destruction of pathogenic bacteria but innocuous to host cells was considered a possible 'magic bullet' in public health (D'Hérelle 1926).

One decade later, in 1929, Alexander Fleming published his findings revealing the therapeutic benefits of the first antibiotic – penicillin. However, penicillin made its breakthrough only after World War II, when some early phage therapy clinical failures, scientific controversies, and ethical concerns were reported. These facts dictated the end of phage therapy in most Western European countries. Therapeutic use of phages continued, combined with antibiotics or alone, in the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, in Poland, in the Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences, in Georgia, and the former Soviet Union (Sulakvelidze, Alavidze and Morris 2001).

Phages consist of genetic material (DNA, RNA) and proteins responsible for its structure and enzymatic activity. The DNA or RNA molecules can be single-stranded (ss) or double-stranded (ds), and a higher predominance of dsDNA phages are known. Genome sizes vary greatly, ranging from 3.5 kb (ssRNA, phage MS2, *Leviviridae* family) to 497 kb (dsDNA, *Bacillus megaterium* phage G, *Myoviridae* family). The vast phage diversity is also seen in morphologic characteristics. Phages can be tailed, polyhedral, filamentous, or pleomorphic, with some containing a lipid envelope or lipids as part of their particle wall. With the massive phage sequencing projects' advent, many taxonomic changes have occurred in the last few years. Several new phage families, sub-families, and genus have been either updated or released. The replication cycles and mechanism of lysins are illustrated in Boxes 1 and 2.

Box 1.
Phage replication cycle

Virulent phages replicate through a lytic replication cycle that causes lysis of the host, while temperate phages resort to a lysogenic cycle where the phage genome stays in a quiescent state in the hosts' genome (Fig. 1).

Both cycles start with the interaction between the phage recognition proteins and the receptors present on the cell surface [i.e., Gram-negative (lipopolysaccharides (LPS), pili, outer membrane proteins), Gram-positive (peptidoglycan (PG), teichoic and lipoteichoic acids)] (Sillankorva and Azeredo 2014). Once the tail's base is positioned correctly, an irreversible connection occurs, followed by a transfer of genetic material into the bacteria through a hole formed in the cell wall.

Following genome insertion, the replication cycle can be either lytic or lysogenic (Oliveira *et al*. 2015). In a lytic cycle, the viral genome overtakes the host's metabolic machinery to synthesize proteins and replicate it. After their production, particles are assembled and released from the cell to restart a new infection cycle. The release involves the holin-endolysin complex at the cytoplasmic membrane level, where holin accumulates innocuously until activation at an allele-specific time, forming micron-scale holes. The produced soluble endolysin can then escape from the cytoplasm to degrade the peptidoglycan (Pires *et al*. 2017).

The lysogenic cycle follows the initial lytic cycle until integrating the phage genome into the bacterial DNA. When cells multiply, the phage genome is passed to daughter cells that will contain the viral genome. Stress, treatment with mutagenic agents, or exposure to ultraviolet light can cause the genome release and consequent adjustment to the lytic replication cycle (Sillankorva and Azeredo 2014).

Box 2.
Exogenous application of lysins

Lysins are phage-encoded peptidoglycan hydrolases that degrade the bacterial cell wall. They can be involved in the phage entry into the bacterial cell, by generating a small wall through which the phage tail tube crosses the cell envelope to eject the phage genetic material, thereby acting from the outside (from without). The nomenclature used to classify this type of lysins is diverse, including virion-associated peptidoglycan hydrolases, virion-associated lysins, tail-associated muralytic enzymes, tail-associated lysins, exolysins, structural lysins, or ectolysins (São-José 2018). In contrast, endolysins are peptidoglycan hydrolases produced at the end of the phage lytic replication cycle. They generally act after the bacterial inner cell membrane pore-forming holins by attacking the peptidoglycan (PG) layer. The disruption caused by holins and endolysins results in hypotonic lysis and subsequent phage progeny release from infected bacteria (Fischetti

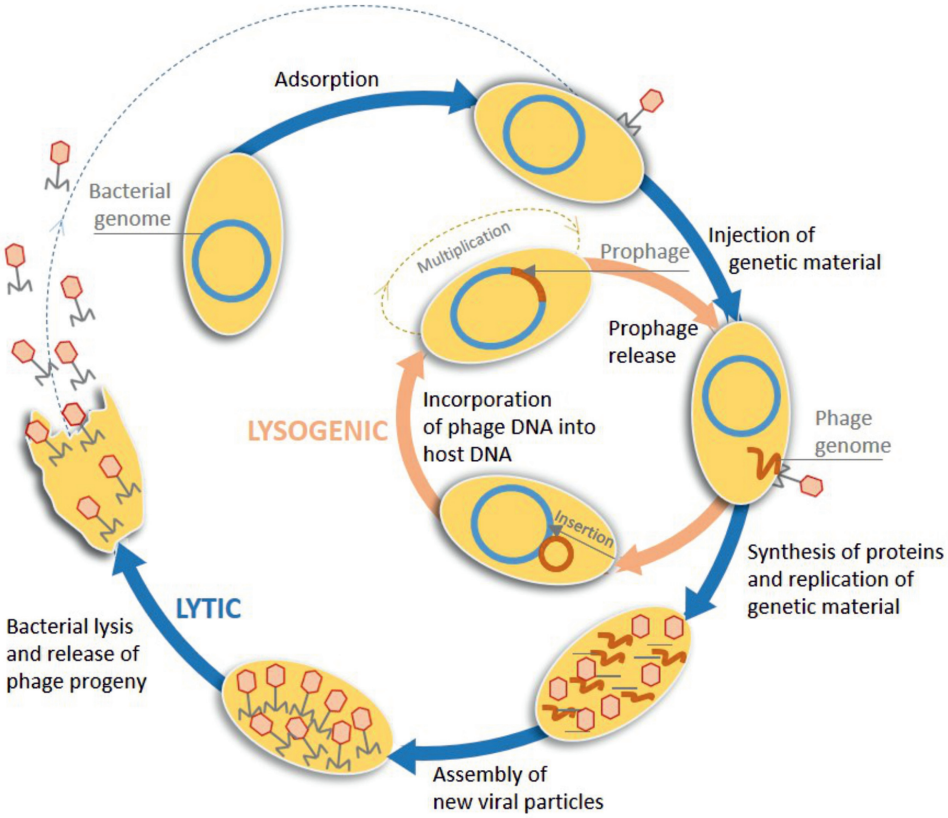


Figure 1. The lytic and lysogenic life cycles of phages. The blue arrows represent the steps to complete a lytic life cycle, and the orange arrows represent the lysogenic life cycle.

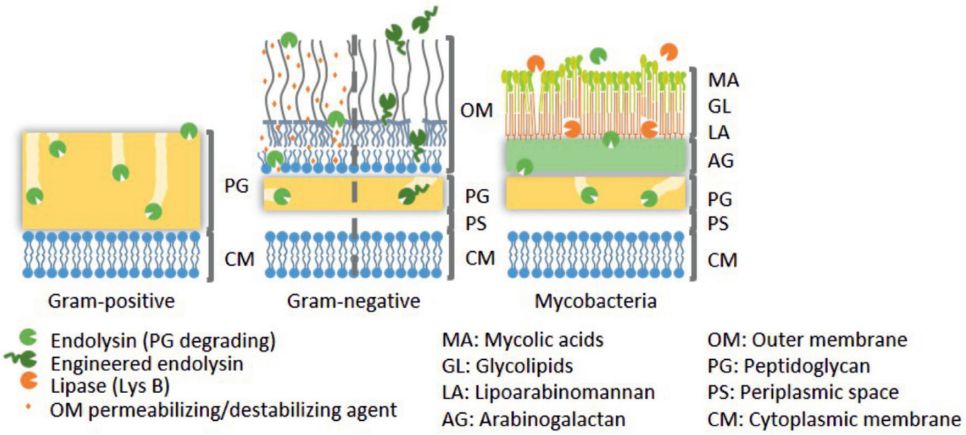


Figure 2. The action of endolysins on Gram-positive, Gram-negative bacteria, and Mycobacteria.

2008; Catalão *et al.* 2013). The ability of recombinantly produced endolysins to kill bacteria when applied exogenously was demonstrated *in vivo* already in 2001 (Nelson, Loomis and Fischetti 2001).

The exogenous application of endolysins to Gram-positive bacteria causes rapid lysis upon contact with the PG, causing cell death by "lysis from without" (Fig. 2). In Gram-negative bacteria, an outer membrane prevents endolysins' access to the PG (Fenton *et al.* 2010). Strategies that weaken the outer membrane of Gram-negative pathogens include outer membrane permeabilizers (e.g., EDTA and organic acids (Oliveira *et al.* 2014)) and recombinant fusion of endolysins with LPS-destabilizing peptides (engineered endolysins) (Briers *et al.* 2014). A few endolysins of Gram-negative pathogens have an intrinsic outer membrane permeabilizing behavior, mostly due to their positively charged regions that interact with the LPS molecules (Oliveira *et al.* 2016; Pires *et al.* 2016). The antibacterial action of these endolysins does not require additional cell permeabilizing strategies. Mycobacteria have a structurally different cell wall compared to both Gram-positive and Gram-negative bacteria. This cell wall consists of a mycolyl–arabinogalactan–PG complex, and, in order for it to disrupt, Mycobacterium-infecting phages

produce two different lytic enzymes. The LysA degrades the PG layer while LysB cleaves the linkage of the mycolic acids (MA), glycolipids (GL), and lipoarabinomannan (LA) mesh and the arabinogalactan layer (AG) (Gerstmans *et al.* 2016). According to a few authors, LysA and LysB together act synergistically, where LysB sensitizes Mycobacteria for the action of LysA. LysA alone can cause growth inhibition; however, its action alone is insufficient to cause cell lysis (Gerstmans *et al.* 2016; Catalão and Pimentel 2018).

BACTERIAL INFECTIONS IN HUMAN SYSTEMS

The human body is constituted by trillions of bacteria that colonize the gut, skin, nasal passages, mouth and more. Some are commensal, co-existing without harming humans, but others are harmful due to their metabolites. Bacteria enter through several routes and spread in our bodies, causing infection. Treatment of bacterial infections has, for many decades, relied on antibiotics. However, we face global antimicrobial resistance that poses severe health threats (Ho *et al.* 2019). In 2017, the World Health Organization (WHO) developed a list of antibiotic-resistant bacteria prioritized to research and develop new antimicrobials (Tacconelli *et al.* 2017). The list includes critical, high, and medium priority pathogens, including, for instance, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecium*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. CDC released in 2019 a list of urgent, serious, and concerning pathogens, but this only covers the threats in the United States (Redfield 2019). The two lists diverge in their pathogen seriousness, but overall, all are distributed into the other categories. Although WHO does not list *Mycobacterium tuberculosis*, this pathogen has an established global priority status (Tacconelli *et al.* 2017).

Antimicrobial agents face many barriers upon their administration by a given route. The physical barriers include aspects such as those related to particle size to reach a site of infection (e.g., alveoli <2 μm), permeability and particle retention at different organs. The chemical challenges that can transfigure the success rely on degradation due to, for instance, changes in pH and enzymes present in different fluids that have an oxidative effect (e.g., catalase, peroxidase). Many different immunological responses can also affect the circulation half-life upon recognition by myeloid (e.g., neutrophils, macrophages, dendritic cells) and lymphoid cells (e.g., T cells, NK cells). These primary immune cells and others that can initiate foreign bodies' removal will be highlighted in light of the precise human system discussed.

There are also biological barriers that prevent infections. For instance, the human skin's commensal microbiota and other factors inhibit pathogen colonization. Pathogen eradication from different mucosal surfaces is not achieved easily due to their tendency to adhere and form biofilms. In biofilms, cells are surrounded by a thick matrix consisting of extracellular polymeric substances secreted by microorganisms, eDNA, proteins, and other components. For instance, polymicrobial infections by *Streptococcus pyogenes* and *Moraxella catarrhalis* are commonly a cause of pharyngotonsillitis treatment failure (Brook 2017). Bacterial biofilms are observed in many human infections, including respiratory tract infections (RTI) (Hall-Stoodley *et al.* 2006; Sanderson, Leid and Hunsaker 2006; Moreau-Marquis, Stanton and O'Toole 2008), GI tract infections (GITI) (Beloin, Roux and Ghigo 2008; Pachori, Gothalwal and Gandhi 2019; Milho, Silva and Sillankorva 2020), and many skin wound infections (Percival *et al.* 2012; Clinton and Carter 2015; Oliveira et al. 2017, 2018). This sessile lifestyle, assumed for the growth and survival of bacteria in surfaces, is a significant cause of antimicrobial agent failure in biofilm-related infections (Mittal *et al.* 2018; Ho *et al.* 2019).

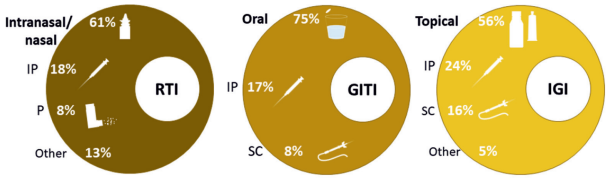


Figure 3. Administration routes of phages and endolysins used *in vivo*. Phage and endolysin treatment routes used in respiratory tract infections (RTI), gastrointestinal tract infections (GITI), and integumentary infections (IGI). The value indicates the percentage that a given administration route was used to deliver free and encapsulated phages and endolysins (literature works from 2009–2020). IP – intraperitoneal, P – pulmonary, SC – subcutaneous.

CHALLENGES OF IN VIVO ADMINISTRATION OF PHAGES AND LYSINS

Animal models have been used in different phage and endolysin studies of human diseases. Also, a few compassionate treatments and case series performed in humans have been reported. Their delivery has been done using different routes (Fig. 3).

The choice of a specific delivery route is made according to the disease, but it often diverges between administration to animals and humans. An example of this is the IP administration with limited use in human patients but wide use in animal models (Fig. 3). Most studies reported in this review used rodents to assess a given treatment's efficacy and potential side effects (e.g., inflammations, irritation). Rodents are the most common mammal used in experimental studies due to their availability, laboratory handling, the reduced cost compared to other mammals, and high reproductive rates. Additionally, rodents and human genomes share a set of closely related genes, and, on average, the protein-coding regions between these genomes are 85% identical (Makałowski, Zhang and Boguski 1996). The data from animal models are used to extrapolate the protection in humans. Each administration route has its challenges and benefits that will be briefly detailed.

Pulmonary administration challenges

Drug inhalation through the mouth and into the airways is a current standard for human respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and infection. Inhaled drugs enter through the oral cavity avoiding the severe nasal cavity barriers (discussed below), resulting in higher concentrations reaching the lungs (El-Sherbiny, El-Baz and Yacoub 2015). Pulmonary administration is safe and causes a rapid local therapeutic action, with drugs reaching the lungs quickly. The delivery route enhances the accumulation of antimicrobials in the lungs and higher retention, maximizing efficacy. Additionally, there is a minimal entry of drugs into the bloodstream and accumulation in other organs, along with limited systemic and side effects (Weers 2015). However, only a small number of antimicrobials can be inhaled (Weers 2015). Inhalants are commonly gaseous, aerosols, nitrites, and volatile solvents, but nanotechnological methodologies have increased the number of antimicrobials available for delivery (e.g., encapsulation into liposomes). After

passing the oral cavity, antimicrobial delivery is challenged by the immunological response of different defense mechanisms that keep the antimicrobials out of the lungs (Newman 2017; Ho *et al*. 2019). Particles can be inactivated, degraded, and cleared by the respiratory system due to the different secretory cells releasing polymeric mucin glycoproteins, apically localized motile cilia in the trachea that transport and eliminate them, and the phagocytic elimination by macrophages in the alveoli, neutrophils, dendritic cells, among other types of cells (Newman 2017). Another obstacle for pulmonary delivery is the particle size that needs to reach, for instance, the alveoli, where bacterial colonization occurs. Besides, improper use of an inhaler device or skipping the specified treatment regimen can also result in an unsuccessful outcome.

Nasal/intranasal administration challenges

The nasal/intranasal delivery is often used in animal models and frequently chosen for allergic and infectious rhinitis local treatment. The nasal/intranasal delivery is rapid, non-invasive, easy, and convenient with reduced side effects. It is promising for low molecular weight drugs, increasing their bioavailability and preventing them from being degraded in the GI tract. With its high vascularity and permeability, the nasal mucosa makes this route desirable for systemic drug administration. However, antimicrobials' intranasal delivery can be compromised by several challenging chemical and immunological barriers in the RS's diverse organs. The obstacles start upon entry due to the nasal valve's physical characteristics, the airflow conditions that can prompt a rapid uptake or deposition of particles, elimination due to mucociliary clearance, and permeation through the viscous mucus layer (Merkus *et al*. 1998). After passing the nasal cavity, particles can be degraded, inactivated, and cleared in the same manner as in pulmonary administration. Intranasal treatment's efficacy is also defied by the particle size that needs to reach distinct organs. Besides, nasal congestion (e.g., cold, allergies) may contribute to a limited delivery of antimicrobials, and the routine use of this delivery route may cause irritation and damage to the nasal mucosa.

Oral administration challenges

Oral delivery is preferred over many delivery routes for providing a painfulness administration with minimal invasiveness and cost-effectiveness. Orally administered therapeutic compounds reach the systemic circulation and are widely distributed to all the tissues and organs (Homayun, Lin and Choi 2019). It can accommodate various types of drugs within different formulations (tablets, capsules, liquids) to obtain a good drug bioavailability and distribution to the intended site of infection (Sosnik and Augustine 2016). Some drugs may be taken on an empty stomach, while others require food in the stomach and gut for drug absorption. Some drugs should also not be mixed with other specific drugs (Bushra, Aslam and Khan 2011). Despite the high degree of stability and accurate dosage of these formulations, drugs given orally have limitations due to their uptake throughout the GI tract and the low pH encountered in the stomach (Mei *et al*. 2013). Before the capillaries, the drugs encounter barriers such as the mucus barrier, the passage through tight junctions, enzymes (e.g., lingual and pancreatic lipase, amylase, pepsin, trypsin), salts, bile, and the epithelial cells of the GI tract (Lundquist and Artursson 2016). The mucus acts as a barrier to pathogens and foreign substances. Its secretion hinders the transport of drugs and, due to its physicochemical properties, it can inhibit drug permeability and decrease the residence time and dosage of the drugs (Leal, Smyth and Ghosh 2017). Peptides are essential components of food, and the GI organs harbor enzymes that can degrade them. Thus, peptides and peptide-based medications (e.g., insulin) do not survive the GI tract, severely affecting drugs' stability and dosage (Gavhane and Yadav 2012). The epithelial cell barrier transports molecules from the lumen to the underlying tissue compartment. In the presence of toxins secreted by intestinal pathogens, the epithelial permeability is increased, and the drug concentrations in the mucosa can be changed (Hua 2020). Besides, GI absorption can be very unpredictable (Hua 2019) since foods and other drugs in the GI tract may alter the gut pH, gastric motility, and the rate and extent of drug absorption (Abuhelwa *et al*. 2017).

Intravenous administration challenges

The IV administration allows drug distribution throughout the body within seconds, being the fastest drug delivery route. The IV route results in a 100% bioavailability of hydrophilic drugs (Pang, Yang and Zhai 2014; Stanisic *et al*. 2018) but cannot be used to deliver lipophilic drugs (Stanisic *et al*. 2018). This route allows a constant plasma concentration by controlling the administration rate (Intravenous Drug Administration 1994), and the drug delivery benefits bypassing the GI absorption barriers, avoiding the first-pass drug effect (Intravenous Drug Administration 1994; Pang, Yang and Zhai 2014). However, high concentrations of drugs delivered rapidly can elicit toxic effects, making this the most potentially hazardous administration route (Maddison, Page and Dyke 2008), but can be halted by stopping the infusion (Intravenous Drug Administration 1994). IV administration increases the risk of infection and limits self-administration. It is mostly used when a quick onset of action is required, such as anesthesia or an emergency, or when oral administration is not possible due to the inherent physicochemical properties of the drug or patient factors (unconsciousness, vomiting, among others) (Maddison, Page and Dyke 2008). Upon administration, drugs can encounter several obstacles that can compromise efficacy, such as opsonization followed by phagocytosis predominantly by macrophages. Hemorheological/blood vessel flow rates and pressure gradients can also limit the action of drugs. Moreover, cellular internalization, endosomal escape, and drug efflux pumps that confer resistance to the therapeutic are other challenging biological barriers (Blanco, Shen and Ferrari 2015).

Skin administration challenges

The skin has been an attractive path for delivering drugs via antimicrobial lotions, gels, patches, creams, and ointments. These are generally inexpensive in comparison with other therapies. The transdermal route is non-invasive, allows self-administration, and has minimal or no first-pass effects (Prausnitz and Langer 2008). The main challenges are the therapeutic compound permeation and possible skin irritation. The leading causes of irritation (e.g., irritant contact dermatitis and allergic contact dermatitis) are a change in skin pH, disturbance of the stratum corneum barrier, immunological response, bacterial proliferation, and the pharmaceutical ingredient's specific chemical properties. Skin irritation can be minimized by modifying the drugs to include corticosteroids or perform a pretreatment on the skin surface with corticosteroids before skin

delivery. The pH at the skin surface (pH 4.1 to 5.8) usually inhibits colonization by pathogenic bacteria, but its increase can escalate the risk of acquiring bacterial infections (Proksch 2018). The primary mechanisms of early immune defense account for the action of antimicrobial peptides, which have broad antibacterial, antifungal and antiviral activity (Yamasaki and Gallo 2008).

Some drugs can be administered systemically across the skin but often encounter tissue proteases or the reticuloendothelial system and are sent to the spleen and liver for degradation. Also, the production of antibodies against these agents is a possibility, promoting their destruction.

Intraperitoneal administration challenges

The IP route is a current practice when delivering drugs to laboratory animals. It is considered an easy and quick treatment, inducing little stress in the animals. The benefits of drug injection in the animals' peritoneum include a larger surface area for delivering large amounts of drugs (Bajaj and Yeo 2010). Nonetheless, the injection procedure can cause puncturing of the intestine or other abdominal organs, and repeated administration causes peritonitis. Although its extensive use in lab animals, particularly in rodents, its use in humans is minimal and only performed in a few patients with adrenal lymphoma (Yang *et al.* 2020) and GI cancer (Sumida *et al.* 1999; Lauer *et al.* 2018). Due to this reason, the challenges of drug delivery via IP will not be detailed.

ENCAPSULATION OF PHAGES AND LYSINS

Phages and lysins have a clinical significance in therapeutics; however, their delivery may be compromised due to their degradation and clearance by different defense mechanisms upon entry into our bodies. For instance, phages for GI infections will be subjected to harsh conditions that include enzymatic degradation and inactivation by the GI tract's low pH conditions. The encapsulation benefits include protection from enzymatic and chemical degradation, mechanisms to evade clearance by the immune system (Pison *et al.* 2006; Moreno-Sastre *et al.* 2015; Lim *et al.* 2016), improving shelf-life, transportation, administration conditions, and optimizing their retention at the infection site (Puapermpoonsiri, Spencer and van der Walle 2009; Singla et al. 2015, 2016a; Loh *et al.* 2020), among others. Additionally, encapsulated drugs can release antimicrobials in a controlled manner or triggered by a specific event. The direct delivery to the infection site increases bioavailability, decreases the number of therapeutic doses needed, reduces side effects, lowers the negative impact on the commensal microbiota, and reduces the likelihood of bacterial resistance emergence. Specifically, the local delivery favors phage contact with the host bacteria, accelerating phage replication and consequent bacterial lysis (Bodier-Montagutelli *et al.* 2017). Nano to macro-sized particles can be modified to have mucoadhesive properties, target specific cells, and increased half-time. Several methods and materials can be used to accomplish these purposes [see (Loh *et al.* 2020) for a detailed review on these topics]. Until today, phages have been encapsulated in more delivery vehicles than lysins (Fig. 4). Data from encapsulated phages and lysins are mainly from *in vitro* assays, limiting the delivery route's conclusions on *in vivo* efficacy. The administration route challenges that free phages and lysins face can be sensed by the human system differently when these antibacterials are delivered in encapsulated formulations.

INFECTIONS IN THE RESPIRATORY SYSTEM

The respiratory system, responsible for the gas exchange between the body and the external environment, is a significant portal for pathogen entry (Louten 2016). Several non-infectious diseases (such as rhinitis, asthma, and lung cancer) and infectious diseases of bacterial or viral origin affect this system (Swai *et al.* 2009; Scherließ 2019). The respiratory system is divided into the upper respiratory tract (URT) and the lower respiratory tract (LRT) (Fig. 5).

Typically, pathogens reach the lungs through aspiration of secretions or inhalation of contaminated droplets (Mandell 2015; Lanks, Musani and Hsia 2019). RTI infections are classified as upper (U) or lower (L), according to their localization. The respiratory microbiota colonizes the URT in higher densities (Man, De Steenhuijsen Piters and Bogaert 2017), triggering upper, lower, or disseminated respiratory infections. However, pathogens' multiplication is challenged by the inhabiting microbiota. These commensal microorganisms often act as gatekeepers resisting the overtake of areas by mediating host immunity, competing for nutrients or adhesion sites, and secreting inhibitory substances (Man, De Steenhuijsen Piters and Bogaert 2017; Esposito and Principi 2018). These efforts are frequently insufficient, and pathogens eventually overcome these barriers and colonize and infect the different organs (Man, De Steenhuijsen Piters and Bogaert 2017; Esposito and Principi 2018; Khan, Petersen and Shekhar 2019). URTI is the most common symptomatic infection and the reason for medical consultation (Francis and Butler 2010). Most URTI are of viral origin; nevertheless, several bacteria can be implicated (Table 1). For instance, *S. pyogenes* (Group A *Streptococcus*) cause inflammation of the pharynx, its soft tissues, or both (pharyngitis and tonsillitis) (Esposito *et al.* 2004), and less commonly laryngitis (Tebruegge and Curtis 2018). Bacteria also infect the sinuses, leading to sinusitis (Brook 2016), and the most common pathogens are the Gram-positive *S. pneumoniae* and the Gram-negative *H. influenzae*. Viral URTI can trigger ear infections by bacteria inhabiting the URT. In these cases, the bacterial species colonizing the nasopharynx ascend to the middle ear inducing otitis media infection (Silva and Sillankorva 2019).

According to the WHO, LRTI was the deadliest communicable disease in 2016, causing 3.0 million deaths worldwide (World Health Organization 2018). Tuberculosis, a preventable and treatable disease caused by *M. tuberculosis*, affects mostly the lungs and is spread through airborne particles. This bacterium killed nearly 1.2 million people globally in 2018, remaining the leading cause of death from a single infectious agent (World Health Organization 2019a). Pneumonia, an acute infection of the lungs, is a significant cause of morbidity and mortality, accounting for 15% of deaths in children under five years old (Lanks, Musani and Hsia 2019; World Health Organization 2019b). Although the bacteria that most commonly elicit pneumonia are *S. pneumoniae* and *S. aureus*, numerous other pathogens can be involved (Table 1). CF is an autosomal recessive lung disease affecting nearly 70 000 patients worldwide (Hügel *et al.* 2020). Patients with this disease characteristically produce a viscous, thick mucus that impairs mucociliary clearance, resulting in frequent infections (Rubin 2007). *P. aeruginosa* is the primary airways affecting pathogen (Table 1), known to form biofilms within the mucus, persisting in the airways and establishing chronic antibiotic-resistant infections (Magalhães *et al.* 2016; Velino *et al.* 2019). COPD is exacerbated and predispose to pulmonary infections, with emphysema and chronic bronchitis being mostly caused by *H. influenza* (Alikhan and Lee 2014;

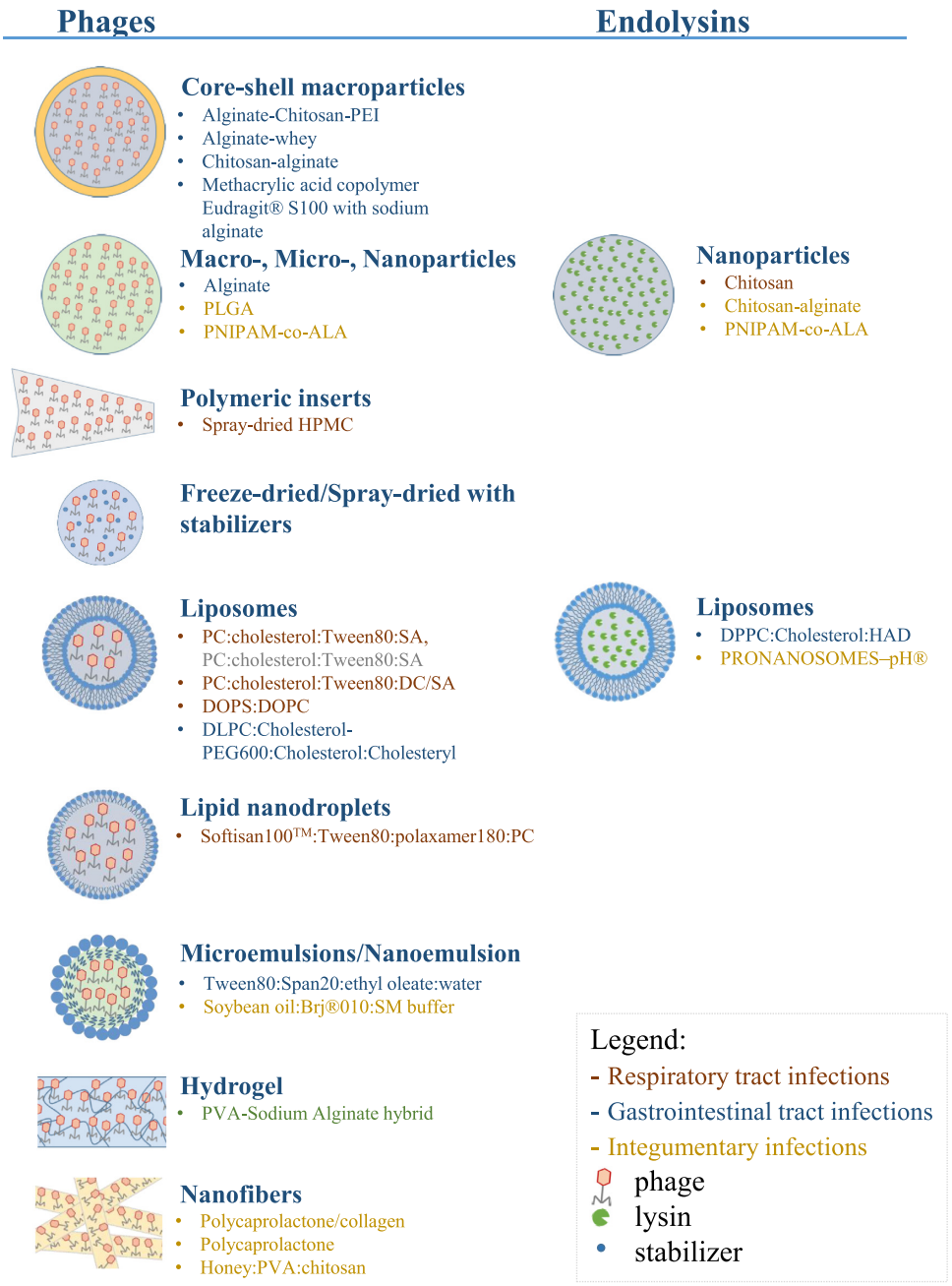


Figure 4. Encapsulation of phages and endolysins in different delivery vehicles to treat RTI, GITI and IGI. Nano to macro size representation of vehicles is not to scale. The colors of the systems addressed are presented at the lower right corner.

Ahearn, Gallo and Murphy 2017). COPD manifests by breathlessness, chronic cough, and mucus production due to persistent airflow reduction (Labaki and Rosenberg 2020).

RTI treatment with antibiotics resort to oral or IV administration, or both, and often, combinations of different antibiotics for prolonged uses. Inhalation can also be used for local and systemical administration of, for instance, aerosols, bronchodilators, corticosteroids, decongestants, some antibiotics, vaccines, among others.

Use of free phages and lysins *in vivo*

Although the conventional oral and IV routes are used in humans, experiments with phages and lysins for RTI in animal models have elected primarily the nasal/intranasal delivery routes (Fig. 3, Table 2) resorting to nebulization and intranasal instillation of liquid phage formulations. The nasal/intranasal delivery has been chosen due to the ease of use, rapid action, and low induction of stress to animals.

Even though many factors can compromise phage therapy's success, there is growing evidence, dating since early in the 20th century, that their use for treating human respiratory infections is positive [see (Abedon 2015)]. Many *in vivo* works have reported a successful phage treatment of different bacterial species (e.g., *P. aeruginosa*, *Klebsiella pneumoniae*, *S. aureus*) using two animal models (mouse, sheep) and infection models [e.g., pneumoniae, ventilator-associated pneumoniae (VAP), sinusitis] (Table 2). Many studies highlight the safety of using phages and their efficacy in decreasing the number of infected animals and even deaths (Debarbieux *et al.* 2010; Morello *et al.* 2011;

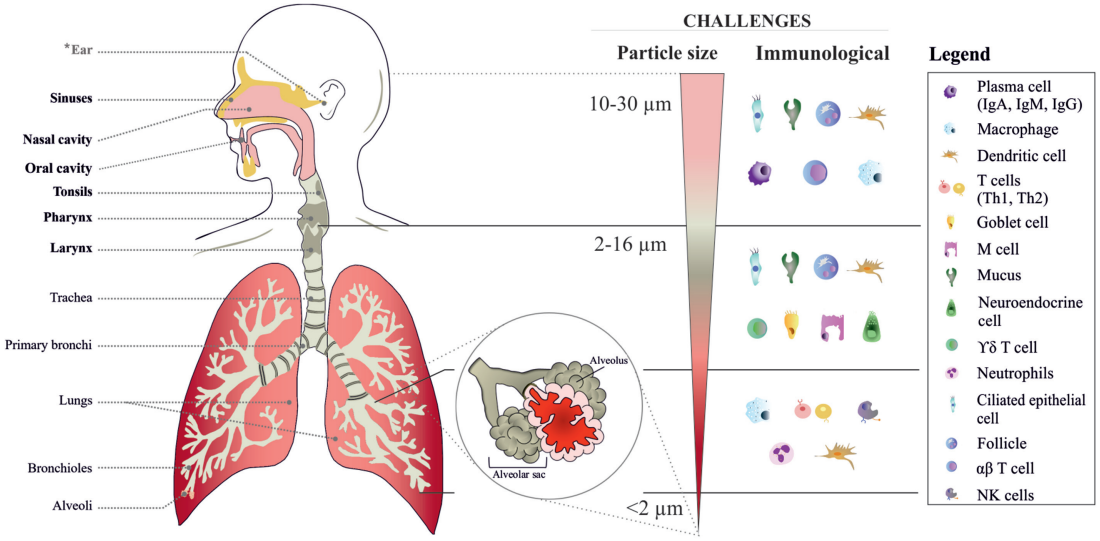


Figure 5. Schematic representation of the respiratory system and the main challenges that phages and endolysins may face. In bold are highlighted the URT, and in plain text are the LRT organs, and in the circle, an approximated look of the alveolus and the respective duct and sacs. On the right side, due to their significant importance, are highlighted the particle dimensions effectively needed for reaching the infection site, and the immunological barriers. ∗ Ear is represented due to the association of middle ear infections with URT infections.

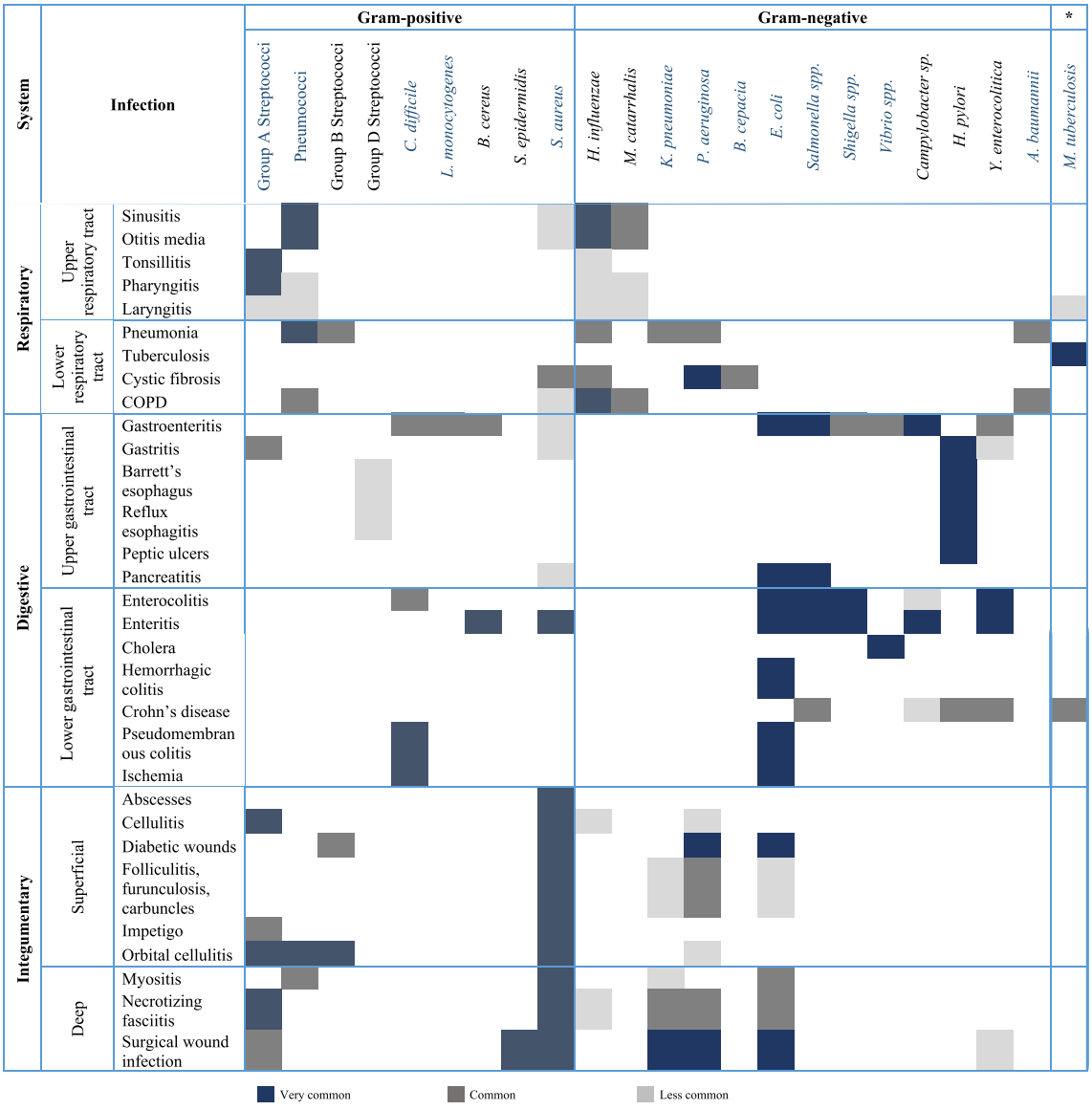
Alemayehu *et al.* 2012; Henry, Lavigne and Debarbieux 2013; Drilling et al. 2014, 2017; Fong *et al.* 2019; Ooi *et al.* 2019).

Upon administration, the spatial distribution of phages and lysins within the lungs can be heterogeneous, and one could think that they might not reach, in high enough concentrations, the areas where the pathogens are residing. Nonetheless, studies using phages targeting *P. aeruginosa* in mice infection models of pneumonia, lung infection, and rhinosinusitis, using IP, intranasal, and topical delivery routes, have shown that phages do efficiently reach the site of infection where they can further multiply and control the bacterial infection (Debarbieux *et al.* 2010; Morello *et al.* 2011; Alemayehu *et al.* 2012; Henry, Lavigne and Debarbieux 2013; Fong *et al.* 2019). Also, the delivery of liquid phage formulations through nebulization is an effective administration route (Semler *et al.* 2014; Prazak *et al.* 2020). Many *in vitro* studies also support nebulization as an administration mode for delivering liquid phage formulations for different pathogens (e.g., *Burkholderia cepacia* (Golshahi *et al.* 2008), *P. aeruginosa* (Cooper, Denyer and Maillard 2014; Sahota *et al.* 2015; Astudillo *et al.* 2018; Leung *et al.* 2019; Lin *et al.* 2019), and *M. tuberculosis* (Carrigy *et al.* 2017; Leung *et al.* 2019).

The success of phages in treating RTI relies on their nanometer size to reach even the pulmonary alveoli (Fig. 5). However, the therapeutic effect may be depended on the phage concentrations applied and period of application, which reportedly varied from a single dose per day (10^5-10^9 PFU per mL) (Morello *et al.* 2011; Alemayehu *et al.* 2012; Pabary *et al.* 2016; Forti *et al.* 2018) to twice a day dose strategy (10^8-10^{10} PFU per mL) (Fong *et al.* 2019). In general, increasing the multiplicity of infection (MOIs) enhances the RTI treatment outcome, and although some studies have shown a certain level of reduction with low MOIs [e.g., MOIs 0.05-1.0 (Henry, Lavigne and Debarbieux 2013; Forti *et al.* 2018)], many argue that the treatments are ineffective or less with MOIs below 10 (Debarbieux *et al.* 2010; Alemayehu *et al.* 2012; Semler *et al.* 2014). This may hypothetically be due to a higher yield of phages, when higher MOIs are used, which end up reaching the infected area (e.g., lungs or sinus), despite many phages ending deposited throughout uninfected URT and LRT areas. However, these are mere assumptions since there is no evidence of the phage deposition events published. Also, the repetition of dosages may have a similar effect, increasing the number of phages available to infect a specific bacterial species causing infection. However, it is difficult to assess if a single dose is more efficacious than multiple doses since none of the studies reports this. Nonetheless, a single dose of highly concentrated phages, timely administered, should suffice to guarantee an increase in their concentration, without the need of an extra dosage, due to the self-replicating characteristic of phages. However, when low concentration phage preparations are used, it may be worth using more dosages to achieve a similar effect. According to animal studies, the phage administration timing is pivotal and should be applied as fast as possible to avoid bacterial multiplication. This is easy to accomplish using animal models, but not that easily translated to human treatments due to the need of verifying the efficacy of a phage or combination of phages in a collection (if this exists), and if these are not effective, it may even require isolation of more phages. All these steps require time, which will allow an infection to evolve.

Pathogens colonize and reside in biofilms in the sputum and within the airways of persistent lung infections such as CF, COPD, and non-CF bronchiectasis. These biofilms are often encircled by dead or dying neutrophils that could not penetrate the 3D structure to phagocyte the bacteria (Bjarnsholt *et al.* 2009). There is a shred of promising evidence that phages may surpass the biofilm matrix barrier of bacteria causing an RTI *in vivo* (Drilling *et al.* 2014), and further works are welcome. For instance, histological sectioning of biofilm aggregates in the sputum might shed some light on phages' interaction with the 3D structures. A far more advanced understanding of phage-biofilm interactions is reported *in vitro* in artificial sputum medium biofilm models and a CF bronchial epithelial cell line model that resemble CF patients' lung environment. These *in vitro* results are promising, reducing bacterial levels by 3–4 log units (Alemayehu *et al.* 2012; Waters *et al.* 2017) and decreasing the total biofilm biomass substantially (Forti *et al.* 2018; Jeon, Park and Yong 2019). It is well known that the biofilm matrix is an impor-

Table 1. Examples of the most common bacterial infections in the respiratory, digestive, and integumentary systems and their causing organisms.



Bacterial species in blue font color have been used as a target in phage or lysin studies using free and encapsulated delivery (Tables 2 and 3). Bacterial species in black font color have not yet been addressed by phage and lysin studies.
*Bacteria not classified as Gram-positive or Gram-negative.

tant barrier having a detrimental effect on antibiotics due to the numerous anionic and cationic molecules (e.g., proteins, glycoproteins, and glycolipids) that can chain charged antimicrobial agents, limiting their spread to the inner layers of biofilms (Dincer, Uslu and Delik 2020). The biofilm matrix can also accumulate a high degree of enzymes (e.g., β-lactamases in *P. aeruginosa* biofilms), leading to increased antibiotic hydrolysis (Anderl, Franklin and Stewart 2000; Bagge *et al.* 2004). Also, eDNA, present in the biofilm matrix, due to its anionic charge, chelates cations and causes an acidification of the biofilm, which induces a cascade of events leading, ultimately, to changes in the bacterial outer membrane (e.g., lipopolysaccharide in *P. aeruginosa*), lowering penetrability for positively charged molecules, and promoting the growth of antibiotic-resistant phenotypes (Wilton *et al.* 2016).

Most antibiotics do not cause problems when properly used, but side effects do occur at some frequency for any class of drugs used. Their adverse reactions range from mild allergic reactions to serious adverse events in a patient and antibiotic-dependent way. One of these side effects includes the increase of inflammatory marker levels. For instance, the impact on cytokines [e.g., tumor necrosis factor α (TNF-α), interleukin (IL) 6 (IL-6)] caused by the antibiotic erythromycin may negatively influence specific host defense mechanisms during pneumococcal pneumonia and are a disadvantage for infection clearance (Schultz *et al.* 1998). Amoxicillin also has caused upregulation of TNF-α, IL-6 and IL-10, and induced a slower downregulation than the natural event in a rat model of acute otitis media, inhibiting an efficient bactericidal activity (Melhus 2001). A few *in vivo* phage studies have shown a reduction of inflammatory marker levels TNF-α

Table 2. *In vivo* models used in free phages or lysins treatment of respiratory, digestive, and integumentary infections.

System	Reference	Phage/ endolysin	Route	Dosage	Animal/ Model	Target organism											
						P. aeruginosa	*S. aureus*	*B. cepacia complex*	*K. pneumoniae*	Group A *Streptococcus*	*S. pneumoniae*	*A. baumanii*	*Salmonella sp.*	*E. faecalis*	*E. coli*	*V. cholerae*	*C. difficile*	*S. sonnei*
PHAGES																		
Respiratory	(Anand *et al.* 2020)	VTCCBPA43	IN	SD	Mouse/ pneumonia				●									
	(Prazak *et al.* 2020)	Aerophages cocktail	P	SD	VAP		●											
	(Jeon, Park and Yong 2019)	Bϕ-R2096	IN	SD	Mouse/ pneumonia							●						
	(Jeon and Yong 2019)	Bϕ-R656, Bϕ-R1836	IN	SD	Mouse/ pneumonia	●												
	(Fong *et al.* 2019)	CT-PA cocktail	T	MD	Sheep/ rhinosinusitis	●												
	(Lehman *et al.* 2019)	AB-SA01 cocktail	IN	MD†	Mouse/ pneumonia		●											
	(Prazak *et al.* 2019)	2003, 2002, 3A, K	IV	MD	Mouse/ VAP		●											
	(Forti *et al.* 2018)	PYO2, DEV, E215, E217	IN	SD	Mouse/ lung infection	●												
	(Hua *et al.* 2018)	SH-Ab15519	IN	SD	Mouse/ lung infection							●						
	(Drilling *et al.* 2017)	NOV012 cocktail	IN	MD	Sheep/ sinusitis		●											
	(Waters *et al.* 2017)	PELP20	IN	SD	Mouse/ lung infection	●												
	(Jeon *et al.* 2016)	phage Bϕ-C62	IN	SD	Mouse/ lung infection							●						
	(Pabary *et al.* 2016)	3 different cocktails	IN	SD	Mouse/ lung infection	●												
	(Wang *et al.* 2016)	IME-AB2	IN	MD	Mouse/ pneumonia							●						
	(Cao *et al.* 2015)	1513	IN	SD	Mouse/ pneumonia				●									
	(Drilling *et al.* 2014)	CTSA cocktail	IN	SD*	Sheep/ sinusitis		●											
	(Semler *et al.* 2014)	5 different phages	P or IP	SD	Mouse/ lung infection			●										
	(Henry, Lavigne and Debarbieux 2013)	11 different phages	IN	SD	Mouse/ lung infection	●												
	(Alemayehu *et al.* 2012)	ϕMR299-2, ϕNH-4	IN	SD	Mouse/ lung infection	●												
	(Morello *et al.* 2011)	P3-CHA, PAK-P3	IN	SD	Mouse/ lung infection	●												
	(Debarbieux *et al.* 2010)	PAK-P1	IN	SD	Mouse/ lung infection													
	(Carmody *et al.* 2010)	BcepIL02	IN or IP	SD	Mouse/ lung infection			●										
	(Chhibber, Kaur and Kumari 2008)	SS	IP	SD	Mouse/ pneumonia				●									
Digestive	(Bao *et al.* 2020)	vB_SenM-PA13076	IP	SD	Mouse/ bacterial challenged								●					
	(Dissanayake *et al.* 2019)	Foodborne outbreak pill	O	MD	Mouse/ bacterial challenged										●			
	(Vahedi *et al.* 2018)	Specific EPEC phage	O	SD	Mouse/ bacterial challenged										●			
	(Yen, Cairns and Camilli 2017)	ICP1, ICP2, ICP3	O	SD	Mouse/ bacterial challenged											●		
	(Galtier *et al.* 2017)	LF82_P2, LF82_P6, LF82_P8	O	SD	Mouse/ bacterial challenged										●			
	(Nale *et al.* 2016)	7 different phages	O	SD	Hamster/ acute infection												●	
	(Mai *et al.* 2015)	5 different phages	O	SD	Mouse/ bacterial challenged													●
	(Tanji *et al.* 2005)	SP15, SP21, SP22	O	SD*	Mouse/ bacterial challenged										●			
	(Chibani-Chennoufi *et al.* 2004)	JS4, JS94.1	O	SD	Mouse/ bacterial challenged										●			
Integumentary	(Rouse *et al.* 2020)	5 different phages	T	SD/MD	Mouse/ wound infection							●						
	(Rahimzadeh *et al.* 2020)	Recombinant Nano phage	T	MD	Rat/ burn wounds		●											
	(Nath *et al.* 2019)	ϕpsbhu-1, ϕpsbhu-15, ϕpsbhu-17	IP	SD	Mouse/ burn wound	●												
	(Ding *et al.* 2018)	JD007	SC	SD	Mouse/ dermal abscesses		●											
	(Khalid *et al.* 2017)	Eff7, Eff10 and Eff11	T	SD	Rabbit/ wound infection	●												
	(Chadha, Katare and Chhibber 2016)	5 different phages	T	SD	Mouse/ burn wound				●									
	(Regeimbal *et al.* 2016)	AB-Army1, AB-Navy1–4	IP, T	SD	Mouse/ wound infection							●						
	(Kusradze *et al.* 2016)	vB-GEC_Ab-M-G7	ND	SD*	Rat/ wound infection							●						
	(Shivaswamy *et al.* 2015)	38	T	SD	Rat/ diabetic wounds							●						
	(Holguín *et al.* 2015)	ΦPan70	SC	SD/MD	Mouse/ burn wound	●												
	(Seth *et al.* 2013)	*S. aureus*–specific phage	T	SD*	Rabbit/ wound infection		●											
	(Kumari, Harjai and Chhibber 2010a)	Kpn5	IP	SD	Mouse/ burn wound			●										
	(McVay, Velásquez and Fralick 2007)	Pa1; Pa2, and Pa11	IM, SC, IP	SD	Mouse/ burn wound	●												
LYSINS																		
Respiratory	(Bae *et al.* 2019)	SAL200	IN	SD	Mouse/ pneumonia		●											
	(Corsini *et al.* 2018)	Cpl-1, Cpl-7S, Cpl-711	IN	SD	Mouse/ nasopharyngeal						●							
	(Xia *et al.* 2016)	LysGH15	IN	SD	Mouse/ pneumonia		●											
	(Doehn *et al.* 2013)	Cpl-1	P	SD	Mouse/ pneumonia						●							
	(Witzenrath *et al.* 2009)	Cpl-1	IP	MD	Mouse/ pneumonia						●							
	(McCullers *et al.* 2007)	Cpl-1	IN	MD†	Mouse/ acute otitis media						●							
	(Loeffler 2001)	Pal	IN	SD	Mouse/ nasopharyngeal						●							
	(Nelson, Loomis and Fischetti 2001)	C1 lysin	IN, O	SD	Mouse/ nasopharyngeal colonization					●								
Digestive	(Cheng *et al.* 2017)	LysEF-P10	IP, SC	SD (IP) and MD (SC)	Mouse/bacterial challenged									●				
Integumentary	(Chopra, Harjai and Chhibber 2016)	MR-10	SC	SD	Mouse/ burn wound		●											
	(Totté, van Doorn and Pasmans 2017)	Staphefekt SA.100	T	MD	Case series: 3 patients with chronic and recurrent dermatoses		●											

IP – intraperitoneal, IV – intravenous, SC – subcutaneous, IM – intramuscular, T – topical, IN – intranasal, O – oral, P – pulmonary, ND – Not defined, SD – single dose, MD – once or twice a day dose applied for a period of consecutive days, ∗ single dose applied during consecutive days, † two doses applied only in a single day

and IL-6 (Debarbieux *et al*. 2010; Morello *et al*. 2011; Hua *et al*. 2018; Jeon, Park and Yong 2019), and also of the macrophage inflammatory protein 2 (MIP-2) (Carmody *et al*. 2010)), providing proof that phages reduce the infection. Also, a reduction of inflammatory foci after phage treatment has been visualized by microcomputed tomography (Wang *et al*. 2016).

Mucus, a complex hydrogel biopolymer barrier mostly composed of mucin glycoproteins, is one of the major challenges to transmucosal drug delivery (Leal, Smyth and Ghosh 2017). The binding of antibiotics to mucin and the consequent reduction of antibiotic efficacy has been reported (Huang *et al*. 2015; Samad *et al*. 2019). However, phages' binding to mucin has been linked to an increased phage abundance relative to bacterial cells in mucosal environments. The binding of phages to mucin glycoproteins occurs via immunoglobulin-like (Ig-like) protein domains displayed on their capsids. The phage adherence to mucus has been proposed as a non-host-derived layer of immunity mediated by phages (Barr *et al*. 2013; Van Belleghem *et al*. 2019). The increased encounter rates between phages and bacteria are mediated by increased bacterial motility in addition to the phage interaction with mucin (Joiner *et al*. 2019).

The effect of macrophage, neutrophils and dendritic cell phagocytosis mechanisms for eliminating phages upon administration may result in their engulfment, retention of lytic activity, and in some circumstances, cause virion particle disintegration. Different mechanisms can promote phages' uptake into macrophages and lead to macrophage activation (Krut and Bekeredjian-Ding 2018). These events have been shown mostly *in vitro*, with phages (T2 phage) remaining briefly active in macrophages and rabbit peritoneal neutrophils but being inactivated once transported to lysosomes (Aronow *et al*. 1964; Ivanenkov, Felici and Menon 1999) and inactivated by dendritic cells due to the removal of the phages' outer coat (phage T4) (Barfoot *et al*. 1989; Kaźmierczak *et al*. 2014). *In vivo* results are scarce but show phages (e.g., T4) circulating in the blood are cleared by macrophages resulting in decreased phage treatment efficacy (Kaur *et al*. 2012; Hodyra-Stefaniak *et al*. 2015). Bacteria have evolved to escape the immune system using multifaceted methods. For instance, *S. aureus* uses a Trojan-horse strategy, hiding in dysfunctional neutrophils, where it survives and proliferates, waiting for the neutrophils' eventual death so that they are released at different locations causing further infection (Leliefeld *et al*. 2018). They have also found ways to survive and propagate inside macrophages (e.g., *Mycobacterium tuberculosis, Legionella pneumophilia*) by antagonizing the autophagy machinery [reviewed in (Mitchell, Chen and Portnoy 2017)]. It is unclear how phage phagocytosis could kill internalized bacteria, but many antibiotics successfully penetrate and act on intracellular bacteria. Understanding bacterial internalization, or suspicion of such fact in an infection, and of the available antibiotics capable of penetrating and treating such infections is critical in clinical decisions [see review (Bongers *et al*. 2019)].

Trials for treating lung infections using *P. aeruginosa* phage formulations are in the pipeline of companies and projects, such as the AB-PAO1 from AmpliPhi Biosciences, PneumoPhage project, and Phage4Cure project (Phage4Cure; PneumoPhage; Kaul *et al*. 2019). To our knowledge, AmpliPhi has not shared the proposed route of administration of their phage preparation; however, the two latter projects aim at delivering phages by inhalation to treat acute RTI.

The first use of endolysins prophylactically and therapeutically was carried out in mice models of nasopharyngeal colonization by group A, C, and E *Streptococcus* (Nelson, Loomis and Fischetti 2001) and upper respiratory tract colonization by group A *Streptococcus* (Loeffler, Nelson and Fischetti 2001). The works used the C1 and the Pal endolysins, delivered by oral and nasal or pharyngeal and nasal administration, respectively, and both protected mice from infection. Nasopharyngeal and nasal carriage models are commonly used to investigate colonization and impact on infection establishment, evaluation of antimicrobial efficacy, vaccine development (Kiser, Cantey-Kiser and Lee 1999), and were also used to reduce or clear *S. pneumoniae* using endolysins (Cpl-1, Cpl-7S, Cpl-711) (Loeffler, Nelson and Fischetti 2001; Corsini *et al*. 2018). Recently, MSlys endolysin, resembling Pal, showed activity against different serotypes of *S. pneumoniae* and *in vitro* conditions close to those found in the middle ear (Silva *et al*. 2020). Cpl-1 endolysin, studied in a mice model mimicking otitis media triggered by a viral infection (McCullers *et al*. 2007), reduced nasal colonization, preventing mice from developing otitis media. Endolysins have also been used for treating pneumonia in mice models (Table 2). The *S. pneumoniae* Cpl-1 endolysin, administered by injection or aerosolized, rescued mice from fatal pneumonia (Witzenrath *et al*. 2009; Doehn *et al*. 2013), and intranasally administered endolysins (SAL200, LysGH15) reduced *S. aureus* loads in the lungs of mice and improved the histological damage (Xia *et al*. 2016; Bae *et al*. 2019). Several other endolysins against respiratory pathogens (e.g., *P. aeruginosa, K. pneumoniae, A.baumannii*) have shown high *in vitro* bactericidal capacity against planktonic and biofilm cultures, including multidrug-resistant isolates (Vázquez, García and García 2018).

Cytokines (e.g., interleukin 8 (IL-8), interleukin 1β (IL-1β), lactate dehydrogenase (LDH), IL-6, among others) were studied after endolysin application in mice models of pneumonia (Witzenrath *et al*. 2009; Doehn *et al*. 2013; Xia *et al*. 2016; Bae *et al*. 2019). These pro-inflammatory cytokines are predominantly produced during an inflammatory reaction by activated macrophages. The quantification of cytokines was done to evaluate if intracellular components' release might have pro-inflammatory or destructive effects in the animals studied. This pro-inflammatory effect was observed in an endocarditis infection model, where the rapid pneumococcal lysis and probably the sudden release of cell wall fragments with the high-dose regimen of Cpl-1 resulted in increased cytokine secretion (Entenza *et al*. 2005). Overall, the cytokine levels increased in non-treated animals but not in the endolysin-treated group in the pneumonia models. Nonetheless, in one study, comparable cytokine levels amongst the non-treated and treated groups of animals at all time-points assessed were reported (Bae *et al*. 2019). In another, the levels of IL-1β and IL-6 were high in non-infected animals, which had received endolysin therapy, but none of the other cytokines were evaluated (Doehn *et al*. 2013). This later increase in IL-1β and IL-6 was possibly due to the endolysin recombinant production in *Escherichia coli*, and the contaminating LPS might elicit this response even though endotoxin was not detected ($\leq$0.01 EU/μg).

In vitro delivery of encapsulated phages and endolysins

Different bacteria have been targeted with encapsulated phages and lysins, and most studies aim to control lung pathogens. The reasons behind encapsulation include creating delivery systems for a controlled release with improved storage and product stability, improved pharmacokinetics, and biodistribution. Also, encapsulation has been performed to attenuate the immune host's clearance and protect from neutralizing antibodies (Fig. 5).

For the treatment of LRTI, the aerosolization of therapeutic agents by inhalation to the lungs can be accomplished

using nebulizers, pressurized metered-dose inhalers (pMDIs), and powder inhalers (DPIs) (Moreno-Sastre *et al.* 2015; Newman 2017). Nebulizers are somewhat large and expensive devices, requiring long nebulization times and cleaning and disinfection following their use. DPIs are cheaper, smaller, more comfortable to transport, and easy to operate. Dry powders are also favored over liquid formulations due to their increased shelf life without refrigeration (Vandenheuvel *et al.* 2013; Velino *et al.* 2019).

For pulmonary delivery, the particles' aerodynamic diameter (ad) plays a critical role (Tsuda, Henry and Butler 2013). Tiny particles (a_d <1 μm) can be expelled during exhalation. Large particles are retained in the pharynx and larynx by inertial impaction. Particles with an a_d <5 μm reach the lungs, and a_d <2 μm get deposited in the alveolar epithelium. The particle deposition is affected by device and inhalation parameters (inhaled flow rate, inhaled volume, and breath-hold pause) (Moreno-Sastre *et al.* 2015; Newman 2017).

For an effective delivery of phages and endolysins using DPIs, phage formulations have been dried using freeze-drying and spray-drying techniques along with sugars (e.g., trehalose, sucrose, and lactose) (Merabishvili *et al.* 2013; Leung *et al.* 2016) that stabilize and preserve phage infectivity (Table 3). Freeze-drying, or lyophilization, produces stable dry powders for DPI devices (Moreno-Sastre *et al.* 2015). Freeze-dried *S. aureus* or *P. aeruginosa* phages have been encapsulated into biodegradable poly(DL-lactic co-glycolic acid (PLGA) microspheres with an a_d of 3.30 (*P. aeruginosa*) and 3.57 μm (*S. aureus*) (Puapermpoonsiri, Spencer and van der Walle 2009). Phage release began within the first 30 minutes and continued for approximately six hours. Freeze-drying does not affect the phages' lytic activity; however, the technique can cause a small decrease in their titer, even when dried together with stabilizing agents (Alfadhel *et al.* 2011; Golshahi *et al.* 2011). The formulations produced can also have a shorter shelf life than expected (less than seven days at both 4 and 22°C), and median a_d values too large for a successful deposition in the lungs. For instance, only 30% of inhaled freeze-dried phage particles with an a_d of 3.4 μm reached the lungs (Golshahi *et al.* 2011).

Based on the solvent's evaporation from a liquid or suspension, spray-drying is a widely used single-step method for producing dry powders with appropriate deposition characteristics in the lungs (Moreno-Sastre *et al.* 2015). Most studies report good aerosol characteristics for pulmonary delivery of the respirable phage powders produced. For instance, *B. cepacia* complex and *P. aeruginosa* phages spray-dried with sugars at low temperatures (40–45°C) prevented phage inactivation and resulted in a_d <3 μm, which is considered suitable for delivery to the lungs (Matinkhoo *et al.* 2011). Trehalose has shown better preserving properties than lactose, and dextran 35, protecting *P. aeruginosa* and *S. aureus* phages from temperature and shear stress throughout the process (Vandenheuvel *et al.* 2013). Spray-drying provided protection (low titer reduction) to pseudomonal phages and good aerosol performance regardless of the different virion particle morphologies (Chang *et al.* 2017). The same research group further showed that the *P. aeruginosa* phages (dried with lactose and leucine) presented no *in vitro* toxicity to human epithelial and macrophage cells and significantly reduced the bacterial loads after 24 hours of treatment, decreasing lung damage in animals (Chang *et al.* 2018). Spray-drying of a combination of this phage with ciprofloxacin using leucine with or without lactose as excipients had a strong synergistic *in vitro* antimicrobial effect (Lin *et al.* 2019).

Dry phage powders have remained stable when stored between 0 and 22% of relative humidity (RH) conditions at 4°C, even after 12 months. However, an RH of 60% destroyed the phages in just three months (Leung *et al.* 2017). The temperature of storage additionally varies according to the phage and formulation used. Storage of dry phage powders for one year without significant loss of activity can be accomplished if the powders are vacuum packed and stored at 4°C or 20°C (Leung *et al.* 2018).

Phages can also be encapsulated into biopolymeric structures particles or liposomes, and in this case, PDIs and nebulizer devices can also be adopted. Phage-loaded PLGA microparticles' (phage-MPs) efficacy for RTI treatment via dry powder inhalation was analyzed *in vitro* and *in vivo* (Agarwal *et al.* 2018). PLGA microparticles prepared by water-in-oil-in-water (w/o/w) double emulsion methodology, with ammonium bicarbonate as effervescent, were incubated with a cocktail of three to five *P. aeruginosa* phages for their adsorption to the microparticles. These phage-MPs exhibited reduced internalization by macrophages and killed *P. aeruginosa* cells in synthetic viscous sputum and biofilms, and the inhaled phage-MPs caused no damage to mice lung tissues. Furthermore, the phage-MPs reduced bacterial loads and inflammation levels, rescuing mice from pneumonia in a mouse model mimicking CF (CFTR gene knockout).

Encapsulation of phages into liposomes has been explored to target intracellular bacteria, improve phage pharmacokinetics and biodistribution and protect them from neutralizing antibodies (Table 3). The encapsulation of the model phage λeyfp and the mycobacteriophage TM4 into giant unilamellar liposomes (≈5 μm) showed that liposome-associated phages are internalized by eukaryotic cells more proficiently than free phages (Nieth *et al.* 2015). *K. pneumoniae* phages encapsulated into cationic liposomes presented a mean size of 576.9 nm with an encapsulation efficiency of 92% (Singla *et al.* 2016b). The liposomes were stable at 4°C for 11 weeks, causing no significant reduction in the number of encapsulated phages. The liposome-entrapped phages had improved pharmacokinetics, compared to free phages, in a mouse model of pneumonia. The IP administration reduced *K. pneumoniae* in the lungs even three days after bacterial challenge, in contrast with free phages that were effective only up to 24 hours after establishing the infection. The prophylactic administration of these particles 48 hours before infection protected mice from pneumonia (Singla *et al.* 2015). Phages entrapped in liposomes can be retained longer in different organs. These can be detected up to day 6 in the mice's lungs, whereas free phages became undetectable after 36 hours (Singla *et al.* 2016b). Moreover, their encapsulation into liposomes provides complete protection from neutralizing antibodies, while free phages were neutralized within three hours. Additionally, liposomes with encapsulated phages lysed 94.6% of the intracellular *K. pneumoniae,* while free phages only killed 21.4% of intracellular bacteria (Singla *et al.* 2016a).

A w/o/w multiple emulsion system developed for nebulization purposes, integrating small-sized lipid nanodroplets with aqueous cores containing *P. aeruginosa* phage particles, reached an encapsulation efficiency of 89.2% and stability for nearly one year at 4°C (Table 3) (Rios *et al.* 2018). These emulsions showed no significant cytotoxic effects against lung cell lines. The cytotoxicity of freeze-dried *P. aeruginosa* phage formulations containing stabilizers evaluated in three cell lines (A549, HEK239, THP-1) and mice models caused no toxicity and thus were considered safe (Chang *et al.* 2018). Cytotoxicity of lipid nanodroplets with *P. aeruginosa* phages, also evaluated in A549 and V79 cell lines, presented low toxicity, with 80% of the cells preserving their viability (Rios *et al.* 2018).

The inflammatory response can vary whether phages are delivered free or encapsulated. For instance, phages entrapped

Table 3. Recent studies of encapsulation of phages and lysins for the treatment of respiratory, digestive, and integumentary infections.

System	Reference	Phage/ endolysin	Delivery system (*in vivo model*)	Stabilizer							Study		Proposed route	Target organism								
				Lactose	Trehalose	Sucrose	Leucine	Dextran	Mannitol	Other or NA	*In vitro*	*In vivo*		*P. aeruginosa*	*S. aureus*	*B. cepacia complex*	*M. tuberculosis*	*K. pneumoniae*	*S. pneumoniae*	*E. coli*	*Salmonella sp.*	*C. difficile*
PHAGES																						
Respiratory	(Lin *et al.* 2019)	PEV20	Spray-dried powder	●			●				●		P	●								
	(Leung *et al.* 2018)	PEV2, PEV40	Spray-dried powder		●		●				●		P	●								
	(Rios *et al.* 2018)	JG004	Softisan100™:Tween80:polaxamer18							●	●		P	●								
	(Agarwal *et al.* 2018)	ØPaer22, ØE2005-C, Ø109	Freeze-dried powder of PLGA polymeric microparticles (mouse/ lung infection)							●	●	●	P	●								
	(Chang *et al.* 2018)	PEV20	Spray-dried powder (mouse/ lung infection)	●			●				●	●	P	●								
	(Chang *et al.* 2017)	PEV1, PEV20, PEV61	Spray-dried powder	●	●					●	●		P	●								
	(Leung *et al.* 2017)	PEV2	Spray-dried powder		●		●		●		●		P	●								
	(Singla *et al.* 2016b)	KPO1K2	PC:cholesterol:Tween80:SA liposomes (mouse/ phage biodistribution and toxicity)							●	●	●	IP					●				
	(Singla *et al.* 2016a)	KPO1K2	PC:cholesterol:Tween80:DC/SA liposomes (mouse/ immune							●	●	●	IP					●				
	(Leung *et al.* 2016)	PEV2	Spray freeze-dried & spray-dried		●		●		●		●		P	●								
	(Nieth *et al.* 2015)	TM4	DOPS:DOPC liposomes			●					●		P				●					
	(Singla *et al.* 2015)	KPO1K2	PC:cholesterol:Tween80:SA liposomes (mouse/ lung infection)							●	●	●	IP					●				
	(Merabishvili *et al.* 2013)	ISP	Freeze-dried powder		●	●					●		P		●							
	(Vandenheuvel *et al.* 2013)	LUZ19, Romulus	Spray-dried powder	●	●			●			●		P	●								
	(Alfadhel *et al.* 2011)	NCIMB 9563	Freeze-dried HPMC polymeric insert						●		●		P									
	(Golshahi *et al.* 2011)	KS4-M, ΦKZ	Freeze-dried powder	●						●	●		P	●		●						
	(Matinkhoo *et al.* 2011)	KS4- M, KS14, ΦKZ/D3 cocktail	Spray-dried powder with stabilizers (and surfactant)		●		●				●		P	●		●						
	(Puapermpoonsiri, Spencer and van der Walle 2009)	*S. aureus* phage, *P. aeruginosa* phage	Freeze-dried powder into PLGA polymeric microspheres							●	●		P	●	●							
Digestive	(Vinner *et al.* 2019)	Felix O1	Spray dried methacrylic acid		●						●		O								●	
	(Śliwka *et al.* 2019)	T4	Alginate macrospheres with stabilizer						●		●		O							●		
	(Otero *et al.* 2019)	UAB_Phi20	DLPC:Cholesterol-PEG600:Cholesterol: Cholesteryl							●	●	●	O								●	
	(Vinner and Malik 2018)	Felix O1	Methacrylic acid copolymer Eudragit® S100 with sodium alginate							●	●		O								●	
	(Vinner *et al.* 2017)	CDKM9	Methacrylic acid copolymer Eudragit® S100 with and without							●	●		O									●
	(Rastogi *et al.* 2017)	T4	Tween80:Span20:ethyl oleate:water microemulsion (rat/ bacterial							●		●	T							●		
	(Moghtader, Eğri and Piskin 2017)	T4	Alginate beads coated with chitosan and polyethylene imine (PEI)							●	●		O							●		
	(Tang *et al.* 2015)	K	Alginate-whey protein microspheres							●	●		O		●							
	(Kim, Jo and Ahn 2015)	*E. coli* O157:H7	Chitosan-alginate microspheres							●	●		O							●		
Integumentary	(Kaur, Gondil and Chhibber 2019)	MR10, Kpn5, PA5	Wound dressing of PVA-Sodium Alginate hybrid hydrogel membrane							●	●	●	T	●	●			●				
	(Rubalskii *et al.* 2019)	PA5	Fibrin glue							●	●		T	●								
	(Cheng *et al.* 2018)	T4	Polycaprolactone/collagen nanofibers (rabbit/ biodegradation studies)							●	●	●	T							●		
	(Chhibber, Kaur and Kaur 2018)	MR-5 and MR-10	PC:cholesterol:Tween80:SA liposomes (mouse/ diabetic wound							●	●	●	T		●							
	(Chhibber, Kaur and Kaur 2018)	MR-5 and MR-10	PC:cholesterol:Tween80:SA liposomes (mouse/ diabetic wound							●	●	●	T		●							
	(Chadha, Katare and Chhibber 2017)	KØ1, KØ2, KØ3, KØ4, KØ5	PC:cholesterol:Tween80:SA liposomes (mouse/ burn wound							●	●	●	IP					●				
	(Chhibber, Shukla and Kaur 2017)	MR-5 and MR-10	PC:cholesterol:Tween80:SA transfersomes (rat/ thigh infection)							●	●	●	IP		●							
	(Nogueira *et al.* 2017)	vB_Pae_Kakheti 25	Wound-dressing of polycaprolactone (PCL) nanofibers (non-woven textile)							●	●		T	●								
	(Sarhan and Azzazy 2017)	PS1	Honey:PVA:chitosan nanofibers							●	●	●	T	●								
	(Esteban, Jenkins and Arnot	K	Soybean oil:Brj®010:SM buffer							●	●		T		●							
	(Hathaway *et al.* 2015)	K	PNIPAM-co-ALA nanospheres							●	●		T		●							
	(Kumari, Harjai and Chhibber 2010b)	Kpn5	Hydroxy propyl methyl cellulose hydrogel							●	●	●	T					●				
LYSINS																						
Respiratory	(Gondil *et al.* 2020)	Cpl-1	Chitosan nanoparticles (mouse/ immune stimulation studies)							●	●	●	-						●			
	(Gondil, Harjai and Chhibber 2020)	Cpl-1	Chitosan nanoparticles (mouse/ pneumonia)							●	●	●	IN						●			
	(Vipra *et al.* 2012)	P128	Hydroxyethyl cellulose, propylene glycol and glycerin hydrogel								●		T		●							
	(Paul et al. 2011)	P128	Hydrogel (rat/nasal colonization)								●	●	T		●							
Digestive	(Bai *et al.* 2019)	BSP16Lys	DPPC:Cholesterol:HAD liposomes			●					●		-							●	●	
Integumentary	(Kaur *et al.* 2020)	LysMR-5	Chitosan-alginate nanoparticles							●	●		-		●							
	(Portilla *et al.* 2020)	LysRODI	PRONANOSOMES–pH® liposomes							●	●		-		●							
	(Hathaway *et al.* 2017)	$CHAP_k$	PNIPAM-co-ALA nanospheres							●	●		T		●							

IP – intraperitoneal, T – topical, IN – intranasal, O – oral, P – pulmonary.

in liposomes improved the inflammatory response by reducing the inflammatory cytokines IL-1β and TNF-α in treated mice compared to placebo and free phage-treated groups (Singla *et al.* 2015).

Endolysin encapsulation studies with the potential to be used against respiratory infections are also reported (Table 3). Chitosan nanoparticles loaded with the Cpl-1 pneumococcal endolysin were produced to increase their *in vivo* bioavailability. The Cpl-1-loaded nanoparticles had an approximate size of 100 nm, with about 55% of the endolysin being efficiently encapsulated. After 24 hours of incubation, more than 70% of the endolysin was released from the nanoparticles, with an initial burst release followed by a constant release. Cpl-1 chitosan nanoparticles were shown to have mucoadhesive properties and low cytotoxicity to lung epithelial cell lines, causing an insignificant increment in antibody titers in mice immune studies (Gondil *et al.* 2020). Later, the same group evaluated the same encapsulated endolysin in an animal model of pneumoniae (Gondil, Harjai and Chhibber 2020b). The treatment reduced bacterial colonization in the lungs, lowered inflammation levels, and decreased cytokines (IL-1β, TNF-α, IL-10, IL-12). The P128 chimeric lysin (tail-associated muralytic enzyme of staphylococcal phage K and the cell-wall binding domain of lysostaphin) was incorporated in a hydrogel containing hydroxyethyl cellulose, propylene glycol, and glycerin as the main excipients, being effective in the eradication of *S. aureus* isolates from the nares of healthy people, contributing to a decreased risk of infection (Vipra *et al.* 2012). Previously, a P128 hydrogel (composition not described) was reported to effectively decolonize *S. aureus* from rat nares (Paul *et al.* 2011). Furthermore, the safety and effectiveness of P128 in eradicating *S. aureus* from the human nostrils were demonstrated in a phase I/II clinical trial (NCT01746654).

INFECTIONS IN THE DIGESTIVE SYSTEM

The digestive system comprises the GI tract and the accessory organs of digestion (Fig. 6). The GI tract starts from the mouth to the anus, in which a long muscular tube connects several organs, with an individual microbiota pattern that varies throughout the tract (Greenwood-Van Meerveld, Johnson and Grundy 2017; Dieterich, Schink and Zopf 2018).

The muscular tube along the GI tract is coated with mucus mainly composed of mucin glycoproteins (Ma, Rubin and Voynow 2018). Mucin serves as a lubricant in chyme transport through the tract, preserves intestinal homeostasis, and acts as a barrier against harmful molecules and microbial infections (pathogenic bacteria, viruses, and parasites) (Kebouchi *et al.* 2020). The alteration in the mucus' composition and structure by antibiotics opens doors for microbial colonization and consequent diseases (Stecher and Hardt 2008; Baümler and Sperandio 2016; Round and Palm 2018). Once microorganisms adhere to the epithelium, they are known for altering the mucus' secretion (Probert and Gibson 2002; Macfarlane and Dillon 2007). Many diseases of the digestive system are associated with the entry of bacteria (Table 1), viruses (e.g., rotavirus, norovirus, or adenovirus), and parasites (e.g., *Giardia* spp. and *Cryptosporidium* spp.) (Sell and Dolan 2018; Eslick 2019).

Several diseases can affect the GI tract, including non-infectious diseases such as inflammatory bowel disease, irritable bowel syndrome, diverticulitis, ischemic colitis, and colorectal cancer (Sell and Dolan 2018). Bacterial GI infections include upper and lower GI tract infections (Table 1).

GI infections kill approximately 2.2 million people every year in the world (WHO 2016). These numbers can be aggravated due to person-to-person, fecal-oral, environmental, and airborne routes (Fletcher, McLaws and Ellis 2013). Proper food preparation and enteric precautions must be considered (PHLS Advisory Committee on Gastrointestinal Infections 2004).

Generally, antibiotic treatment is not recommended for acute watery diarrhea since this is often of viral etiology, and bacterial diarrheas improve spontaneously (Kim *et al.* 2019). The inappropriate use of broad-spectrum antibiotics to treat such infections may contribute to antibiotic tolerance and aggravate GI symptoms (e.g., acute diarrhea, vomiting, and nausea) due to the motility, permeability, and microbiota changes induced by the antibiotics (Maxwell *et al.* 2002; Tulstrup *et al.* 2015). The onset and symptomatology of antibiotic-associated diarrhea, irritable bowel syndrome, pseudomembranous colitis, and increased susceptibility to subsequent disease are some side effects of the antibiotic treatment (Keeney *et al.* 2014).

Use of free phages and lysins *in vivo*

Promising free phage therapy results in animal models and human patients with GI infections are known (Table 2), and the main delivery route has been the oral (Fig. 3). Phages are present in the gut from early infancy till late adulthood. It is estimated that the abundance is as high as the bacterial host they target, are unique in each individual and go through drastic changes during their development (Breitbart *et al.* 2008; Reyes *et al.* 2010; Manrique *et al.* 2016). Although orally administered phages survive the gut transit and are recovered in fecal samples of different animals and humans, the concentrations are quite low [see (Dąbrowska 2019) and references cited therein]. Upon entry through the alimentary tract, phages have to withstand different absorption times at varied pH ranges, affecting the pH-sensitive phage particles. For instance, if the transit of phages takes as much time as it normally does for food digestion, phages may be up to 4 hours in contact with pH values between 1 and 3 (Fig. 6) and will certainly be inactivated.

Studies demonstrate minimal or no gut microbiota distortion when phage therapy is applied in animal models. For example, a comparative study in *E. coli* O157:H7 infected mice between orally administered antibiotic (ampicillin) and a phage cocktail showed better bacterial clearance using the antibiotic (79%) than the phage formulation (54%). However, ampicillin caused two adverse side effects - weight loss and a noticeable distortion of the gut microbiota that only returned to normal after ten days (Dissanayake *et al.* 2019). These may seem light side effects, yet when continuous antibiotic prophylaxis is needed, these effects can worsen and cause severe damage to the immune system. When orally administered to *Shigella*-challenged mice, ampicillin and ShigActive™ (Intralytix, Inc., USA) phage formula evidenced that both treatments effectively reduced *Shigella* (Mai *et al.* 2015). However, ShigActive™ had less impact on gut microbiota and promoted long-term safety (no side effects, no distortions in the gut microbiota), evidencing the product's safety and efficacy for this type of GI infection.

The IP application of phage PA13076 reduced 2.5 $\log_{10}$ of *S.* Enteritidis cells in the blood, intestine, liver, spleen, and kidney of *Salmonella*-challenged mice within 24 hours of treatment. The blood circulation allowed phage distribution into five organs, promoting the survival of infected mice, with titers remaining above 10^4 PFU/g for at least 72 hours. This work shows that phage PA13076 passes the epithelial barrier, entering into extraintestinal sites (Bao *et al.* 2020). Nonetheless, phages can be seen as potential invaders and reduced by the spleen and liver's

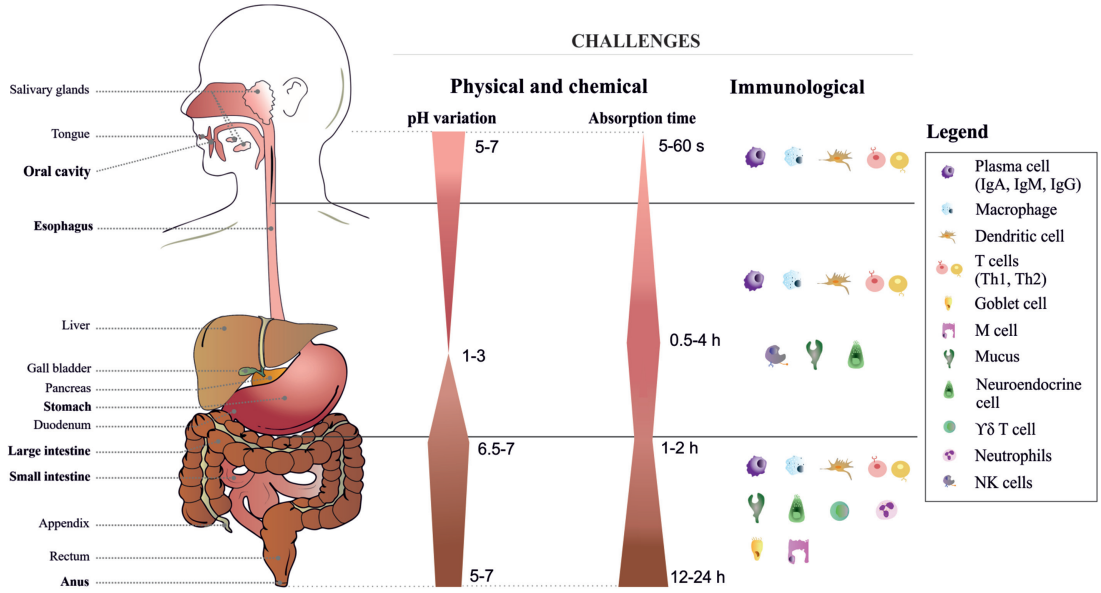


Figure 6. Schematic representation of the digestive system and the main barriers for phage and endolysin delivery. In bold are the essential organs and, in plain text, the accessory organs. On the right side are the challenges for delivery, highlighting the physical and chemical (pH variation and time of absorption) and immunological barriers.

reticuloendothelial system. The liver's reticuloendothelial system includes Kupffer cells, and the spleen includes a large pool of B cells that have been shown responsible for phage clearance (Inchley 1969; Srivastava, Kaido and Carrier 2004). About 99% of T4 phages were reported to be eliminated from the circulation in less than 1 hour after injection, with Kupffer cells inactivating phages four times as fast as splenic macrophages and degrading phage protein twice as rapidly, in the early hours after the start of treatment (Inchley 1969). Similar results have been reported for T7 phage, which was neutralized in the blood to 1% of the initial concentration used (Srivastava, Kaido and Carrier 2004). This study proved that the host immune system reacted with B-cell dependent immunoglobin leading to the phages' neutralization. Studies have demonstrated an improvement in the phage half-life through phage capsid modifications which makes them able to evade the reticuloendothelial system prolonging their circulation and consequently improving the therapeutic effects (Merril *et al.* 1996; Serwer and Wright 2018). Phage mutants that remain longer in circulation can be obtained following adaptation procedures with serial passages in bacterial-challenged mice. Phages in patients with a deficient or suppressed immune system seem to have a prolonged circulation due to their impaired immunity function (RES and other B cells-mediated immunity) (Bearden *et al.* 2005; Borysowski and Górski 2008).

According to phages' involvement in the human gut, it has been reported that they influence the bacterial organization of the microbiome, where they also play an anti-inflammatory and immunomodulatory role in the gut immune response. Phage interactions with the gut cells and lymphoid tissue produce pro-inflammatory cytokines and reduce the overproduction of reactive oxygen species maintaining a healthy gut microbiota [reviewed extensively in (Lusiak-Szelachowska *et al.* 2017; Carroll-Portillo and Lin 2019; Gutiérrez and Domingo-Calap 2020)].

Clinical trials testing phage therapy for GI diseases have been published. The randomized, double-blind, placebo-controlled crossover trial NCT03269617 tested the effectiveness of PreforPro, a cocktail of 4 phages (LH01-*Myoviridae*, LL5-*Siphoviridae*, T4D-*Myoviridae*, and LL12-*Myoviridae*) on 43 healthy adults with mild to moderate GI distress (Febvre *et al.* 2019; Gindin *et al.* 2019). The treatment was safe and tolerated by all the participants. Although no significant GI improvement (e.g., gastric function, small intestine pain, and colon pain) was observed compared with placebo, additional studies on the microbiota modulatory potential of phages for use as a dietary supplement and therapeutic agent were encouraged. In another trial (NCT00937274), T4 *E. coli* phage or ColiProteus phage cocktail (NPO Microgen, Russia) or placebo were administered to 120 Bangladeshi children (4-24 months of age) with severe diarrhea (Sarker *et al.* 2016). Phage administration caused no significant side effects, but the treatment also did not improve the diarrheal outcomes. The intestinal *E. coli* phages did not amplify, and the titers remained low. The replication threshold of T4 is 10^3 CFU/mL, and the bacterial concentrations present were not sufficiently high for the *in vivo* replication to occur once administered orally. Tweaking the formula to contain a higher titer could improve the outcome. It is also important to highlight that only half of the patients that underwent treatment contained phage-sensitive *E. coli* in stool samples. Adaptation of the phage cocktail to kill isolates from the Bangladeshi tested should have been done to achieve greater coverage. Successful case reports and clinical trials without FDA-defined phases demonstrate that phages are safe. However, there is no single successful phase II trial, providing evidence that phage therapy's future path should reckon that customized single phages or phage cocktails have more advantages than non-patient-customized formulations. Despite these previous trial outcomes, a phase I/II clinical trial (NCT03808103) is currently recruiting participants to test the safety and efficacy of EcoActive™ (Intralytix, Inc., USA), targeting adhesive invasive *E. coli* (AIEC) to improve Crohn's disease without affecting the intestines' natural microbiota. For this trial, 30 male or female participants, ≥ 18 years of age with inactive Crohn's disease, will be engaged.

There is only one *in vivo* study using lysins (Table 2). In this work, a single IP administration of 5 μg of LysEF-P10 endolysin was enough to protect mice from lethal vancomycin-resistant Enterococci (VRE). LysEF-P10 presents a broad bactericidal range against antibiotic-sensitive *Enterococcus faecalis* strains and lysed multidrug-resistant strains, including VRE (Cheng *et al.* 2017). The administration did not induce IgM and IgE, which presents a low risk of allergy if repeated administration of LysEF-P10 is needed. Also, no inflammation or mast cell activation in major organs was observed after single or multiple doses. Antibodies against the endolysin were formed after mice treatment; although, preliminary *in vitro* experiments show that anti-LysEF-P10 specific antibodies did not neutralize LysEF-P10's bactericidal activity. Overall, these results are promising and should encourage further research with different lysins for bacterial GI infections. It is important to have sufficient pre-clinical data and, hopefully, also clinical interest to move studies forward to safety and efficacy studies in human volunteers.

In vitro and *in vivo* evaluation of encapsulated phages and lysins

Phages and phage-encoded lysins need to pass through the stomach and intestines to lyse the target bacteria. In there, they will find a unique environment characterized by a low pH in the stomach (pH 1–2 up to pH 4–5) (Fig. 6) and by the presence of pancreatic enzymes and bile salts in the small intestine (Beasley *et al.* 2015). These conditions can compromise phages' viability and stability by modifying the phage's structural components and nucleic acids (Ackermann, Tremblay and Moineau 2004; Jończyk *et al.* 2011; Ly-Chatain 2014). Besides, the *in vivo* GI tract conditions (e.g., peristaltic motion, complex microbiota, and diverse individual diets) can profoundly impact the phages and lysins, and the response may even be phage morphology dependent. Different phages have distinct stability at different pH ranges (Jończyk *et al.* 2011), and to increase their survival in the acidic conditions, these can be administered together with antacids or other acid-neutralizers shortly after feeding or encapsulating the phage within a protective carrier (Verthé *et al.* 2004; Brüssow 2005; Tanji *et al.* 2005).

The protective potential of encapsulation against low gastric pH has been shown in several studies (Table 3). Encapsulation in natural biopolymeric matrices is gaining attention since they are mainly insensitive to the stomach's acidic environment (Fig. 4) (Dini *et al.* 2012). T4 phage entrapped in mannitol-alginate dry macrospheres decreased only slightly in titer in an acidic environment simulating the gastric fluid contrarily to nonencapsulated phage (Śliwka *et al.* 2019). Phage K also remained more stable when encapsulated into alginate-whey protein microspheres, maintaining viability even at a pH of 2.5 (Tang *et al.* 2015). Dry powder phage preparations make storage, transportation, and application easier to accomplish (Vinner *et al.* 2019) but require optimization since the dehydration process can cause a loss in viability (Kim, Jo and Ahn 2015; Moghtader, Eğri and Piskin 2017; Vinner *et al.* 2017; Vinner and Malik 2018).

A certain amount of free phages in the circulatory system activates macrophages that remove them to the liver and spleen (Inchley 1969; Uchiyama *et al.* 2009). Encapsulation can protect phages from the host immune system delaying this activation and phage clearance. For instance, the small intestine contains M-cells with high transcytosis capacity, few lysosomes, and a thinner mucous glycocalyx (He *et al.* 2019). These characteristics favor the access, uptake, and transport of positively charged particles, such as the liposome capsules, enhancing the systemic phage bioavailability (Li *et al.* 2016; Yu *et al.* 2016). Encapsulation in liposomes also increases phage stability after oral delivery, improves their retention in the mouse stomach, persisting there, and in the intestinal membrane for extended periods (Otero *et al.* 2019).

INFECTIONS IN THE INTEGUMENTARY SYSTEM

The integumentary system is composed of the skin, consisting of three layers and its appendages that include hair follicles, nails, sebaceous, and sweat glands (eccrine and apocrine) (Fig. 7).

The skin is the largest organ in the human body, providing a physical protective barrier to internal organs against environmental stresses (e.g., ultraviolet radiation, physical damage, temperature variations) and assault by foreign agents or toxic substances (Grice and Segre 2011; Diegel, Danilenko and Wojcinski 2018). It also excretes waste, regulates the temperature through sweat, and further supports all underlying tissues (Baker 2019). Skin is constituted by a complex but harmless and beneficial microbiota, including bacteria, fungi, and viruses (Grice and Segre 2011). Each individual possesses a unique and specific skin microbiota depending on the exposure to different settings during infancy and adulthood when it stabilizes (Ying *et al.* 2015; Oh *et al.* 2016). However, under stress, this symbiotic relationship disrupts changing into a dysbiotic relationship resulting in integumentary infections (IGI) (Abdallah, Mijouin and Pichon 2017). Primary bacterial colonizers of the skin such as *Staphylococcus epidermidis* (related to nosocomial infections derived from contamination of medical devices such as catheters or heart valves) (Otto 2009), *S. aureus* (atopic dermatitis) (Jagadeesan *et al.* 2014; Totté *et al.* 2016), *Corynebacterium* spp., *Brevibacterium* spp., *Micrococcus* spp., and *Acinetobacter* spp. have also been reported to cause skin diseases (Kloos and Musselwhite 1975; Blaise *et al.* 2008; Howard *et al.* 2012). Pathogenic bacteria can contribute to persistent inflammation and hinder chronic wound healing (Table 1) [see (Pinto *et al.* 2020) for a more detailed review of chronic wounds, economic burden, incidence, and microorganisms]. For instance, *S. pyogenes*, *Enterococcus* spp. or *P. aeruginosa* are known to colonize many burn wounds, and *S. aureus* is the most frequently isolated bacterium in diabetic foot ulcers (Polavarapu, Ogilvie and Panthaki 2008; Otta, Debata and Swain 2019).

Use of free phages and lysins *in vivo*

Commonly, antibiotics are used to treat bacterial infections that cause IGI (Table 1). Alternative treatments using phages have been suggested to kill specific bacteria (Table 2) using different routes (Fig. 3).

Studies of topical phage treatment were efficacious in the treatment of skin infections in animal models. Topical application of phages significantly decreased infection, the period of epithelization, and wound contraction in *A. baumannii*-challenged uncontrolled diabetic rats when compared with antibiotic-treated and the control groups (Shivaswamy *et al.* 2015), or decreased wound size in mice infected with *A. baumannii* without adverse effects (Rouse *et al.* 2020). The combination of topical phage therapy with sharp debridement in *S.*

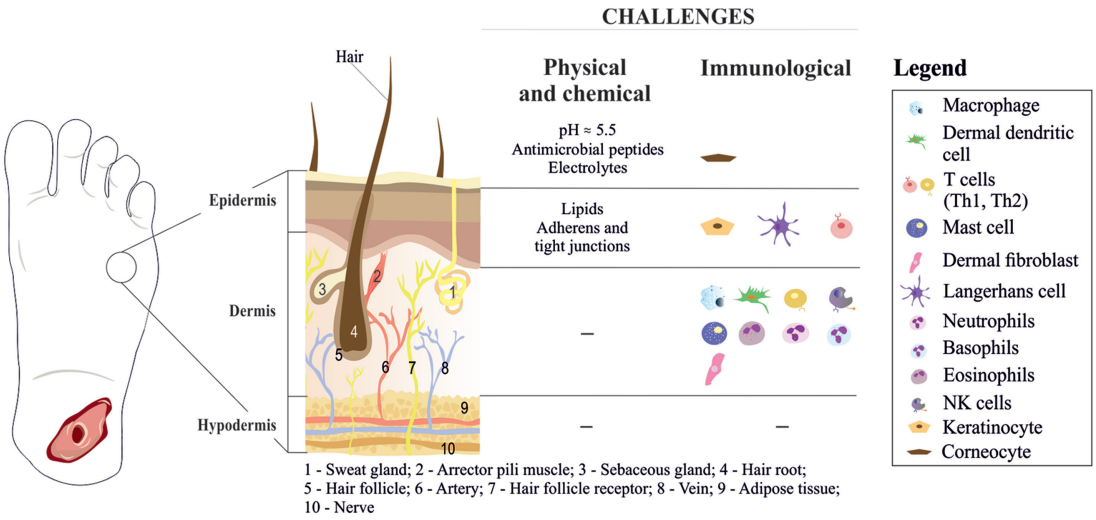


Figure 7. Schematic representation of the integumentary system and the main challenges for phage and endolysin delivery. On the left side, a representation of a foot ulcer and the different layers of healthy skin. On the right side are highlighted the primary physical, chemical, and immunological barriers.

aureus biofilm-infected wounds (rabbit ear model) disturbed the extracellular biofilm matrix and increased the phages' penetration into the biofilm layers (Seth *et al.* 2013). In treating a mouse model of a *P. aeruginosa* burn infection, intraperitoneal administration was more effective than intramuscular or subcutaneous administration (McVay, Velásquez and Fralick 2007). This is explained by the pharmacokinetics studies of phage delivery to the blood, spleen, and liver, as intraperitoneally administered phages were delivered at a higher dose, earlier, and for a more sustained period. Intravenous phage administration immediately after the bacterial challenge was effective in *K. pneumoniae* infected mice burn wounds (Kumari, Harjai and Chhibber 2010a).

Concluded clinical trials and case reports have demonstrated the success of phage therapy treatment in wounds. One case study used a Pyo bacteriophage preparation (NPO Microgen, Russia) to treat two patients with diabetic foot ulcers colonized by Methicillin-Resistant *Staphylococcus aureus* (MRSA). The treatment of this type of infection is challenging for diverse reasons, including reduced microcirculation of the area to be treated, presence of antibiotic-resistant bacteria, a biofilm-related infection that acts as a barrier to antimicrobials, among others. Healing of both wounds took 21 days to 4 weeks, and at the end of the treatment, both patients had no signs of MRSA infection and showed a notable healing improvement of the ulcers (Morozova *et al.* 2018). Although phages have a significant role in the treatment outcome, some steps are necessary before phage application, including debridement to remove necrotic tissue and an antiseptic solution that does not compromise the phage titers (Morozova *et al.* 2018). It is also important to do continuous microbiological monitoring and only stop treatment once the bacteria has decreased in high orders of magnitude (above 3–4 orders of magnitude) or are even absent. Another commercial topical preparation of staphylococcal phage Sb-1 was used to treat nine patients with diabetes, toe ulcers (osteomyelitis, gangrene) infected by *S. aureus* (Fish et al. 2016, 2018). The phage treatment was effective contrarily to the inadequate responses obtained for the antibiotic treatment, including culture-directed oral antibiotics (e.g., levofloxacin, piperacillin/tazobactam) and IV treatment, with a few patients having been considered for amputation (Fish *et al.* 2018). Sb-1 treatment (0.7 cc) was applied to the ulcerated feet once a week for seven weeks. Osteomyelitis increased the difficulty of successful treatment delaying the ulcer healing. However, complete healing of these was achieved within two months with no recurrence, at least after one year. A phase I clinical trial using *P. aeruginosa*, *S. aureus*, and *E. coli* phages, including 42 patients with chronic venous leg ulcers, showed that phages did not cause undesired side effects were safe to use (Rhoads *et al.* 2009). The wounds healed quickly (12 to 24 weeks). This report also shows that two patients who dropped out of the study and were not given the full treatment saw an effective reduction in the affected area. These case reports, where some of the patients had been offered phage therapy as a last resource before amputation, prove that effective wound healing is possible even when antibiotics fail to do so.

PhagoBurn (NCT02116010), a randomized, controlled, double-blind phase I/II trial, was stopped in 2017 due to the insufficient efficacy of the phage cocktail's low doses. The study design aimed to compare the efficacy and tolerability of a cocktail of *P. aeruginosa* phages with the standard of care for patients with burns. Even though the trial ended earlier than expected, significant advances in phage therapy were achieved, particularly regarding the scientific discussion that led to phage compassionate use approval and the first GMP-like phage production (Ministere de la defense 2017; Jault *et al.* 2019).

There are currently two approved clinical trials recruiting individuals. PhagoPied (NCT02664740, phase I and II) will recruit 60 adults over 18 years of age with type 1 or type 2 diabetes, and a wound below the ankle mono-infected with MRSA. The trial will evaluate the topical application of a phage cocktail associated with a standard treatment compared to treatment placebo only. Sterile compress dressings impregnated with a phage solution (10^7 PFU/mL) will be administered thrice (days 0, 7, and 21), while the control group will receive sterile compress dressings impregnated with a placebo solution. The other trial is a phase I, randomized, open-label, active-controlled trial (NCT04323475) and aims to assess the safety and tolerability of phage cocktail-SPK to prevent and treat burns susceptible to infection or infected by *S. aureus*, *P. aeruginosa*, or *K. pneumoniae* species. This cocktail containing 14 phages (final concentration 10^5 PFU/cm^2 of a burned area) will be used as an adjunct to the standard therapy (Xeroform primary dressing, a Melolin interface, and a crepe or Kenacomb for participants with diagnosed or suspected local

infections) on 12 participants ≥18 years of age with second-degree burns covering less than 10% of total body surface area with no need of surgical intervention.

Endolysins are also suggested as an effective strategy for targeting multidrug and biofilm-forming bacteria commonly present in wounds (Chopra, Harjai and Chhibber 2016; Totté, van Doorn and Pasmans 2017). LysGH15, a lysin derived from the staphylococcal phage GH15, and apigenin, a flavonoid with recognized anti-inflammatory and antioxidant activity in chronic inflammation and skin inflammation, were added into an emollient ointment commercially prepared, named Aquaphor, to form a LysGH15-api-Aquaphor ointment (Cheng *et al.* 2018a). The product exhibited bactericidal activity against the target bacterium in an MRSA-infected mouse model, improving wound healing by inhibiting hemolysis and reducing the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ), which are involved in cell proliferation, inflammation and immunity. Increased levels of these cytokines are common when an infection occurs. The ointment reduced bacterial counts in 4 days and helped to accelerate wound healing.

In a case study including three patients with chronic *S. aureus*-related dermatoses, a recombinant endolysin (Staphefekt SA.100) was topically administrated (Totté, van Doorn and Pasmans 2017). No resistance induction was observed, and the results indicate that this treatment improved eczema and decreased pustules within the first month. Improvements continued until the end of the treatment, suggesting Staphefekt SA.100 might be an attractive alternative for traditional antibiotic therapy. Staphefekt SA.100 in a cetomacrogol-based cream was also tested in a multi-center intervention study double-blind and randomized design (NCT02840955) (Totté *et al.* 2017). A hundred participants with moderate and severe atopic dermatitis were treated topically for 12 weeks, either with Staphefekt SA.100 or with placebo treatment (cetomacrogol-based cream alone). The data collected aimed to detect the effect of long-term anti-staphylococcal therapy with Staphefekt SA.100 on corticosteroid use and follow the clinical symptoms and quality of patients' life. The study concluded that the endolysin was well tolerated but had no topical corticosteroid-sparing effect in atopic dermatitis patients. However, a lack of compliance with the treatment and the application of concomitant products might have masked the clinical benefit (de Wit *et al.* 2019).

The combined use of phages and endolysins has demonstrated good antibacterial activity against *A. baumannii* (Wu *et al.* 2019). Phage vB_AbaP_PD-6A3 (PD-6A3) and its encoded endolysin Ply6A3 applied by IP injection to mice with lethal *A. baumannii* sepsis were successfully rescued. The formulation also shows therapeutic potential against clinical multidrug-resistant *A. baumannii* strains.

The direct or indirect influence of the mammalian host immune system on phages (Fig. 7) has been described in several wound healing studies. For instance, an *A. baumannii* phage mixture (AB5075) administered to wounded mice was safe, effective, and caused no adverse reactions. However, the treatment down-regulated the levels of cytokine/chemokine responsible for the maturation and function of monocytes (e.g., G-CSF, IL12 (p40), IL-13, among others), which further decreased after a second administration (Rouse *et al.* 2020), reducing the risk of tissue damage. The AB5075 treatment also induced Ig2a and Ig2b antibodies, which continuously increased after a second administration, and when tested *in vitro*, these showed phage neutralizing action. However, the pro-inflammatory (e.g., IFNγ and TNFα) and anti-inflammatory (IL-4 and IL-10) markers were below detectable levels due to the decreased bacterial numbers in the wound bed. Whole blood samples of animals from untreated and treated groups revealed no significant differences in the number of immune cell populations and the frequency of T-cells, B-cells, dendritic cells, and macrophages. One hypothesis for these similarities may be due to the commensal phages present in the human body that continuously stimulate the immune responses. In another study, phage JD007 prevented dermal abscesses in *S. aureus* (MRSA) challenged-mice through bacterial growth inhibition, causing no severe immune responses (Ding *et al.* 2018). No significant differences were observed in the IFN-γ and TNF-α and cytokine (IL-1β and IL-6) levels in the prevention, infection, and control groups. However, once the infection was established (1 h infection), the phage treatment resulted in higher IL-1β, IL-6 levels, which may lead to fever. Phage therapy also elevated IL-8 levels that activate T cells in the infection site to clear the bacterial pathogen and possibly also the phage. A single IP injection of phage Kpn5 in a mice model of burn wound infection by *K. pneumoniae* B5055 controlled the infection and decreased the mortality (73.3% survivors) (Kumari, Harjai and Chhibber 2010a). Samples of infected sera and lungs from mice treated with the phage had lower pro-inflammatory cytokines (IL-1β, TNF-α) and anti-inflammatory cytokine IL-10 than untreated mice, where cytokine levels in sera and lungs increased gradually over the 72 hours of analysis.

The use of phages clears bacteria and helps balance the host immune system responses avoiding inflammatory damages. Besides, in cases where phage therapy is topically applied, these are less likely to be rejected by the immune system (Kumari, Harjai and Chhibber 2010b). Nonetheless, antimicrobial peptides, the primary skin, which include peptides such as defensins and cathelicidins, are known to increase after skin infection, inflammation or injury, and act on host cells to stimulate cytokine production. The antiviral activity of defensins is known towards adenovirus, papillomavirus, human immunodeficiency virus, and herpes simplex virus [see (Yamasaki and Gallo 2008)], but, to our knowledge, their activity on phages is unknown. Studies on non-infected wounds could shed some light on the role of antimicrobial peptides in skin homeostasis and dynamics in the presence of phage formulations. Also, the role of lipids, such as ceramides, cholesterol, and fatty acids in the intracellular stratum corneum, on phages and lysins is unclear. Skin lipids are known to have antimicrobial characteristics, and they also serve as skin barriers to drug and xenobiotics penetration. Nonetheless, these skin lipids might not have an antiviral action since phages for *Propionibacterium acnes* have already been isolated from lipid-rich skin areas such as the forehead and nostrils (Marinelli *et al.* 2012; Liu *et al.* 2015).

In vitro and *in vivo* evaluation of encapsulated phages and lysins

Encapsulation has been a means to overcome several barriers related to skin delivery (Table 3). Phages have been encapsulated to promote a controlled release and to enhance viable phage persistence at the wound site. Endolysins' encapsulation has aimed to increase their survival at pH values close to five and be thermally-triggered at temperatures similar to those in infected wounds. For instance, a polyvinyl alcohol (PVA)-sodium alginate (SA) hydrogel wound dressing incorporating phage MR10 and antibiotic (minocycline) applied on mice with burn injuries provided a wound healing environment, with the skin surface being able to absorb the phages and antibiotics, which then took care of the local infection (Kaur, Gondil and Chhibber

2019). The antimicrobial dressing reduced the bacterial counts significantly, caused wound contraction, and reduced inflammation. Also, polycaprolactone/collagen I nanofibers (PCL-ColI), with four varying PCL and ColI concentrations, and incorporating phage T4 eliminated *E. coli* infection and established different wound hemostatic time and bleeding (Cheng *et al.* 2018b). The PCL-ColI B (30%:70%, w/w) membrane showed the best *in vitro* antibacterial effect, and the *in vivo* biocompatibility assays of the materials demonstrated that it had the highest degradation time. One week after the implantation in mice, inflammatory and necrotic cells were observed in the surroundings, and eight weeks after, myofibroblast and hair follicle tissues were recovered. Although these are promising results, *in vivo* efficacy of the membranes with incorporated T4 phage remains unknown.

Liposome-encapsulated *K. pneumoniae* phages administered intraperitoneally to mice (wound model) maintained their viability and bioactivity, reaching higher bacterial reductions in blood and major organs (Chadha, Katare and Chhibber 2017). The phage encapsulation protected phages from the immune system, leading to six times longer circulation than the non-encapsulated phages. Thus, immunogenic attenuance reduces cytokine levels (IL-1β and TNF-α) compared to baseline and is a highly significant benefit of encapsulation.

In another study, transfersomes, cationic liposomes composed of phosphatidylcholine, Tween-80, and stearyl amine were employed to enhance permeability (Chhibber, Shukla and Kaur 2017). The intramuscular application of an MRSA phage cocktail in soft tissue infections mediated rats resulted in faster healing of the infection (7 days) than in untreated animals (20 days). In general, the transfersome-entrapped phage cocktail showed better persistence and stability than free phages.

In a study, a group of *E. coli* challenged-rats treated topically with phages in microemulsions demonstrated that these permeated the skin layers rescuing above 83% of the mice from death (Rastogi *et al.* 2017). Additionally, the treated animals showed no sign of tissue damage, lesion, or necrosis, contrarily to the control (saline-receiving) groups, which engendered an inflammatory response against the infection. The authors also measured the fluorescence generated from the reaction of IL-6 with their antibodies, observing a higher intensity of immunofluorescence in treated than non-treated *E. coli* challenged-rats. This result was possibly due to toxin release upon lysis of bacteria, which caused the expression of IL-6 and generated fluorescence, absent in the non-challenged group treated with phages.

To date, there are no encapsulated lysin studies reporting immunogenicity results. However, it is noticeable that the encapsulation process improves lysins' therapeutic outcomes by gaining more stability, shelf life, and increased therapeutic efficacy. An example of this is a study that used an endolysin derived from phage MR-5 (LysMR-5) incorporated in alginate-chitosan nanoparticles (Kaur *et al.* 2020). The nanoformulation caused no detrimental effects on endolysin's physiochemical properties, representing a promising delivery system for the treatment of S. aureus infections. Poly(N-isopropyl acrylamide) (PNIPAM) nanoparticles, used to entrap the truncated CHAPK endolysin and the lysostaphin, responded upon thermal triggered control S. aureus. The skin surface temperature of healthy individuals is around 32°C, but when a wound becomes infected, the temperature increases by approximately 3.6°C (Fierheller and Sibbald 2010). The PNIPAM nanoparticles released the antimicrobials only at temperatures indicative of infection. Besides, CHAPK and lysostaphin's combination acted synergistically, fastening the control and response time for MRSA treatment (Hathaway *et al.* 2017). As described earlier, biofilm formation is a significant obstacle to treatment. Nonetheless, the *S. aureus* endolysin LysRODI encapsulated in pH-sensitive liposomes (loading efficiency of 47%) maintained activity and was released from the liposomes to reduce effectively planktonic and biofilm bacterial counts at pH 5 (Portilla *et al.* 2020).

FUTURE PERSPECTIVE AND CLINICAL PATH

Many bacterial species that cause the type of infections described herein have not been addressed by current phage and endolysin studies (Table 1). Most of these species are fastidious, anaerobic, or spore-forming bacteria, requiring particular nutrients for growth, and additional equipment (such as CO_2 incubator with or without O_2 range control, anaerobic incubator), among other limiting factors. Virulent phages are known for some of these bacterial species, such as the spore-forming *Bacillus cereus* (Lee *et al.* 2013) or the microaerophilic *Campylobacter spp.* (Orquera, Gölz and Hertwig 2012). However, other species are not covered, to date, by any virulent phages. This hampers the eventual use of phage therapy in the clinical setting. A virulent phage infects metabolically active bacterial hosts by attaching to cell surface structures that act as receptors for phages. These structures are absent in spores, and as such, are one of the reasons pointed for the deficiency in isolating phages for infecting spore-forming bacteria (Goh, Riley and Chang 2005). Moreover, the increased proportion and diversity of prophage carriage by certain strains and species may contribute to temperate selection over virulent phages (Hargreaves and Clokie 2014). Although virulent phages have been preferred, the advances in sequencing technologies and synthetic biology tools have started exploring modified temperate phages (Monteiro *et al.* 2019). Soon, it is conceivable that new collections gathering synthetically built phages can be established, or be added to existing phage collections (e.g., American Type Culture Collection, the Félix d'Hérelle reference center for bacterial viruses), and made available to the community, in a similar way to naturally isolated phages.

The recombinant expression, production, and purification can be a challenge for many proteins. *E. coli* is the most popular endolysin expression platform due to its cost-effectiveness and convenience, with different strains available and compatible plasmids. However, proteolytic degradation and protein misfolding are common. For instance, expression in *E. coli* of LysK results in insoluble inclusion body formation due to the adverse formation of disulfide bonds, which hinder their application (Kashani *et al.* 2017; Love *et al.* 2018). Advances in expression systems and techniques will certainly increase the range of lysins available for different species. The application of engineering techniques has also allowed and will continue to build novel lysins with increased antibacterial activity, specificity, among other characteristics. Endolysins against Gram-positive pathogens have been successfully applied in the clinical setting, with a product classified as a class 1 medical device (Staphefekt^{TM}) already available for human use in intact skin. Importantly, the CF-301 (exebacase) lysin recently completed with success a phase II clinical trial (NCT03163446) that evaluated its safety, tolerability and efficacy in patients with *S. aureus* (including MRSA) bacteremia, and will enter phase III studies. This is a major advantage compared to phages, which have not provided successful large clinical trial efficacy outcomes. Endolysins' application to Gram-negative bacteria has been increasingly studied, from endolysins combined with OM-permeabilizing agents,

endolysins with intrinsic OM permeabilizing activity, and engineered endolysins such as Artilysins. With some of them moving into preclinical development, toxicity concerns due to the release of LPS during the bactericidal lysis, pharmacokinetic issues due to the cell wall's complexity, and immunogenicity need to be systematically addressed (Ghose and Euler 2020).

The use of animal models provides evidence of the safety and efficacy of the therapeutic use of phages and lysins, and their use is increasing and has become vital for immunogenicity studies. The studies do not always predict the therapeutic outcome reliably in humans. However, due to the difficulties, including funding challenges, in pursuing the different phases of well-designed randomized controlled clinical trials in humans, animal experimentation is used by research groups with access to animal facilities, FELASA accredited personnel to perform the experiments or funding to outsource these assays. The rigid regulatory framework has also hindered human testings. Although they are approved for use in food or food surfaces, the unusual pharmacology of phage products that might result in unexpected therapeutic outcomes hinders their more generalized approval for human therapy. Characteristics such as the narrow host range, bacterial resistance, immunogenicity, pharmacokinetics, and economic viability are among the main challenges for the approval. The pharmacokinetics and pharmacodynamics of phages and lysins are still poorly understood. Compared to antibiotics, both resemble in their antibacterial characteristic, but phages have diffusional problems due to their bigger size, limiting the administration of high phage concentrations. Size and dosage are particularly limiting factors when systemic treatments are considered. With low concentrations of phages allied with the many barriers discussed herein that are responsible for their clearance, there will be fewer opportunities for a phage:bacterial interaction, which can decrease the therapeutic outcome. Nonetheless, if phages do reach the infected site, they will replicate, increasing the available phage dosage. This is why encapsulation is so important, maximizing the dosage that reaches the infected area while minimizing the clearance by the immune system so that they may, in the future, be considered viable antibiotic replacements for bacterial infections. Lysins do not have dosage and size limitations, and the approval path may be more similar to that of antibiotics. Their large-scale production and purification can even benefit from existing processes and equipment used, for instance, to produce insulin which is nowadays also produced recombinantly in either *E. coli* or *S. cerevisiae*, similarly to most lysins. This factor can be particularly attractive for pharma companies. However, lysins are prone to proteolysis degradation, and their chemical structure may affect tissue penetration if systemically administered. Nonetheless, this can be circumvented using chimeric lysins capable of intracellular transduction, as reported for *S. aureus* using PGH-CPP (Röhrig *et al.* 2020). Additionally, a few studies have shown that lysins bind to plasma proteins affecting pharmacokinetics (Peng *et al.* 2017). These also are reasons why encapsulation of lysins should be considered for therapeutic applications.

A few patents for phage therapeutics have been granted, and a few clinical trials performed, but no approval by FDA or EMA has been attributed so far. However, today, there are ways to use phages in personalized medicine, including their compassionate use as an experimental therapy, as unapproved therapeutic drugs but only in Australia, France, and Belgium [reviewed in (Pinto *et al.* 2020)]. Since lysins are proteins and not biological entities as phages, their approval should be more straightforward once there is evidence of the pharmacokinetic and pharmacodynamic data that will provide more information about their interaction with the human body. The many *in vitro* works with the encapsulated formulas report highly promising outcomes; however, there is a massive lack of *in vivo* experimentation.

The existing literature is still scarce on works describing encapsulated phages and lysins. Most phage encapsulation and characterization studies date from this last decade. It is worth highlighting that the encapsulation of lysins for clinical environments started only in 2017. This area will foreseeably see significant advances soon due to the benefits that encapsulation technologies provide, including improving their delivery, survivability, and shelf-life. Developing strategies that can deliver these agents, at optimum concentrations, to infections in targeted organs and tissues is critical. More *in vivo* studies are compulsory to demonstrate the efficacy of these encapsulated delivery vehicles and their safety. The delivery systems in use (e.g., liposomes and biopolymeric particles) and the potential discovery of new systems can further enhance distribution in the body, prevent degradation, and reduce their clearance rate.

This review focuses on phage and lysin applications for treating infections in three systems: the respiratory, the digestive, and the integumentary. The future use of phages and lysins for respiratory infections may include their intranasal or topical administration for URTI, such as sinusitis, but the administration route for LRTI is not straight-forward. Even for antibiotics, which are usually administered intravenously to treat lung infections, it is unclear if inhalation could be used as primary or adjuvant (Russell *et al.* 2016). Inhaled antibiotics are commonly used to treat chronic lung infections, such as CF or COPD. This administration mode may be an attractive way to deliver phages for such conditions due to its non-invasiveness and cost-effective option (e.g., minimizes healthcare costs and travel-related expenses) that the patient can apply at home. However, supposing that this is the choice prescribed by a physician, the patients must comply with the recommendations, including duration and treatment frequency. Follow-up consultations should be scheduled to check the outcome and possible continuation or discontinuation of the treatment.

Encapsulation of phages and lysins for treating infections in the digestive system is the foreseeable step for a successful antibacterial treatment. Caging these antibacterial agents as long as necessary in pH-responsive and mucoadhesive carriers of nano- to macroparticle size will avoid early degradation by enzymes upon administration or along the path to the site of infection in the GI tract where they must be fully available and effective for antibacterial action. Although encapsulation has been carried out using some mucoadhesive polymeric materials (e.g., alginate, chitosan), there are many natural, synthetic, biocompatible and biodegradable mucoadhesive polymers that remain unstudied. It is important to improve our understanding of the mucoadhesive materials that can be used for phage and lysin encapsulation to design novel gastroretentive delivery systems (e.g., mucoadhesive tablets and nano and microparticles) and intestinal delivery systems (mucoadhesive patches). Besides carrying phages and lysins, regulating gut microbiota by encapsulating them with probiotics or even prebiotics (e.g., in core-shell particles for dual delivery) may further facilitate food processing but have additional health benefits to humans in individuals with bacterial digestive infections. With the advances in drug delivery, many delivery routes are gathering attention. Skin delivery is part of these systems that are increasingly used for topical and systemic distribution of substances but faces skin penetration limitations that need improvements. While phage and endolysin inclusion in carriers

is advancing at a slow pace, R&D on permeability enhancers and physical means for delivery are still rare. For instance, oncogenic drug and vaccine research has advanced significantly in investigating physical means to deliver the substances transdermally by sonophoresis, electroporation, and thermal ablation. However, these physical techniques may inactivate the phages and lysins but could be focused on future research. Transfollicular delivery is becoming an exciting route for macromolecules due to its several benefits. These include hair follicles crossing different skin layers and even subcutaneous fat, providing access to deeper compartments. The accumulation of substances in the hair follicle canal keeps a constant diffusion to the surrounding epithelia. Additionally, the substances can reach follicular and perifollicular cells, cross capillary walls, and reach the blood system. The substances can also be drained to the lymph nodes and from there reaching the systemic compartment. The path to more generalized use of phages and lysins may also involve a more comprehensive understanding of the mammalian immune system, phagocytes in particular, during the course of these treatments. It is also key to continue understanding the role of phages and lysins in biofilm-related infections and even combine them with other agents. For instance, in respiratory infections, it would be interesting to address the use of phages and lysins together with commonly used mucolytic agents to evaluate if these promote simultaneously bacterial death and mucus clearance in this type of infection.

We live in a critical time where a pandemic is devasting lives, significantly affecting the wellbeing, including mental health, of the general population, sometimes making them take the wrong choices, including the misuse and over-use of prophylactic antibiotics. Antibiotics do not treat viral infections (e.g., COVID-19 and flu), despite their increasing throughout the pandemic months. Reports conducted by WHO/Europe show that some patients believed that by taking antibiotics prophylactically, they would avoid a COVID-19 infection (Manohar, Loh and Leptihn 2020; WHO 2020). Although estimates show that 15% of the severely affected COVID-19 patients develop a bacterial co-infection needing antibiotics to resolve the bacterial infection, over 75% of COVID-19 afflicted patients receive antibiotherapy. The adoption of this redundant measure may cause a vast increase in antibiotic-resistant bacterial strains. In this sense, phage and lysin therapy could be made readily available as standard therapy minimizing foreseeable antibiotic-resistance complications. The high mortality rates in pulmonary diseases caused by severe viral outbreaks due to a secondary bacterial infection are alarming (Zhou *et al.* 2020). Around 50% of the fatalities so far of the SARS-CoV-2 pandemic are caused by an untreated or untreatable secondary bacterial infection that was frequently hospital-acquired and caused by multidrug-resistant species (Cox *et al.* 2020; Vaillancourt and Jorth 2020). Phages and phage-encoded lysins can be potential alternatives or complement conventional antimicrobials to treat these secondary bacterial infections.

CONCLUDING REMARKS

Phages and lysins have killing mechanisms distinct from antibiotics, which consents their use in patients with antibiotic-resistant pathogens or unable to receive them due to adverse reactions to antibiotics. Phages have a narrow spectrum of activity, leaving the commensal flora intact, causing few or no side effects upon administration. Although bacteria can become resistant to phages, there are approaches to circumvent, including administering formulations containing multiple phages and synergistic interactions with other agents (e.g., antibiotics, natural compounds, and lysins) and genetic modification (e.g., receptor-binding protein) to minimize their emergence. On the other hand, lysins are among the most rapid antibacterial agents described, and, to date, bacteria have not been able to elicit any resistance mechanism. Despite these advantages, both phages and lysins can be inactivated. In many research fields, we have witnessed a change from free to encapsulated strategies that offer several benefits, as discussed in this review. Nonetheless, the clinical path to generalized use of phages has to start from raising awareness among physicians that phages and lysins have antibacterial characteristics, even in the presence of antibiotic- and multidrug-resistant bacterial infections. The National Institute of Allergy and Infectious Diseases (NIAID), part of the National Institutes of Health, recently granted their first series of awards focused solely on phage therapy research. Paraphrasing NIAID Director Anthony S. Fauci, M.D. "With these awards, NIAID is supporting research needed to determine if phage therapy might be used in combination with antibiotics-or replace them altogether-in treating evolving antibiotic-resistant bacterial diseases." This statement and NIAID's funding initiative are fundamental for rigorous research. Hopefully, funding schemes similar to the one launched by NIAIDs will be available in other parts of the globe to support research and validate phage and lysin therapeutics for bacterial infections, particularly multidrug-resistant bacterial infections.

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AUTHOR CONTRIBUTIONS

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