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REVIEW

Bacteriophage therapy against plant, animal and human pathogens

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ABSTRACT Bacteriophages are specific infective agents of various bacteria. They can be divided into various groups according to their life cycle. The lytic phages kill their host cells and this property can be applied for selective elimination of pathogenic bacteria. The first bacteriophage treatment was described one hundred years ago, and phage therapy had been extensively used till the Second World War. Upon appearance of antibiotics, the medical application of phages retrograded in most parts of the world. In the last decades, owing to the costs of development of new antibiotics and rapid emergence of multidrug-resistant bacteria, this old approach was revitalized and phage-based treatment was legalized from the middle of the last decade. Here, we summarize the current knowledge on phage therapy, its advantages and potential drawbacks. The application of phages against plant pathogens, especially *Erwinia amylovora* is discussed. Moreover, the current status of phage therapy against food-borne, animal and human pathogens is also presented. Among these, special focus is set on phages of *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes*. Phage cocktails against *L. monocytogenes* and *E. amylovora* have been already commercialized.
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History of bacteriophage therapy

Bacteriophages (phages) are viruses, which are obligate intracellular parasites of bacteria. The total global population of tailed phages, being the most abundant organisms on Earth, is estimated in the order of 10^{31} viral particles (Waldor et al. 2005). According to the 2014 release of Virus Taxonomy by the International Committee on Taxonomy of Viruses, seven orders were established: *Caudovirales* (3 families), *Herpesvirales* (3 families), *Legamenvirales* (3 families), *Mononegavirales* (3 families), *Nidovirales* (3 families), *Picornavirales* (3 families) and *Tymovirales* (3 families). Seventy-eight virus families could not be assigned to these orders (http://www.ictvonline.org/virusTaxonomy.asp). The tailed phages constitute the largest group, the order *Caudovirales*. On the basis of length and contractibility of the tails, it has three families: *Myoviridae*, *Siphoviridae* and *Podoviridae.* Bacteriophages can be divided into various groups according to their life cycle (Fig. 1); they can productively infect the host bacterium resulting in more viruses or can get into a latent state when their genomes are integrated into the host cell DNA. In the latter case, the virus is multiplied with the host genome. During the productive infection, phages can lyse the cells or virions can be released without cell lysis (Waldor et al. 2005). The lytic phages are able to kill the target cells while the lysogenic/temperate phages become part of the genome of the host cells and stay there for a time. Under special environmental conditions, the life cycle of these phages might switch to productive phase, the phage genome is cut off from the host DNA, packed into a protein shell and the mature phages lyse the cells. Phage therapy is simply destroying the pathogenic bacteria by their natural infecting agents, bacteriophages. For pathogen elimination, the most important viruses are the lytic phages. Lysogeny must be avoided since it can lead to unwished gene transfer. Consequently, the exclusively lytic phages can be considered as real biocontrol agents. Nevertheless, temperate phages can be often converted into obligately lytic ones.

The utilization of phages to destroy bacterial infections is nearly as old as the discovery of the bacteriophages. The English bacteriologist Frederick Twort published the first observation of a transmissible bacterial lysing agent for *Staphylococcus* (Twort 1915). In 1917, a French-Canadian microbiologist, Felix d'Herelle discovered an invisible antagonist of

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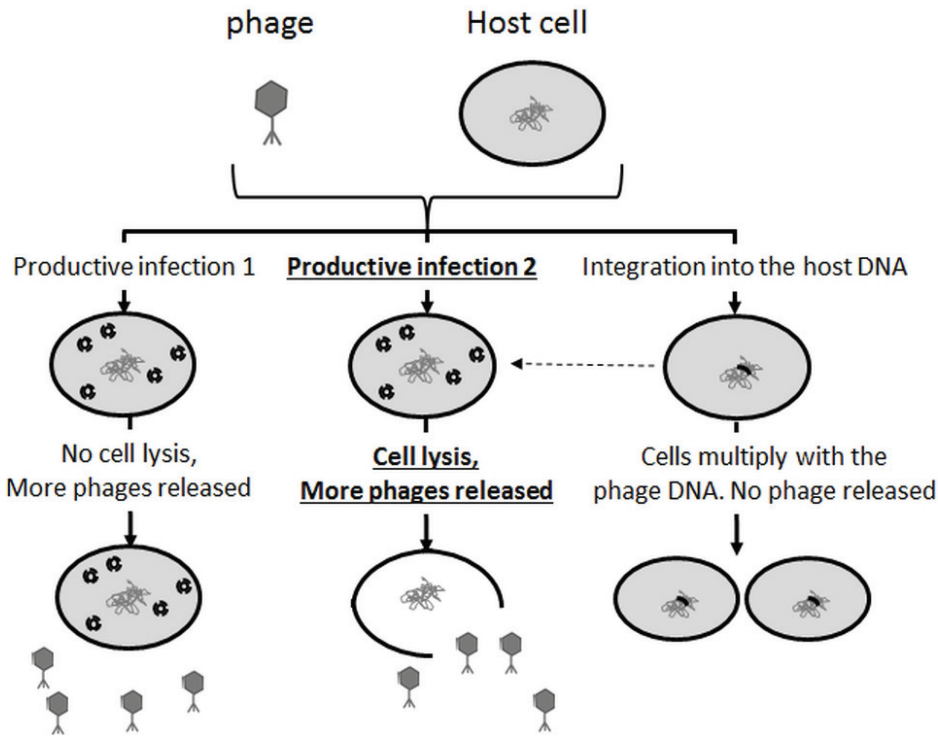

Figure 1. Life cycles of bacteriophages. The therapeutically applicable phage life cycle is set in bold.

dysentery bacillus. A little bit later, Bruynoghe and Maisin (1921) applied bacteriophages to eliminate staphylococcal cells from skin, thus this work was the first public application of phages to treat infectious diseases. Phage therapy has been expanded in the 1920s-1940s for numerous pathogenic bacteria and at that time at least three commercial anti-staphylococcal phage preparations were available on the market produced by Eli Lilly, E.R. Squibb and Sons and Swan-Myers (Straub and Applebaum 1933). Later, upon the emergence of broad-spectrum antibiotics, the application of phages was forfeited and the virus-related researches were directed into rather theoretical aspects in Western Europe. Nevertheless, in the Soviet Union (mainly in Georgia) and Poland, phage therapeutical treatments were continued and widely practiced, and both theoretical and applied projects on this topic were going on. Unfortunately, the articles focusing on phage-based biocontrol were published in Russian: therefore, they were not readily available/understandable for Western scientists. Moreover, these studies were not performed systematically, and in numerous cases they were not well-controlled.

However, after a long latent period, owing to the emergence of multidrug-resistant bacteria and the increasing cost/time of development of new antibiotics and antimicrobial drugs, the interest was redirected towards phage therapy (Chanishvili 2012a, 2012b).

Advantages and limitations of bacteriophages versus other approaches

The application of phages has numerous substantial advantages (Villa and Veiga-Crespo 2010):

1) phages can be easily propagated, moreover, they can be produced at the site of the potential application, at the site of infection;

2) their production is fast and cheap;

3) bacteriophages are specific for their host cells, they do not destroy other beneficial microbes in the normal microbiota;

4) their application has minimal – if any – side effects; one of the most comprehensive human safety tests, performed by Delmont Laboratories, demonstrated only minor side effects in the patients after 12 years (Kutter and Sulakvelidze 2005). In that study, various administration techniques were applied and compared: the phages were introduced to humans intranasally, topically, orally, subcutaneously and intravenously;

5) they are not, or just minimally allergenic – although, it was shown that numerous antibodies could interact with phages, very few of them could neutralize them (Kutter and Sulakvelidze 2005);

6) they can be introduced into the patients through dif-

ferent routes;

7) their usage can be combined with a number of treatments and drugs, including other phages, phage cocktails or antibiotics;

8) development of novel cocktails against resistant bacteria is faster and cheaper than that of new antibiotics or other antimicrobials;

9) the application range of bacteriophages is much broader than that of antibiotics: they can be applied, *e.g.*, as protective materials in food supplements, in milk industry, in pharmacology as eye drops, in toothpastes, in cleaning solutions (Villa and Veiga-Crespo 2010). The US Food and Drug Administration (FDA) has approved bacteriophages as "generally recognized as safe" (GRAS) agents to control *Listeria monocytogenes* on ready-to-eat meat and poultry products (US FDA 2006).

In spite of the uncountable beneficial properties of phage-based biocontrol, the application of antibacterial phage therapy still faces challenges and problems to be solved: (i) the phages might have too narrow host range: on the one hand, the target specificity has advantages (see "point 3"), but on the other hand, the serotype specificity might reduce their effectivities, which is unwanted; (ii) safe propagation of phages specific for pathogenic strains should be solved on non-pathogenic host; (iii) standardized purification of pathogen-free phages suitable for treatments; (iv) the potential immunogenicity of phages in the human body; (v) the pharmacokinetics of phage treatment, application of phages against encapsulated or internalized bacteria or biofilms (Hermoso et al. 2007).

The release of various pro-inflammatory components, such as *e.g.*, endotoxins and peptidoglycan from the lysed bacterium might also cause problems. In principle, these components could result in a septic shock and multiple organ failure (Nau and Eiffert 2002). Fortunately, many carefully performed experimental studies justified that there were no remarkable side effects after robust lysis of infecting bacteria. Moreover, every treatment aiming to kill the pathogens might be accompanied by this risk, thus it is not specific for phage therapy.

In spite of the potential drawbacks, the phage-based biocontrol and bacteriophage therapy are very promising approaches, therefore, research studies and well-controlled applications in this field are encouraged by international organizations. In the next sections, several examples for phages applicable against plant, animal and human pathogens are discussed.

Phages against plant pathogens

Bacteriophages have been isolated and characterized against many plant pathogenic bacterial species, the most important ones being *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Pseudomonas syringae* pv. *tomato*, *Ralstonia solanacearum*, *Xanthomonas arboricola* pv. *juglandis*, *Xanthomonas arboricola* pv. *pruni*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris* pv. *vesicatoria* (Stonier et al. 1967; Ghei et al. 1968; Lin et al. 1994; Gill et al. 2003; Greer 2005; Bae et al. 2012; Dömötör et al. 2012; Romero-Suarez et al. 2012; Yamada 2013; Chae et al. 2014).

Application of bacteriophages against plant pathogenic bacteria as biopesticides opens possibilities for the control of devastating bacterial diseases, against which no effective measures exist. Therefore, besides the scientific importance, an economic interest also girdles around the results of characterization and application of phages infecting plant pathogenic bacteria. Application of phages as biopesticides has several unique features originating - at least partly - from the environment of usage.

The number of phages located in the soil is estimated to be 1.5×10^8 g soil, which is about 4% of the total bacterial population that could be found here (Ashelford et al. 2003). Phages on the field face themselves with the unfavorable circumstances of the rhizosphere, which comprises the soil and the environment surrounding the roots of plants. The most factors which can negatively influence the disease control in the rhizosphere are summarized in the review of Jones et al. (2007). Phages can be trapped in biofilms (Storey and Ashbolt 2001), adsorbed to soil particles reversibly and inactivated by low pH present in the soil (Jones et al. 2007). The rate of diffusion through the soil matrix is low and changing, as it depends on the presence of free water (Jones et al. 2007). Physical objects can hinder phages to reach host bacteria. A serious problem is that a high number of host bacteria and infectious phages are needed in order to start a chain reaction of bacterial lysis (Jones et al. 2007).

The phyllosphere provides a harsh environment for bacteriophages, where they will be inactivated rapidly (Jones et al. 2007). The most limiting factor is UV irradiation (Ignoffo and Garcia 1994), however, high and low pH, exposure to high temperature, desiccation and being washed away by rain can also seriously decrease the number of phages able to infect host bacteria (Civerolo and Keil 1969; Ignoffo et al. 1989; Ignoffo and Garcia 1992; Balogh et al. 2003). How a biopesticide can be applied under these unfavorable circumstances will be detailed on the example of *E. amylovora* phages.

E. amylovora, a Gram-negative bacterium, member of *Enterobacteriaceae*, is the causative agent of a devastating wilt disease - called fire blight - of some rosaceous plants (van der Zwet and Beer 1995). Apple, pear and quince should be highlighted as being economically especially important among its host plants. The infection cycle begins in spring with elevation of temperature when *E. amylovora* colonizes open blossoms, multiplies and infect the trees through natural

openings (van der Zwet and Beer 1995). The bacterium overwinters at the margin of cankers and becomes active again in the spring (van der Zwet and Beer 1995). Control of fire blight is a hard challenge especially in countries where streptomycin is not registered. An alternative solution for the treatment of this disease is the application of bacteriophages.

Phages against *E. amylovora* were isolated mostly from the soil surrounding infected trees but a few isolates originated from infected aerial tissues (Erskine 1973; Ritchie and Klos 1977; Schnabel and Jones 2001; Gill et al. 2003; Born et al. 2011; Dömötör et al. 2012; Meczker et al. 2014; Yagubi et al. 2014; Lagonenko et al. 2015). *E. amylovora* phages were extensively characterized (Gill et al. 2003; Born et al. 2011; Dömötör et al. 2012; Born et al. 2014; Meczker et al. 2014; Yagubi et al. 2014; Lagonenko et al. 2015). All *E. amylovora* phages belong to *Caudovirales* with all three of its families (*Podoviridae*, *Myoviridae* and *Siphoviridae*) represented. Several phages have a narrow host range (Gill et al. 2003) but others are able to infect *Pantoea agglomerans* (Gill et al. 2003) and other members of the *Enterobacteriaceae* family, as well (Kovács et al., unpublished results). Their genome sizes vary between 30 and 271 kb (Dömötör et al. 2012; Meczker et al. 2014; Yagubi et al. 2014; Lagonenko et al. 2015). Plant and field trials (Erskine 1973; Schnabel et al. 1999; Svircev et al. 2010; Nagy et al. 2012) have shown the potential applicability of these phages against *E. amylovora*. Schnabel et al. (1999) registered a 37% decrease of fire blight symptoms in a field study where a mixture of three phages was used to spray on a marked strain of *E. amylovora*. The success of several other trials (Ritchie and Klos 1977; Schnabel and Jones 2001) was limited due to the fact that the number of phage populations declined rapidly in the absence of the host pathogen because of unfavorable circumstances in the phyllosphere. Schnabel and Jones (2001) suggested applying a nonpathogenic (mutant) *E. amylovora* strain together with the administration of phages to maintain the number of the latter ones, however, this would be too risky due to the chance of reversion to a virulent phenotype. Svircev et al. (2010) advocated applying *Pantoea agglomerans*, a nonpathogenic epiphyte abundant in fruit orchards, together with *E. amylovora* phages. A *P. agglomerans* strain which could be infected by many phages (32 from the tested 54) and can produce as high titer as $>10^8$ plaque-forming units (PFUs)/ml of viruses was selected through a sequential process. The co-application of this saprophyte with *E. amylovora* phages can maintain a high level of viruses until the pathogenic host is present in the phyllosphere (Lehman 2007).

Another possibility to protect phages against UV is the utilization of UV-protecting agents as adjuvants, as it was performed in the case of two patents (Hu P0700600 and Hu P1300407) applying a cocktail of extensively characterized phages together with the adjuvant. Based on successful authority trials with this formulation (publication of results is in progress) a new phage-based pesticide has been authorized in Hungary since 2012. This is the first anti-*E. amylovora* phage therapeutic product which was marketed in a country. This product has been applied on apple, pear and quince fields and user's satisfaction was mainly positive. Negative results were partly connected with improper usage of the biopesticide. The application of this product was difficult because it had to be sprayed after sunset, three times during blooming, and contact with copper had to be avoided. Also freezing of phages had to be prevented, but the flasks containing the active components should be kept cooled. In several cases, the water, which was used for the dilution of the pesticide, was polluted with heavy metals which decreased the number of infective viruses, as demonstrated during laboratory trials (Kovács, unpublished results).

An important question is whether phages can be taken up by the plants and can reach *E. amylovora* also inside the plant, or they can act only as contact agents. Preliminary results showed that phages can pass into the plants when sprayed into the phyllosphere and will be translocated towards the roots in apple seedlings (Kovács et al. 2012). More recently these results were supported by new trials and the possibility of phage uptake through the roots was also proved (Nagy et al. 2015). The severity of symptoms caused by *E. amylovora* decreased due to the penetrated and translocated phages in apple seedlings (Nagy et al. 2015). However, whether the fact that phages can enter the plants and will be translocated has any significant anti-*E. amylovora* effect in the orchard, in the case of apple trees, should be clarified. A further question which should be elucidated is the mechanism of the uptake and systematic movement of bacteriophages, taken into consideration that the cell-to-cell movements of macromolecular complexes and viruses are hindered by the size exclusion limit of plasmodesmata, which obviously would exclude bacteriophages. Plant viruses developed strategies (usually their genomes encode virus movement proteins) to overcome the barrier of plasmodesmata (Benitez-Alfonso et al. 2010), however, genes coding for similar proteins have not been found in the case of any sequenced genomes of phages infecting plant pathogenic bacteria.

Two phage-therapeutic pesticides have been registered and marketed worldwide until now. The first was registered by OmniLytics in the USA against *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*, and the second one by Enviroinvest in Hungary against *E. amylovora*. The broad application of phage-based products in crop protection is seriously influenced by the registration procedure of these biopesticides. According to the legal regulations of the EU (1107/2009 EC), a bacteriophage cocktail which will be altered in only one of its components should be reregistered, which would need significant time and capital investment. Taken into consideration that pathogenic host bacteria in the orchard may acquire resistance against the applied phage-

based pesticide rapidly after the treatment (Jones et al. 2007), the need of changing the composition of phage cocktails is an important factor, which cannot be followed by the current registration process. It would be crucial to change the relevant rules in such sense that the need of characterization of phages prior on site application should be standardized and changing the phage composition of pesticides should be allowed.

Based on the data presented in this paper, application of bacteriophages is a promising tool for the control of plant pathogenic bacteria on the fields.

Phages against human/animal (food-borne) pathogens

Staphylococcus aureus and Pseudomonas aeruginosa

Bacteriophages have specific scientific interest in view of their ability to kill multidrug-resistant (MDR) bacteria without disturbing the normal microbiota (Gandham 2015). In human health care, *S. aureus* and *P. aeruginosa* belong to the most frequent MDR pathogens causing both nosocomial and community-acquired infections (Nordmann et al. 2007; WHO 2012). The emergence of these MDR pathogens is increasingly reported worldwide (Chastre 2008).

Bacterial infections are often difficult to treat or untreatable and can be life-threatening. In humans, *S. aureus* can cause endocarditis, osteomyelitis, meningitis, pneumonia, septicemia and toxic shock syndrome (Lowy 1998). *P. aeruginosa* is related to infections in humans mainly in predisposed immunocompromised patients due to chemotherapy, transplantations and HIV-infection. It can cause respiratory tract, urinary tract, ear, eye and burn wound infections (Lyczak et al. 2000).

Phages against *S. aureus* and *P. aeruginosa* are widespread and have been extensively studied (Hayashi and Nakayama 2004; Ceyssens and Lavigne 2010; Deghorain and Van Melderen 2012; Xia and Wolz 2014). They were firstly used for the typing of clinical isolates of these two bacterial species (Blair and Williams 1961; Wentworth 1963; Bergan 1978).

All known *S. aureus* phages are double-stranded DNA phages belonging to the order *Caudovirales* (Deghorain and Van Melderen 2012; Xia and Wolz 2014). They can be further classified into three major families based on tail types: *Podoviridae* (very short tail), *Siphoviridae* (long, non-contractile tail) and *Myoviridae* (long, contractile, double-sheathed tail). According to the genome size of *S. aureus* phages, three categories (which correlate with the morphological classification) can be established: podoviruses have the smallest genomes (16-18 kbp), siphoviruses show intermediate genome sizes (39-43 kbp) and myoviruses contain the largest ones (120-140 kbp) (Kwan et al. 2005).

The majority of the described *P. aeruginosa*-specific phages belong to the order *Caudovirales.* Representatives can be found in all three families of *Caudovirales*. Some *P. aeruginosa* phages have single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA) and can be classified into the families *Inoviridae* or *Leviviridae* (Hayashi and Nakayama 2004; Ceyssens and Lavigne 2010). In contrast to phages of *S. aureus*, the genome sizes of most *P. aeruginosa*-specific phages are uniformly distributed from 35 to 75 kbp (Kwan et al. 2006) with the exception of the large PhiKZ-like viruses which have genomes of 210 to 280 kbp (Krylov et al. 2007).

The first report on the medical application of phages was published by Bruynoghe and Maisin (1921). They treated skin disorders caused by *S. aureus*. Injection of staphylococcal phages resulted in improvement within 48 hours and reduction in pain, swelling and fever. Both *S. aureus* and *P. aeruginosa* were among the most common targets of phage therapy and are still common today. Several companies in Europe and the USA (L'Oréal, Eli Lilly, Squibb & Sons, Parke-Davis, Swan-Myers Division of Abbott Laboratories) manufactured and sold therapeutic phage preparations (monophage, cocktail or phage lysate) against these pathogens (Straub and Applebaum 1933; Sulakvelidze et al. 2001).

A wide range of encouraging studies documented the efficacy of specific phages or phage cocktails in both combined phage and antibiotic therapy and phage therapy alone. These early phage therapy trials established methods for the oral, subcutaneous, intramuscular and intravenous application of phage preparations. Phages have also been used in prophylactic and sanitation experiments (Chanishvili et al. 2012b).

The most comprehensive English language report on phage therapy in humans was published by Slopek and co-workers (1987). They treated 550 patients suffering from infections with antibiotic-resistant *Staphylococcus*, *Pseudomonas*, *Escherichia coli*, *Klebsiella*, *Salmonella* and *Streptococcus* between 1981 and 1986. The success rate ranged from 75% to 100% depending on the bacterium. They observed significant improvement in polymicrobial infection as well as in cases where antibiotic therapy was ineffective (Slopek et al. 1987).

The above-mentioned, encouraging case studies had some deficiencies, such as the lack of proper controls, the insufficient purity of phage preparations or the small numbers of patients. This could be a reason why phage therapy is not used or accepted in most parts of the world. In the future, controlled and regulated clinical investigations should be carried out to introduce phage therapy as a routine medical procedure. Phage preparations applied for human clinical trials or therapy must be carefully studied under various conditions to reduce many potential safety concerns. Identification, morphology and biology of phages must be determined using such pro-

cedures as electron microscopy (EM), host range analysis, restriction fragment length polymorphism (RFLP) and one step growth curve analysis. Only those fully sequenced and analyzed phages are appropriate for further use, for which the phage genome and proteome analysis demonstrate that the phages are really virulent (killing bacteria under all conditions), their genomes do not encode toxins and other factors of pathogenicity and virulence and do not have the ability to participate in a process of horizontal gene transfer. Stringent purification of phage preparation is required to remove remaining uninfected cells, post-lysis bacterial ghosts and some other bacterial lysis products such as endotoxins. Ultracentrifugation, a series of filtration steps, or various forms of chromatography can be involved in the purification process. The elaborate quality control must include stability (shelf life), determination of pyrogenicity, sterility and cytotoxicity, ratification of the lack of temperate phages and confirmation of the presence of the expected virion using transmission EM (Merabishvili et al. 2009; Pirnay et al. 2015).

The possible consequences and safety of phage therapy can be estimated from animal studies. Many successful preclinical studies in animals using phage therapy were performed. The most frequently used animal model is a mouse model to establish an effective and advanced phage therapy system against infections caused by *S. aureus* or *P. aeruginosa* (Soothil 1992; Matsuzaki et al. 2003; Wang et al. 2006; Capparelli et al. 2007; McVay et al. 2007; Watanabe et al. 2007; Vinodkumar et al. 2008; Debarbieux et al. 2010; Morello et al. 2011; Alemayehu et al. 2012). Some of these studies have shown phage therapy to be effective in the prevention of infection in mice caused by antibiotic-resistant bacteria (Wang et al. 2006; Capparelli et al. 2007; Vinodkumar et al. 2008). There are some examples for phage therapy using another animal model such as guinea pig (Soothill 1994), rabbit (Wills et al. 2005), dog (Marza et al. 2006; Hawkins et al. 2010) or insect (Heo et al. 2009). The scientists developed methods for the intramuscular, intravenous, intraperitoneal, subcutaneous, intranasal and oral administration of phage preparations. Morello and co-workers (2011) showed that if they adapted the phage to the bacterial strain by repeated subculturing, phages were more effective in the treatment of lung infection in mice than the unadapted ones. Two research groups (Debarbieux et al. 2010; Alemayehu et al. 2012) used bioluminescent *P. aeruginosa* strain in mouse model to monitor in real-time the eradication of the infection by phages. They demonstrated a rapid killing of bacteria in phage-treated animals without the need of sacrificing animals.

These animal studies have offered data that may be useful in rational planning of human clinical trials. To date, very few human clinical trials have been conducted according to Western regulatory standards. Only four phase I and one phase II studies have been carried out and published. Two of the four phase I clinical trials targeted MDR *Pseudomonas* and methicillin-resistant *S. aureus* (MRSA) infections in venous leg ulcers (Intralytix) and burn patients (Belgian military) to evaluate the safety of phages (Kutter et al. 2010; Harper 2012). The former was completed in 2008 at the Southwest Regional Wound Care Center in Lubbock, Texas. This study used "WPP-201", a cocktail of eight phages prepared by the company Intralytix. The cocktail contained two phages active against *S. aureus*, five against *P. aeruginosa* and one against *E. coli* with a concentration of each at 1×10^9 PFU/ml in phosphate-buffered saline solution. Twenty-one patients received phage cocktail and 18 served as control who received sterile phosphate-buffered saline. According to the results of this trial, no adverse effects were observed associated with the application of this multi-bacteriophage preparation (Rhoads et al. 2009). Another phase I safety trial of phage therapy against these two pathogens was carried out in 2009 in Belgium. The studied phage cocktail "BFC-1" consisting of three exclusively lytic bacteriophages was produced by Merabishvili and co-workers (2009). No adverse effects were observed using the phage cocktail at a concentration of 1×10^9 PFU/ml of each of the 3 phage types (Kutter et al. 2010).

The only Phase II clinical trial with a phage therapeutic agent was carried out by the British company Biocontrol Limited in 2007 (Wright et al. 2009). Following promising results in dogs suffering from chronic otitis caused by antibiotic-resistant *P. aeruginosa* (Hawkins et al. 2010), they performed a double-blind placebo-controlled, randomized phase I/II clinical trial in humans for otitis. The used phage cocktail Biophage-PA consisted of six phages. Twenty-four patients with otitis externa were chosen whose infection was caused by *Pseudomonas* strains which were sensitive to at least one of these six phages. Half of the selected patients were treated topically with a single dose containing 10^5 phages of each of the 6 phage types. Another half of the selected patients received 10% glycerol phosphate-buffered saline diluent as placebo. The results of the study revealed that the presence of bacteria and the symptoms of the disease reduced in the treatment group but not in the placebo group. Complete bacterial eradication was not achieved, but this may be due to the relatively low titer of phages used in single dose. There were no remarkable side effects. The increasing number of all test phages *in situ* indicated that active therapy had occurred (Wright et al. 2009).

Based on the above-mentioned results, bacteriophages could play an important role in eliminating MDR bacteria in humans in the future and offer the most cost-effective alternative to antibiotics.

Listeria monocytogenes

Out of the six members of the genus *Listeria* (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*), only two are considered pathogenic: *L. mono-*

Table 1. Numbers and rates of confirmed listeriosis reported cases, EU, 2008-2012 (ECDC 2014).

	2012	2011	2010	2009	2008
Number of confirmed cases	1642	1515	1663	1675	1425
Case rate per 100000 population	0.37	0.31	0.35	0.35	0.30

cytogenes which is able to infect animals and humans, and *L. ivanovii* infecting animals only. *L. monocytogenes* is a Gram-positive, rod-shaped, facultative anaerobic bacterium. It's motile at room temperature, but does not synthesize flagella at 37 °C. This human pathogenic microorganism is a food-borne pathogen and causes listeriosis. The infection can have the following symptoms: gastroenteritis, meningitis, neuro-encephalitis, chorioamnionitis, abortion and neonatal infections. Prominently in immunocompromised patients, the mortality rates are relatively high (Dworkin et al. 2006).

The bacteria from the genus *Listeria* are able to grow at low temperatures and high salt concentrations. These conditions are used to preserve ready-to-eat (RTE) foods, thus making them ideal carriers of *L. monocytogenes*. According to the European Centre for Disease Prevention and Control (ECDC), listeriosis is an uncommon disease in the European Union, with an average of 1500 cases per year from 2008 till 2012 (Table 1). The number of cases remained constant over the years, but with a high case-fatality ratio with a minimum of 12.1%. The disease infected particularly pregnant woman and elderly people over 65 years (Dworkin et al. 2006; ECDC 2014).

Till this date, the genome sequences of 26 *L. monocytogenes* phages have been determined. All of the aforementioned phages belong to the order *Caudovirales*. *Siphoviridae* phages tend to have smaller genomes in the range of 35.64 to 67.17 kb, while *Myoviridae* phages usually have genomes over a hundred kilobases from 131.38 to 138.04 kb. So far, no *Podoviridae* phage capable to infect *L. monocytogenes* has been characterized (Klumpp and Loessner 2013).

Multiple bacteriophages have been studied against *L. monocytogenes* infections. It is really important to conduct safety studies to show that application of single phages or cocktails of multiple phages is not harmful to individuals. In an oral toxicity study, mice were treated with approximately 2×10^{12} phages per kilogram body weight per day for a five day period with bacteriophage P100. The phage preparation has been found safe and well-tolerated. No mortality, morbidity or histopathological changes related to the phage were observed (Carlton et al. 2005).

Phage P100 has been applied to a surface ripened cheese and found to completely eradicate *L. monocytogenes* infection. The effect of the phage was strictly dose dependent. A smaller concentration of 1.5×10^8 PFU/ml – when applied repeatedly – resulted in a 2-3 log decrease in the viable cell counts. A higher concentration of 3×10^9 PFU/ml completely eradicated the infection. The presence of *L. monocytogenes* was monitored for three weeks and regrowth of the pathogenic strain did not occur (Carlton et al. 2005).

P100 was also tested against *L. monocytogenes* in catfish fillet. A dose of 2×10^7 PFU/g of phage P100 was necessary for a significant reduction of the bacteria. This dose reduced *L. monocytogenes* counts by 1.5-2.3 log10/g. 2×10^8 PFU/g was required to achieve a 2 log reduction. Although phage application initially reduced the *L. monocytogenes* counts, it did not inhibit growth during the ten day storage period. The same results were obtained at 4 °C and 10 °C (Soni et al. 2010).

The peptidoglycan cell wall of Gram-positive bacteria is easily accessible by phage-encoded endolysins from the outside. This has been demonstrated in a study, where endolysin LysZ5 was expressed in *E. coli* and its lytic activity against *L. monocytogenes* has been tested in soy milk. The enzyme was able to reduce *L. monocytogenes* to undetectable levels. The reduction could be observed within 20 minutes and at refrigerated temperatures, showing that the enzyme retained its activity even at low storage temperatures (Zhang et al. 2012).

L. monocytogenes is able to form biofilms which renders it extremely resistant to antibiotic-based disinfections. It is crucial to find reliable and cheap procedures for preventing the formation or elimination of these biofilms from metal surfaces, *e.g.*, in food processing plants or hospitals. Phage P100 has been demonstrated to reduce *L. monocytogenes* levels under biofilm-forming conditions. It was effective against multiple serotypes of the bacteria, thus providing a promising tool to disrupt biofilms (Soni and Nannapaneni 2010).

Salmonella species

The genus *Salmonella* contains two species: *S. bongori* and *S. enterica*. *S. enterica* is a Gram-negative rod-shaped facultative anaerobic bacterium. It is an important pathogen of both humans and animals and causes salmonellosis in humans, which is the second most common gastrointestinal infection in the European Union. *Salmonella* bacteria can colonize the intestinal tract of humans and farm animals, but it can also be found in wild birds, reptiles and insects. The disease is mainly transmitted with contaminated food. Although *S. enterica* has six subspecies, 99% of the human infections are caused by only one of the subspecies *S. enterica* subsp. *enterica*. The different serotypes of this subspecies account for different percent of the infections. The most common serotypes in the European Union in 2012 were *S*. enteriditis and *S*. typhimurium. Altogether, these two serovars were

Table 2. Number and rates of confirmed salmonellosis cases, EU, 2008-2012 (ECDC 2014).

	2012	2011	2010	2009	2008
Number of confirmed cases	91029	96682	101589	110179	134581
Case rate per 100000 population	21.82	20.75	21.78	23.94	29.61

responsible for about 60% of the infections. The number of cases has a decreasing tendency, but the pathogen still accounts for ten thousands of infections annually (Table 2). Clinical manifestations of human salmonellosis range from subclinical gastroenteritis to severe bacteremia, meningitis, and other forms of extraintestinal infections. Poultry is one of the most prominent source of infections and therefore a successful treatment in animals could also reduce the number of human illnesses (Dworkin et al. 2006; ECDC 2014).

An oral toxicity study was performed, with bacteriophage wksl3 in mice. A single dose phage of high titer (1.1×10^{11} PFU/kg body weight) was administered to the animals. During the experimental period, no behavioral change or any physical symptom could be observed. No adverse effect on the organ level could be detected at the end of the experiments. These results suggest that phage wksl3 is not toxic for the mice (Kang et al. 2013).

The same bacteriophage was used to treat chicken skin artificially infected with *S. enteritidis*. A significant 3 log decrease in the bacterial counts could be observed after 24 hours at 8 °C. Some growth still occurred on the second day, but no significant growth was observable after it. After the experiment forty-eight cells were recovered and tested for resistance against phage wksl3. All of the tested bacteria remained susceptible to the phage (Kang et al. 2013).

In a previous study, the activity of phage Φ1 against *S. enterica* serovar *paratyphi* B was evaluated. Mice were injected with a lethal dose of bacteria (10^7 CFU/animal) and immediately injected with phage Φ1. While all animals in the control group died within 48 hours, all of the phage treated mice survived. The difference between the two groups could also be observed at the organ levels. The bacterial load of blood and two of the most heavily infected organs (liver and gastrointestinal tract) were also compared. No bacteria from the phage-treated group could be isolated, whereas the control group displayed high bacterial loads (>10^6 CFU/ml in the blood, >10^6 CFU/g in the gastrointestinal tract). However, a successful therapy should be effective when delayed compared to the infection. Therefore mice were infected with a sublethal dose (10^5 CFU/animal) of the pathogen and treated only two weeks later. Phage Φ1 proved to be successful in disinfecting the animals even when the treatment was delayed. A Φ1 phage-resistant strain was isolated from the phage-susceptible strain. This new strain, however, could not infect mice and was cleared from the systems of the animals within 4-7 days. Mice immunized with this strain showed resistance against infection with the pathogenic strain (Capparelli et al. 2010).

Since poultry is one of the most prominent infection sources, a successful treatment in these animals could also reduce the number of human illnesses. The efficiency of three phages against three *S.* enteritidis serotypes in commercially available chickens was evaluated (Atterbury et al. 2007). It has been found that high concentration of the phages (10^{11} PFU) reduced the bacterial number of serotypes *S.* enteritidis and *S.* typhimurium by more than 2 log. The number of serotype *S.* hadar was unaltered in these experiments, however *in vitro* the viable counts of these bacteria were significantly reduced.

The intestinal tract of animals is a very complex environment. In this case, a significantly higher number of phages is required for successful reduction of bacterial counts. Bacteriophage-insensitive mutants could recolonize the intestinal tract after the initial reduction of the bacterial numbers. However, administration of phages just prior to slaughter could still result in a significant reduction of bacterial loads (Atterbury et al. 2007).

Salmonella species are able to colonize solid surfaces (*e.g.*, stainless steel, glass) similarly to *L. monocytogenes*. Two phage cocktails SalmoFresh™ and SalmoLyse™ were tested for their ability to reduce *Salmonella* contamination on the aforementioned surfaces. SalmoFresh™ was able to lyse *S. kentucky* and *S. brandenburg* and therefore, significantly reduced bacterial counts (>99% reduction). This finding is in accordance with previous spot test assays, where these two strains were found susceptible to the phage cocktail. SalmoFresh™ wasn't able to lyse *S. paratyphi* B. Two phages from the cocktail were substituted with ones that were also able to lyse *S. paratyphi* B *in vitro*, thereby constructing SalmoLyse™. This new cocktail was effective against *S. paratyphi* B as well. With these experiments the authors showed that updating phage cocktails for following changes in bacterial populations and bacterial resistance is technically feasible (Woolston et al. 2013).

Currently, phage preparations against *L. monocytogenes* (Listshield™) and *S. enterica* (SalmoFresh™) are commercially available in the USA. Listshield™ is approved GRAS for direct application to fish and shellfish (including smoked varieties, like smoked salmon), fresh and processed fruits, fresh and processed vegetables and dairy products (including cheese) (GRN No. 528). SalmoFresh™ is also in GRAS status for direct application onto poultry, fish and shellfish, fresh and processed fruits and vegetables (GRN No. 435).

Conclusion

Applicability of phages against pathogenic bacteria has been recognized one hundred years ago but phage therapy as an antibacterial treatment retrograded due to the appearance of antibiotics. Upon the emergence and fast spread of multidrug-resistance, nowadays, development of novel antibiotics is not feasible and alternative antimicrobial approaches should be discovered and applied. Phage therapy has many benefits over antibiotics, represents a green solution for elimination of harmful bacterial infections even in those cases when antibiotics are ineffective. This has been realized in the last decades and the innovative research on phage therapy and enzybiotics was revitalized. A decade ago, it was an outstanding milestone, that FDA has approved the application of phages as food additives. However, medical application of phages still requires further systematic studies, carefully performed clinical trials and – very importantly – well-defined regulatory rules. Fortunately, it was understood by the decision makers and research on phage therapy is encouraged in most parts of the world. These facts and events indicate that widespread applications of phage-based antimicrobial agents might be expected in the future.

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