

REVIEW ARTICLE

Bacteriophages and their derivatives for the treatment and control of food-producing animal infections

Carla Carvalho^{a,b} , Ana Rita Costa^a, Filipe Silva^c and Ana Oliveira^a

^aCEB-UM: Centre of Biological Engineering, University of Minho, Braga, Portugal; ^bInternational Iberian Nanotechnology Laboratory (INL), Braga, Portugal; ^cCECAV-UTAD, Animal and Veterinary Research Centre, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal

ABSTRACT

Nowadays, the world is facing an increasing emergence of antibiotic resistant bacteria. Simultaneously, the banning of some existing antibiotics and the lack of development of new antimicrobials have created an urgent need to find new alternatives against animal infections. Bacteriophages (phages) are naturally occurring predators of bacteria, ubiquitous in the environment, with high host specificity and harmless to animals. For these reasons, phages and their derivatives are being considered valuable antimicrobial alternatives and an opportunity to reduce the current use of antibiotics in agri-food production, increasing animal productivity and providing environmental protection. Furthermore, the possibility of combining phage genetic material with foreign genes encoding peptides of interest has enabled their use as vaccine delivery tools. In this case, besides bacterial infections, they might be used to prevent viral infections. This review explores current data regarding advances on the use of phages and phage-encoded proteins, such as endolysins, exolysins and depolymerases, either for therapeutic or prophylactic applications, in animal husbandry. The use of recombinant phage-derived particles or genetically modified phages, including phage vaccines, will also be reviewed.

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Introduction

Livestock production frequently involves raising animals in confined conditions, promoting the easy and fast spread of infectious diseases. To prevent and treat these diseases, antibiotics have been a common practice for decades. Indeed, more than one hundred different antimicrobials have been used in food-producing animals (Hao et al., 2014). In addition to their therapeutic use, some antibiotics modulate the digestive flora and immune system and thus have been used as growth promoters to increase animal growth and feed conversion rates. However, this has been leading to antibiotic overuse, threatening their efficacy due to the emergence and spread of antibiotic resistant bacteria (Page & Gautier, 2012), and causing a negative environmental impact. Currently, the amount of antibiotics used in livestock is by far the largest portion of all antibiotics used worldwide, exceeding those used in human medicine (Mellon et al., 2001), mostly from the same class in both medical fields (Phillips et al., 2004). In 2010, approximately 63,151 tons of antimicrobials were used in livestock worldwide. By 2030, this consumption is expected to increase by 67% (Hollis & Ahmed, 2013, Van Boeckel

et al., 2015). Most of these antibiotics are used for non-therapeutic purposes, as growth promotion and disease prevention (Mellon et al., 2001). Tetracyclines are the most commonly used veterinary antibiotics followed by sulfonamides and macrolides (Kim et al., 2011).

Overall, the continuing increase and emergence of antimicrobial resistance of bacteria, the lack of novel and effective alternative drugs and the banning of antimicrobials as growth promoters in some countries emphasize the urgent need to develop and implement alternative antimicrobials in Veterinary Medicine (Aarestrup et al., 1998).

Bacteriophage and their derivatives are being considered valuable alternative antimicrobial solutions. Bacteriophages (phages) are viruses that specifically infect bacteria, being harmless to humans, animals and plants. Their initial discovery in 1917 by d'Herelle led to promising studies regarding their antimicrobial efficiency, but they were stalled in the United States and Western Europe by the development of antibiotics. Nevertheless, research on phages and their medical applications continued particularly in the former Soviet Union and Eastern Europe (Sulakvelidze et al., 2001).

More recently, with the emergence of multiple antibiotic-resistant bacteria, the interest in phage therapy was renewed (Goodridge & Bisha, 2011; O'Flaherty et al., 2009). Besides using the whole phage particle, it is also possible to use their heterologous expressed proteins with antimicrobial interest (as lysins and depolymerases) (Oliveira et al., 2013; Schmelcher et al., 2012a; Young, 2013, 2014). Furthermore, the possibility of using simple methodologies for the genetic modification of phage genomes offers several opportunities to improve their antimicrobial and safety properties, as well as for their prophylactic use as efficient antigen delivery vehicles (phage vaccines) against viruses.

The phage-based solutions applied to animals or animal models described herein aim to highlight the existence of safe and efficient tools to treat and control infections that cause diseases in food-producing animals. These may decrease the need for antibiotics without impairing productivity, while simultaneously offering environmental protection, by reducing the use of agro-chemicals.

The main agents affecting food-producing animals

There are hundreds of bacteria and viruses causing diseases in food-producing animals, affecting one or more animal species, with the most relevant presented in Table 1.

Bacteria cause a diversity of diseases that vary with the animal species, the agent gateway, the immune response of the animal and the farm sanitation. Likewise, viruses infect specific animals and have outcomes that range from no symptoms to mild and life-threatening diseases. These infectious diseases adversely affect the profitability of farms, either by animal death, fertility decrease and reduction of animal-derived products such as milk, eggs or wool, or by causing low food conversion rates resulting in an increase of food input for the same economic output (Lamy et al., 2012). Furthermore, bacterial and viral diseases in animals have potentially serious impacts on human health. In fact, more than 70% of all emerging human pathogens over the last years have been derived from pathogens originated from animals or products of animal origin (zoonotic) (Jones et al., 2008; Woolhouse et al., 2005). The World Health Organization (WHO, 2015) has estimated that globally 2.2 million people die each year from foodborne and waterborne diarrhoeal diseases alone.

Approximately, 80 types of bacteria cause disease in poultry industry, such as salmonellosis, pasteurellosis,

Table 1. Bacterial genera and virus families that cause disease in food-producing animals (Bradley et al., 2015; FAO, 2015; Merck, 2015; Persson & Bojesen 2015; VetBac, 2015).

	Cattle	Poultry	Pigs	Small ruminants
Bacterial genera				
<i>Actinobacillus</i>	■			■
<i>Actinomyces</i>	■			■
<i>Anaplasma</i>	■			■
<i>Avibacterium</i>		■		
<i>Bacillus</i>	■		■	■
<i>Bibersteinia</i>				
<i>Bordetella</i>		■	■	■
<i>Borrelia</i>	■			■
<i>Brachyspira</i>		■		■
<i>Brucella</i>	■		■	■
<i>Campylobacter</i>	■		■	■
<i>Chlamydia</i>		■		
<i>Chlamydophila</i>	■		■	■
<i>Clostridium</i>		■	■	
<i>Corynebacterium</i>				
<i>Coxiella</i>				
<i>Dermatophilus</i>				
<i>Dichelobacter</i>				
<i>Ehrlichia</i>				
<i>Enterococcus</i>	■	■	■	■
<i>Erysipelothrix</i>		■		
<i>Escherichia</i>	■		■	■
<i>Francisella</i>			■	
<i>Fusobacterium</i>	■			■
<i>Gallibacterium</i>		■		
<i>Haemophilus</i>			■	
<i>Histophilus</i>	■			■
<i>Klebsiella</i>	■			■
<i>Lawsonia</i>			■	
<i>Leptospira</i>	■		■	■
<i>Listeria</i>	■	■		
<i>Mannheimia</i>			■	
<i>Moraxella</i>			■	
<i>Mycobacterium</i>		■		
<i>Mycoplasma</i>		■	■	
<i>Neorickettsia</i>			■	
<i>Nocardia</i>			■	■
<i>Pasteurella</i>		■		■
<i>Peptoniphilus</i>				
<i>Prevotella</i>				
<i>Proteus</i>				
<i>Pseudomonas</i>		■		■
<i>Rhodococcus</i>			■	
<i>Salmonella</i>		■	■	■
<i>Serratia</i>		■		
<i>Staphylococcus</i>			■	■
<i>Streptobacillus</i>		■		
<i>Streptococcus</i>			■	■
<i>Treponema</i>	■			■
<i>Trueperella</i>			■	■
<i>Ureaplasma</i>			■	
<i>Yersinia</i>	■			■
Virus families				
<i>Adenoviridae</i>		■		
<i>Arteriviridae</i>			■	
<i>Asfarviridae</i>			■	
<i>Astroviridae</i>		■		
<i>Birnaviridae</i>				
<i>Bornaviridae</i>	■			■
<i>Bunyaviridae</i>				
<i>Caliciviridae</i>		■	■	
<i>Circoviridae</i>				
<i>Coronaviridae</i>	■			■
<i>Flaviviridae</i>	■			
<i>Herpesviridae</i>	■			■
<i>Orthomyxoviridae</i>		■	■	
<i>Papillomaviridae</i>	■			■
<i>Paramyxoviridae</i>				
<i>Parvoviridae</i>				
<i>Picomaviridae</i>		■	■	
<i>Poxviridae</i>				
<i>Reoviridae</i>				
<i>Retroviridae</i>			■	
<i>Rhabdoviridae</i>				
<i>Togaviridae</i>				

tuberculosis, staphylococcosis, streptococcosis, infectious coryza, necrotic enteritis, avian chlamydiosis, colibacillosis and mycoplasmosis (Hao et al., 2014; Lima et al., 2016; Masdoq et al., 2008; Merck, 2015).

In cattle, mastitis (caused e.g. by *Staphylococcus* spp. or *Streptococcus* spp.) is an important cause of economic loss due to milk waste, treatment costs and premature culling of animals in severe cases (Heikkilä et al., 2012), while respiratory infections (caused e.g. by *Pasteurella* spp., *Mannheimia* spp., *Mycoplasma* spp. or *Pseudomonas* spp.) are associated to significant morbidity and mortality (Royster & Wagner, 2015; Vogel et al., 2001).

In pigs, respiratory diseases are the most important health concern for swine producers nowadays, and are often the result of a combination of primary and opportunistic infectious agents (Brockmeier et al., 2002). Common respiratory diseases where bacteria are responsible for significant economic losses are *Mycoplasma pneumoniae* and pleuropneumonia or *Haemophilus parasuis* infections. *Pasteurella multocida* and *Bordetella bronchiseptica* induce atrophic rhinitis that is also an important predisposing factor for increased respiratory diseases (Brockmeier et al., 2002; Magyar & Lax, 2002; Merck, 2015; Nedbalcova et al., 2006).

Viruses can also cause several diseases with further complications. In fact, the host's response to the viral infection compromises its defense mechanisms, opening the way to secondary bacterial infections (Folkerts et al., 1990; Hodgins et al., 2002). Some examples are: Parainfluenza-3 virus (*Paramyxoviridae*) induced pneumonia, Rotavirus (*Reoviridae*) and Coronavirus (*Coronaviridae*) induced neonatal diarrhea, bovine respiratory syncytial viral pneumonia, infectious bovine rhinotracheitis, ovine progressive pneumonia, contagious ecthyma in sheep and goats, infectious bronchitis in poultry and Avian Gumboro disease caused by the infectious bursal disease virus (*Birnaviridae*) (Chakraborty et al., 2014; Guy, 1998; Kapil & Basaraba, 1997; Merck, 2015; Müller et al., 2003; Saif, 2010; Torres-Medina et al., 1985).

Phages and their derivatives as alternative antimicrobials

Phages and phage proteins namely endolysins, exolysins and depolymerases can present several advantages as antimicrobials against bacterial infections that affect food-producing animals. Despite tested *in vitro*, these potential applications have also been tested *in vivo* (Table 2) with some promising results.

Bacteriophages

Phages are the most abundant entities in the biosphere, and can be isolated from several sources, including alimentary tract of humans and animals, food, soil, water, sewage and other environment niches. In fact, phages can be found in the same environments where their bacterial hosts exist (Bergh et al., 1989; Clokie et al., 2011).

Most phages are very specific, normally infecting single bacterial species or serovar groups, due to their attachment to specific receptors on bacterial surface before injecting their genetic material (Figure 1). Virulent phages follow a lytic cycle, multiplying inside the bacteria and lysing the cell to release new viruses. In contrast, temperate phages follow a lysogenic life cycle, integrating the bacterial genome without causing cell lysis, but still being able to switch to a lytic cycle under certain stimuli. For therapeutic or prophylactic applications, non-transducing virulent phages should be used to avoid the risk of virulence gene transduction and to take advantage of their replication capacity (Gill & Hyman, 2010; Kutter et al., 2010; Kutter & Sulakvelidze, 2005).

Phages have many advantages over antibiotics for therapeutic purposes, including: high specificity that reduces damage caused to the normal host microbiota; replication only at the site of infection; self-limiting and self-dosing capacity i.e. replication only when sensitive bacteria are present; and lower propensity to induce resistance, especially when using a phage cocktail (Loc-Carrillo & Abedon, 2011; Örmälä & Jalasvuori, 2013; Sulakvelidze et al., 2001).

Several *in vivo* studies support the use of phages as an intervention strategy for reducing bacterial infections in food-producing animals.

In poultry, promising results were obtained for phage therapy against *Salmonella enterica* serotypes Typhimurium and Enteritidis, which may cause disease especially in young chicks (Lima et al., 2016). The importance of using phage cocktails and administering high and multiple doses of phages was also demonstrated (Berchieri et al., 1991; Fiorentin et al., 2005; Sklar & Joerger, 2001). Phages given both by coarse spray and in drinking water reduced *Salmonella* Enteritidis intestinal colonization of broiler chicks, showing that these means of phage administration are conceivable and can ease the application and establishment of phage biocontrol in an industrial environment (Borie et al., 2008). The combined treatment with phages and competitive exclusion products was also tested in birds, against *Salmonella* infections, showing synergistic effects (Borie et al., 2009). Other authors found that

Table 2. Summary of *in vivo* studies evaluating bacteriophage and their derivatives against bacteria infecting food-producing animals.

Treatment agent	Animal model	Pathogen	Delivery method	Output	Refs
Bacteriophages	Poultry	<i>S. Enteritidis</i>	Oral gavage, cloacal	Reduction in bacterial loads in cecal tonsils 24 h after treatment	Andreatti Filho et al. (2007)
			Aerosol spray, drinking water	Reduction of intestinal colonization by both delivery methods	Borie et al. (2008)
			Aerosol spray	Reduction of ≈ 3 log CFU in cecal contents when phages were combined with competitive exclusion products	Borie et al. (2009)
			Oral Feed	Reduction of 3.5 log CFU on cecal contents Prevention of horizontal bacterial transmission in the flock	Fiorentin et al. (2005) Lim et al. (2012)
		<i>S. Typhimurium</i>	Oral, feed, drinking water	Reduction of 0.3–1.3 log CFU in cecal contents	Sklar & Joerger (2001)
			Oral, feed	High concentrations of phages reduced mortality of birds	Berchieri et al. (1991)
			Oral	Reduction of <i>Salmonella</i> counts in cecum and ileum; no clear synergism with competitive exclusion products	Toro et al. (2005)
		<i>E. coli</i> (APEC)	Intramuscular	Effective prevention and treatment of septicemia and meningitis	Barrow et al. (1998)
			Aerosol spray, intramuscular	Reduction of bird mortality using intramuscular injection	Huff et al. (2003)
			Intramuscular	Total protection of birds using phage combined with enrofloxacin	Huff et al. (2004)
	Aerosol, drinking water		Reduction of bird mortality using aerosol administration	Huff et al. (2002)	
	Drinking water, spray		Mortality reduced to levels below 0.5% in naturally infected flocks	Oliveira et al. (2010)	
	Swine		<i>S. Typhimurium</i>	Oral	Absence of clear evidences of intestinal decrease of bacteria
		Oral gavage		Reduction of >1.4 log CFU in cecal contents	Callaway et al. (2010)
		Oral, intramuscular		Reduction of bacterial loads in pig tonsils (4 log CFU) and cecum (2 log CFU)	Lee & Harris (2001)
		Oral gavage, feed		Direct feeding of microencapsulated phages led to a reduction of bacterial loads	Saez et al. (2011)
		<i>E. coli</i> (ETEC)	Oral gavage	Microencapsulated phages led to a reduction of bacterial counts (1–3 log CFU)	Wall et al. (2010)
			Feed	Reduction of $\approx 60\%$ of bacterial loads in challenged pigs	Cha et al. (2012)
			Oral	Reduction of the duration and severity of diarrhea	Jamalludeen et al. (2009)
			Oral	Efficient early treatment of ETEC diarrhea in neonatal pigs	Smith & Huggins (1983)
Cows	<i>S. aureus</i>	Intramammary	Non-statistically significant cure rate of 16.7%	Gill et al. (2006a)	
Mice	<i>E. faecium</i>	Intraperitoneal	Higher phage doses rescue 100% of mice from bacteremia	Biswas et al. (2002)	
		Intraperitoneal	Efficient rescue of infected mice using low phage titer	Uchiyama et al. (2008)	
		Intraperitoneal	Protection of mice against infection	Soothill (1992)	
			100% rescue of bacteremic mice	Vinodkumar et al. (2008)	
		Intraperitoneal	Survival rate dependent on the time of administration and phage dose	Wang et al. (2006)	
	<i>K. pneumonia</i>	Intraperitoneal	Effective treatment of infection only with early phage administration	Chhibber et al. (2008)	
		Intraperitoneal, intragastric	Inhibition of infection by phages at low doses; intraperitoneal treatment more efficient than intragastric	Hung et al. (2011)	
	Hamster Mice	<i>C. difficile</i> <i>S. pyogenes</i> , MRSA <i>S. pyogenes</i>	Oral	Rescue most of the animals	Ramesh et al. (1999)
			Intraperitoneal	Protection of 92% of mice from bacteremia	Gilmer et al. (2013)
			Intraperitoneal	Protection of mice from death (90–95%) in a bacteremia model	Lood et al. (2014)
Oral, nasal		Elimination of the pathogen in colonized mice	Nelson et al. (2001)		
<i>S. agalactiae</i> <i>S. agalactiae</i> , <i>S. dysgalactiae</i> , <i>S. uberis</i> <i>S. pneumoniae</i>		Intraperitoneal	Protection of 80% of mice from infection	Oechslin et al. (2013)	
	Intramammary	Reduction of intramammary bacterial loads (1.5–4.5 log)	Schmelcher et al. (2015a)		
Endolysins	Hamster Mice	<i>S. pneumoniae</i>	Aerosol	Reduction in mice mortality of 80%	Doehn et al. (2013)
			Intraperitoneal	Rescue of mice from death due to endolysins synergism	Jado et al. (2003)

(continued)

Table 2. Continued

Treatment agent	Animal model	Pathogen	Delivery method	Output	Refs	
Engineered endolysins	Mice	MRSA	Intravenous, nasal, oral	Reduction of bacterial loads in nasopharyngeal and bacteremia models; longer survival of animals	Loeffler et al. (2003)	
			Nasal, pharyngeal	Reduction of bacterial loads to undetectable levels	Loeffler et al. (2001)	
			Intraperitoneal	Total protection of mice when administration within 24 h; reduced protection in delayed treatment (42% survival)	Witzenrath et al. (2009)	
			Intraperitoneal	Protection of mice from bacteremia	Gu et al. (2011)	
			Intravenous	Increased survival rate and reduction of bacterial counts in the bloodstream	Jun et al. (2012)	
			Intraperitoneal, nasal	Elimination of MRSA from the nares of mice and protection against death	Rashel et al. (2007)	
	Rats	<i>B.anthraxis</i> <i>S. pneumoniae</i>	Intraperitoneal	100% protection from death; no clinical signs of disease	Schmelcher et al. (2015b)	
			Intravenous	Rescue of mice from fatal septicemia Elimination of pneumococci causing endocarditis from blood within 30 min with a high-dose treatment	Schuch et al. (2002) Entenza et al. (2005)	
	Rats, dogs	MRSA	Intravenous	Absence of abnormal reactions to the endolysin	Jun et al. (2014)	
	Mice, rat	MRSA	MRSA	Intraperitoneal	Protection of mice from death with a synergy between the endolysin and oxacillin	Daniel et al. (2010)
				Intraperitoneal	100% protection of mice from death	Yang et al. (2014b)
				Topical	Better performance (3 log CFU reduction) than the antibiotic mupirocin	Pastagia et al. (2011)
Mice, rat		<i>S. pneumoniae</i>	<i>S. pneumoniae</i>	Intramammary	Reduction of 3.36 log CFU when combined treatment with lysostaphin	Schmelcher et al. (2012b)
				Intraperitoneal	50% greater protection of mice compared to the parental endolysin	Díez-Martínez et al. (2015)
				Intranasal, intramammary, intramuscular	Better activity than the parental protein in models of nasal infection, mastitis and osteomyelitis	Becker et al. (2016)
Zebrafish embryos	<i>S. pneumoniae</i> , <i>S. pyogenes</i>	Immersion	Increased survival rate of 95-99%; combination with carvacrol was efficient in killing Gram-negative bacteria	Díez-Martínez et al. (2013)		
<i>C. elegans</i>	<i>P. aeruginosa</i>	Immersion	Improved survival of nematodes with the artilysin (≈63%) in relation to the native endolysin (≈40%)	Briers et al. (2014a)		
Exolysins	Rat	MRSA	Intranasal	Total decolonization of the nares of 44.4% animals	Paul et al. (2011)	
Depolymerases	Poultry	<i>Salmonella</i>	Oral gavage	Reduction of colonization and bacterial penetration in internal organs	Waseh et al. (2010)	
	Rat	<i>E. coli</i>	Intraperitoneal	Reduction of systemic infections and prevention of death in at least 80% of infected animals	Mushtaq et al. (2005)	
Engineered phages	Mice	<i>E. coli</i>	Intravenous	Enhanced bacterial killing of phages when combined with antibiotics	Lu & Collins (2009)	
			Intraperitoneal	Reduced phage toxicity and immunogenicity and improved survival of animals	Matsuda et al. (2005)	

although the combined treatment reduced the bacterial colonization, this reduction was at least as effective as phage treatment alone (Andreatti Filho et al., 2007; Toro et al., 2005). Phages were also effective as a prophylactic treatment for *Salmonella* Enteritidis. Lim et al. (2012) revealed that phages prepared as feed additives significantly prevented horizontal transmission of bacteria from infected to healthy chicks within the same flock, also reducing the environmental contamination level. In fact, 70% of the non-infected chickens treated with phage had no detectable intestinal *Salmonella* three weeks after treatment.

Regarding the control of the avian pathogenic *Escherichia coli* (APEC), Barrow et al. (1998) showed that

phages were highly effective in both prevention and treatment of experimentally induced septicemia and meningitis. Huff et al. (2003) demonstrated that the efficacy of phage treatment is dependent on the route of administration, which in turn defines the phage titer reaching the site of infection. Phages given as an aerosol spray or intramuscular injection immediately after the *E. coli* challenge significantly reduced the mortality of birds but when the treatment was delayed, only the intramuscular injection was efficient. Also phages were more effective when administered as multiple intramuscular injections rather than a single dose. Phages given prophylactically as an aerosol significantly decreased the mortality of birds while when added to the drinking

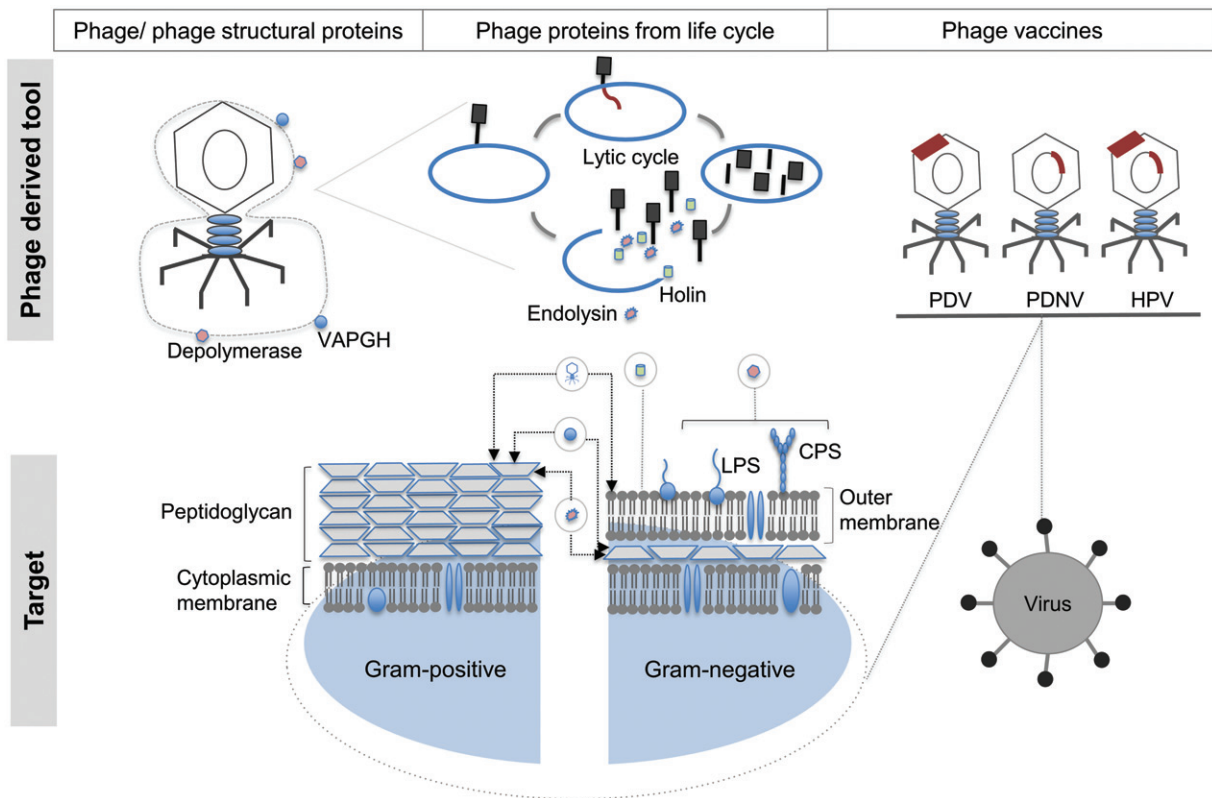


Figure 1. Schematic representation of the interactions between phages and phage-proteins (applied exogenously) and their respective targeted structures on the cell wall of Gram-positive and Gram-negative bacteria. Illustration of phages as antigen delivery vehicles against viruses and bacteria. CPS: capsular polysaccharides; LPS: lipopolysaccharides; PDV: phage display vaccine; PDNV: phage DNA vaccine; HPV: hybrid phage vaccine.

water provided no protection (Huff et al., 2002). The combination of the antibiotic enrofloxacin given in drinking water and the intramuscularly administered phage seemed to totally protect the challenged birds (Huff et al., 2004). In a more recent study, Oliveira et al. (2010) demonstrated the efficacy of a phage cocktail given orally and by spray in controlling *E. coli* infection in naturally infected flocks. The recorded mortality before treatment ranged between 0.7 and 7.8%, depending on the sampled flocks. After phage administration, the mortality decreased to levels below 0.5%.

There are relatively few studies in which phages have been used to control pathogens in swine. Smith & Huggins (1983) performed the first study that demonstrated the efficacy of phages given at the onset of diarrhea (13–16 h after infection) on the treatment of experimental Enterotoxigenic *E. coli* (ETEC) diarrhea in neonatal pigs. After the treatment, none of the pigs died, diarrhea was mild and a 3-log reduction was observed in fecal counts of ETEC. More recently, Jamalludeen et al. (2009) showed that phages given prophylactically or therapeutically to ETEC-infected weaned pigs decreased the duration and severity of diarrhea, without affecting the normal *E. coli* flora. Pigs

were pre-treated orally with sodium bicarbonate to protect the phages from the acidity of the stomach and increase the numbers of viable phages entering the intestinal tract. Also a virulent phage, administered in feed one week before pigs were challenged with ETEC, led to significantly reduced bacterial loads (Cha et al., 2012).

Although a recent report on *Salmonella* control in pigs by Albino et al. (2014) described the absence of clear evidences in the intestinal decrease of *Salmonella* Typhimurium after phage treatment, other studies reported encouraging results, Lee & Harris (2001) observed a significant reduction of *Salmonella* loads in pig tonsils (4-log reduction) and cecum (2-log reduction) after phage administration; other authors showed that administration of a phage cocktail given in feed or by gavage to pigs challenged with *Salmonella* Typhimurium, significantly reduced the bacterial load in the feces (Callaway et al., 2010; Saez et al., 2011; Wall et al., 2010).

Studies of phage efficacy to treat mastitis in lactating cows have also been performed but were not conclusive, requiring further experiments. For example, although an intramammary infusion of phage given to

cows with pre-existing subclinical *Staphylococcus aureus* infection led to a cure rate of 16.7%, the difference between this and the non-treated group was not statistically significant (Gill et al., 2006a). This can be due to phage inactivation in the udder due to milk proteins and fats since considerably lower rates of phages were found in the milk for up to 36 h post-treatment. Moreover, since *S. aureus* attaches to whey proteins and aggregates in milk this can limit phage binding (Gill et al., 2006b; O'Flaherty et al., 2005).

Other animal models have been used for phage therapy experiments, in which phages were efficient in the treatment of *Enterococcus faecium* (Biswas et al., 2002; Uchiyama et al., 2008), *Pseudomonas aeruginosa* (Soothill, 1992; Vinodkumar et al., 2008; Wang et al., 2006), *Clostridium difficile* (Ramesh et al., 1999) and *Klebsiella pneumonia* infections (Chhibber et al., 2008; Hung et al., 2011).

Overall, despite several *in vivo* experiments demonstrate that phages have potential as antimicrobials to control infections in food-producing animals, they still present limitations for therapeutic uses (Ly-Chatain, 2014). For example, although phages are less prone to cause resistance than antibiotics, bacteriophage insensitive mutants (BIMs) still occur, caused by the loss or mutation of the bacterial receptor (Hyman & Abedon, 2010; Walmagh et al., 2012a), by the acquisition of restriction–modification systems or by the development of adaptive immunity through interfering clustered regularly interspaced short palindromic repeats (CRISPR) sequences (Hyman & Abedon, 2010; Labrie et al., 2010). Other restrictions include the release of endotoxins caused by rapid lysis of a large number of bacteria that may cause allergic reactions (Drulis-Kawa et al., 2012; Lawrence, 2010), the possibility of inducing an *in vivo* anti-phage immune response, making it hard to sustain an effective phage concentration (Lawrence, 2010), and depending on the delivery system used, the achievement of an effective phage concentration at the site of infection (Abedon & Thomas-Abedon, 2010).

In order to circumvent these limitations, several factors should be considered before phage application *in vivo*. These include the appropriate selection of phages; the use of phage cocktails with phages targeting different surface receptors of the same target pathogen, which can result in synergistic effects and reduction of the likelihood of emergence of bacterial resistance; and the optimization of the phage/bacteria ratio, timing and route of phage delivery, according to the target pathogen and food-producing animal.

Apart from the biological limitations, regulatory issues are still the major obstacle to phage therapy. The uniqueness of phages as protein-based, live-biological

agents (Loc-Carrillo & Abedon, 2011), offer a challenge to the regulatory agencies, which are struggling to find the best approach to regulate phage products (Keen, 2012). Although phages have been suggested as a “biological medicinal product” (Directive, 2001), requiring clinical trials for each phage strain, or as an “advanced therapy medicinal product” (Directive, 2003; Verbeken et al., 2007), with regulation based on manufacturing processes without mandatory clinical trials (Directive, 2003), no final decision has been taken so far.

Phage-encoded proteins

Recently, phage proteins, as endolysins, exolysins and depolymerases (Figure 1) have been explored as antimicrobial therapies, either alone or in combination with other agents.

Although some authors consider endolysins and exolysins in the category of depolymerases, we will exclude them from this class and mention each one separately.

Endolysins

Endolysins are enzymes produced by phages at the end of their replication cycle to digest the bacterial cell wall peptidoglycan (PG), allowing the release of phage progeny. Most endolysins show a modular organization, comprising a catalytic domain (CD) and a cell wall-binding domain (CBD). The CBD binds to the PG or another cell wall component, allowing the CD to cleave a specific bond in the PG structure. Based on their catalytic activity site on the PG, endolysins can be muramidases, glucosaminidases, transglycosylases, amidases or endopeptidases (Schmelcher et al., 2012a).

These enzymes present several advantages as antimicrobials: they are, in general, very specific to the pathogen and thus do not disturb the normal microflora; so far, no resistance mechanisms or resistant phenotypes were detected after endolysins exposure, probably because these enzymes target essential structural components for bacterial viability and since they are applied exogenously they act on the outside of the cell, thereby avoiding a majority of possible resistance mechanisms (Fischetti, 2005; Loeffler et al., 2001, 2003; Nelson et al., 2001; Schmelcher et al., 2012a; Schuch et al., 2002); no signs of toxicity have been noticed after endolysins' treatment of systemic infections in mouse models (Jado et al., 2003; Loeffler et al., 2003; McCullers et al., 2007; Nelson et al., 2001); and the immune response induced by endolysins apparently does not neutralize their activity or prevent their use to treat systemic infections (Fischetti, 2005; Loeffler et al., 2003).

Most of the work described with endolysins concerns Gram-positive bacteria, because Gram-negative bacteria have a highly impermeable outer membrane (OM) that limits the access of endolysins to the PG, when applied exogenously. Therefore, in order to lyse Gram-negative bacteria, most endolysins require physical treatment or chemical OM disruption agents, namely polycationic agents and chelators (Briers et al., 2008a, 2011; Walmagh et al., 2012b). Another option is to genetically engineer endolysins, as exemplified by the Artilysins (detailed below). Nevertheless, some endolysins have been reported to cross the OM and kill Gram-negative bacteria without chemical or physical treatment. Examples include the *Salmonella* phage endolysin SPN9CC (Lim et al., 2014), the *Bacillus amyloliquefaciens* phage endolysin Lys1521 (Morita et al., 2001a; Orito et al., 2004) and the *Acinetobacter baumannii* phage endolysin LysAB2 (Lai et al., 2011). Most of these enzymes have amphipathic or highly cationic regions that interact with the LPS to cross the OM (Lai et al., 2011; Morita et al., 2001b; Walmagh et al., 2012a).

Some phage endolysins have demonstrated capacity to kill some of the most prevalent Gram-positive pathogens such as *S. aureus* and *Streptococcus uberis* which cause mastitis in cows (Celia et al., 2008; Donovan et al., 2006; Horgan et al., 2009; Obeso et al., 2008) and *Streptococcus suis* which cause endocarditis and septicemia in pigs (Wang et al., 2009).

In animal models, endolysins have shown efficacy in the treatment and protection against a wide range of Gram-positive pathogens that infect animals and humans.

For *Streptococcus pyogenes*, oral administration of endolysin PlyC to colonized mice eliminated the pathogen within 2 h (Nelson et al., 2001). Also an intraperitoneal injection of the endolysin PlyPy (Lood et al., 2014) or PlySs2 (Gilmer et al., 2013) protected mice from death in a bacteremia model of *S. pyogenes* infection or a combined MRSA (Methicillin-resistant *S. aureus*) and *S. pyogenes* infection. For *Streptococcus agalactiae*, repeated intraperitoneal injections of PlySK1249 significantly protected mice from the infection (Oechslin et al., 2013). In a mouse model of bovine mastitis, intramammary infusions of endolysins λ SA2 and B30 led to a significant reduction on the intramammary concentrations of the *S. agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* (except for B30 vs. *S. dysgalactiae*) (Schmelcher et al., 2015a).

On *Streptococcus pneumoniae*, endolysins showed potential to control and treat mucosal and systemic infections. Several methods of delivering endolysin Cpl-1 to infected mouse models were tested with different outcomes. When Cpl-1 was given intravenously, the

bacterial loads were reduced to undetected levels within 15 min. Nevertheless, the treated animals died from the infection, possibly due to the relatively short half-life of Cpl-1 in the blood (15–20 min), which indicates that additional doses of Cpl-1 could be needed to completely eradicate *S. pneumoniae* (Loeffler et al., 2003). Endolysins Cpl-1 and Pal applied intraperitoneally to *S. pneumoniae* infected mice, acted synergistically and rescued mice from death (Jado et al., 2003). Also Witzentrath et al. (2009) showed that when applying endolysin Cpl-1 24 h after bacterial challenge with *S. pneumoniae*, mice were fully protected. However, when treatment was delayed for 2 days, the survival rate reduced to 42%. Cpl-1 was also given by aerosol to infected mice after the onset of pneumonia and reduced the mortality by 80% (Doehn et al., 2013). Cpl-1 was also efficient in the elimination and prevention of acquired *S. pneumoniae* endocarditis in rats (Entenza et al., 2005). The application of the endolysins Pal or Cpl-1 has successfully eliminated *S. pneumoniae* from the nares of mice (Loeffler et al., 2001, 2003).

Phage endolysins have also been shown to be powerful therapeutic agents against multidrug-resistant *S. aureus* infections and protected mice from death when administered intraperitoneally (Gu et al., 2011; Rashel et al., 2007; Schmelcher et al., 2015b). An endolysin-based pre-formulation (SAL200), administered as an intravenous injection in a mouse model of MRSA infection, reduced *Staphylococcus* counts in the bloodstream and prolonged survival of mice (Jun et al., 2012). In a preclinical study with rats and dogs, repeated intravenous treatment with SAL200 did not induce signs of toxicity (Jun et al., 2014). Regarding *Bacillus anthracis*, endolysin PlyG, applied as an intraperitoneal injection, rescued mice from fatal septicemia (Schuch et al., 2002).

Phage endolysins have also shown to act synergistically with other endolysins (Jado et al., 2003; Loeffler & Fischetti, 2003), bacteriolytic enzymes such as lysostaphin (Becker et al., 2008; Schmelcher et al., 2012b) and antibiotics (Daniel et al., 2010; Djurkovic et al., 2005; Rodríguez-Cerrato et al., 2007).

Holins are proteins encoded by phages during the late phase of the lytic cycle and are involved in the permeabilization of the membrane, allowing endolysins to cross into the periplasm and degrade the PG (Wang et al., 2000). The combined use of holins and endolysins might be a promising strategy for antimicrobial therapy. Shi et al. (2012) combined the holin HoISMP and the endolysin LySMP, resulting in an extended spectrum of the endolysin against strains of multidrug-resistant *S. suis* and *S. aureus*.

Due to their modular structure, endolysins can be genetically engineered to display desired functional

properties such as extended host spectra, improved lytic activity, solubility and ability to infect Gram-negative bacteria (Fernandes et al., 2012; Yang et al., 2014a).

The modular engineering of endolysins involves the shuffling of natural lysins domains, generating chimeric lysins (or “chimeolysins”), or the fusion of a natural lysin or part of its domain with another component from a peptide or a protein (artificial lysins or “artilysins”).

Recent studies demonstrated that chimeolysins have increased lytic activity and a host range broader than natural enzymes (Becker et al., 2009; Dong et al., 2015; Mao et al., 2013; Schmelcher et al., 2011).

In vivo studies proved the efficacy of treatment with genetically modified endolysins in animal models against human and animal infections.

The chimeolysins ClyH and ClyS when given as an intraperitoneal injection, protected mice from death by MRSA (Daniel et al., 2010; Yang et al., 2014b). The chimeolysin ClyS, when applied topically, performed better than the antibiotic muciporin in a mouse model of skin colonization/infection with *S. aureus* (Pastagia et al., 2011). An engineered triple-acting staphylolytic endolysin reduced colonization by *S. aureus* in a rat nasal colonization model, surpassing the efficacy of either parental protein. When this protein was modified with a transduction domain and tested in a mouse model of mastitis, it demonstrated significantly improvement on the ability to kill intracellular *S. aureus* (Becker et al., 2016). Also, an intramammary infusion of the chimeric endolysins λ SA2-E-Lyso-SH3b or λ SA2-E-LysK-SH3b reduced *S. aureus* loads by 0.63 or 0.81 log units in a mouse model of mastitis. When λ SA2-E-LysK-SH3b was administered in combination with lysostaphin, a 3.36 log reduction was observed in the *Staphylococcus* loads (Schmelcher et al., 2012b).

A single dose of chimeolysin Cpl-7S significantly increased the survival rate of infected zebrafish embryos for *S. pneumoniae* and *S. pyogenes* (Díez-Martínez et al., 2013). Similarly, a single intraperitoneal injection of the chimeric protein Cpl-711, administered 1 h after *S. pneumoniae* challenge, resulted in about 50% greater protection of mice than with the parental endolysin (Díez-Martínez et al., 2015).

As stated above, the major drawback of endolysins is their limited action against Gram-negative bacteria. This was overcome by engineering these enzymes as outer membrane-penetrating endolysins (Artilynsins). Briers et al. (2014a) fused endolysins with different LPS-destabilizing peptides with polycationic, hydrophobic and amphipathic properties, enabling the enzymes to pass the OM and become active against Gram-negative bacteria. For example, the artilysin LoGT-001 was able to kill *P. aeruginosa* (≥ 5.50 -log), *A. baumannii* (5.18-log), *E.*

coli (2.41-log) and *S. Typhimurium* (1.52-log) cells in 30 min. LoGT-001 was tested *in vivo* in a *Caenorhabditis elegans* infection model with *P. aeruginosa* leading to a survival of $63 \pm 4\%$ nematodes 5 days postinfection, while 90% of the animals that did not receive the Artilysin were killed. Recently, the construction of artilynsins by fusing endolysins with antimicrobial peptides (AMPs) which have a self-promoted uptake mechanism across the OM was reported. Artilynsins Art-085 and Art-175, which result from the fusion of an AMP sheep myeloid 29-amino acid (SMAP-29) peptide with the KZ144 endolysin, were able to cross the OM and reduce *P. aeruginosa* loads by 5-log *in vitro* (Briers et al., 2014b). In two case studies of dogs with otitis, the treatment with Art-085 was able to cure the animals from an infection by *P. aeruginosa*, which was not eliminated with standard antibiotics (Briers & Lavigne, 2015).

Exolysins or VAPGHs (virion-associated peptidoglycan hydrolases)

Exolysins are another type of peptidoglycan enzymes encoded by some phages that, in contrast to endolysins, are present as part of the phage structure and participate in the first step of phage infection. VAPGHs degrade the peptidoglycan, enabling phages to reach the bacterial cytoplasmic membrane thus facilitating DNA injection into the host cell (Letellier et al., 1999). These proteins have been identified in phages infecting both Gram-positive and Gram-negative bacteria (Moak & Molineux, 2004). Few VAPGHs have so far been analyzed, however, some studies show their antimicrobial activity when applied exogenously (Rashel et al., 2008; Rodríguez et al., 2011; Takáč & Blási, 2005).

Like endolysins, VAPGHs have limited activity against Gram-negative bacteria due to the presence of an OM. However, some VAPGH have shown activity *in vitro* against Gram-negative bacteria when used with OM destabilizing conditions (Briers et al., 2008b; Caldentey & Bamford, 1992; Lavigne et al., 2004).

VAPGHs present several advantages as antimicrobials: high thermostability and specificity (Lavigne et al., 2004), a low probability of bacterial resistance, and a modular organization that allows the construction of chimeric proteins with multiple domains to improve their antimicrobial activity.

Rodríguez-Rubio et al. (2012) discovered that truncated derivatives and fusion proteins of HydH5, a VAPGH from a *S. aureus* phage, exhibited high lytic activity against *S. aureus* when compared with the parental enzyme and displayed antimicrobial synergy with the endolysin LysH5. These proteins are promising agents against *S. aureus*, which cause bovine mastitis,

since they were able to prevent its growth in milk (Rodríguez-Rubio et al., 2013). The chimeric protein P16–17, composed of the N-terminal endopeptidase domain of endolysin Lys16 and the C-terminal CBD of VAPGH P17, exhibited antimicrobial activity towards *S. aureus* and augmented the antimicrobial efficacy of the antibiotic gentamicin (Manoharadas et al., 2009). Similarly, the chimeric exolysin P128, composed by a tail-associated muralytic enzyme (TAME) from phage K and a lysostaphin SH3 cell wall-targeting domain, showed a potent anti-staphylococcal activity *in vitro*. Moreover, when tested in a mouse infection model, P128 reduced MRSA colonization of rat nares by 2-log (Paul et al., 2011).

Depolymerases

Depolymerases are phage-encoded proteins that have glycanase-like activity and digest the polysaccharides of the bacterial cell wall, namely lipopolysaccharides (LPS) and capsular polysaccharides (CPS).

Although some of these proteins are soluble and generated during host cell lysis, the majority of the described phage depolymerases are structural proteins, facilitating phage infection (Oliveira et al., 2013).

According to their mode of action, phage depolymerases can be hydrolases if they catalyze the hydrolysis of glycosidic bonds, or lyases if they cleave (1,4) glycosidic bonds by a β -elimination mechanism (Oliveira et al., 2013).

There are still few *in vivo* studies in which depolymerases were applied. Mushtaq et al. (2005) showed that a depolymerase with endosialidase activity (endoE) administered to rats reduced systemic *E. coli* infections, suggesting that the degradation of the bacterial CPS decreased its virulence and sensitized cells to host defenses. Waseh et al. (2010) also demonstrated that the oral administration of a tail spike protein with endorhamnosidase activity reduced *Salmonella* colonization in chickens. This may be attributed to the binding of the protein to the O-antigen hence compromising the structure of LPS and, consequently, reducing bacterial motility and infectivity.

Phage-derived vaccines

Although the importance of innovation on therapeutic approaches is irrefutable, preventing diseases is always preferable to treatment. Vaccines are one of the most important immunomodulators, acting by stimulation of the body's immune system to recognize the agent as foreign, enabling infection control. Therefore, the use of vaccines may decrease the need

for antibiotics, contributing to improve health, welfare and production yields in animal husbandry (Prisco & De Berardinis, 2012).

Conventionally, a vaccine contains the causative agent of the disease, genetically engineered microbes, vectored antigen formulations or naked DNA (DNA vaccines).

Recently, phages are also considered as vaccine-delivery vehicles against bacterial or viral infections, as they possess numerous intrinsic characteristics making them promising tools in this field. Presently, two distinct approaches are proposed, either used independently or combined: phage display vaccines and phage DNA vaccines (Clark & March, 2004a) (Figure 1).

In phage display vaccines, specific proteins or peptides from the antigens of interest are engineered to be expressed on the phage surface, leading to an immunogenic response (Clark & March, 2006). The conception of these vaccines relies on the phage display technology, which allows the fusion of the DNA coding a polypeptide with phage structural protein genes, enabling the expression of the former protein on the phage surface (Sidhu, 2000; Smith, 1985). Some phages were already identified as advantageous for this purpose, as T4, T7, lambda phages and the filamentous M13. The T4 phage allows the display of large peptides on its capsid proteins, Soc (small outer capsid protein; 960 copies per particle) and Hoc (highly antigenic outer capsid protein; 160 copies per particle) (Li et al., 2006). In the T7 phage, the fusion proteins are displayed on protein 10B (5–15 copies) of the major phage capsid (gp10), and in the lambda phage both the tail protein gpV (at least 5 copies) and the head protein gpD (405–420 copies) have been used for displaying foreign peptides (Sundell & Ivarsson, 2014). The M13 phage is a highly versatile system as distinct coat proteins can be used for peptide display. Commonly, the pIII protein is used for low valency display (1–5 copies per phage) and the pVIII for high valency display (up to 1000 copies per phage) (Sundell & Ivarsson, 2014).

In phage DNA vaccines, phages are used as vehicles for the delivery of the foreign antigen DNA incorporated into the phage genome, under the control of strong eukaryotic promoters. Lambda phage vectors are commonly used for this purpose (Clark & March, 2004a). Inside the animal organism, the antigen gene will be expressed in targeted cells from the immune system (Clark & March, 2006), originating an immune response.

Overall, vaccines lead to humoral (antibody-dependent) and/or cellular (cell-dependent) immune responses through the combined role of different groups of cells: antigen-presenting cells (APC), which capture and display antigens to another group, the regulatory T-

lymphocytes (Helper cells – HC), which when activated induce the activity of the effector cells (EC) that will accomplish the elimination of antigens (Siegrist, 2012).

The humoral response is mediated by B-lymphocytes (APC), and might be triggered by immunogenic antigens, as the peptides displayed in the surface of phage display vaccines. After the intervention of HC and EC, antibodies will be released, and memory B-cells will remain for subsequent exposures. In the cellular immune response and in the case of a DNA phage vaccine, macrophages or dendritic cells (APC) will translate the foreign antigen DNA into peptides, inducing through HC the activation of phagocytic cells and cytotoxic T-lymphocytes (remain as memory cells) that will destroy the antigen (Figure 2).

Recent studies reported the possibility of producing a hybrid phage vaccine, combining the two versions of phage vaccines. Here, the DNA vaccine is enclosed within the phage particle and a phage-display variant of the same antigen is present on the phage surface, with specific binding affinity to the APC (Clark & March,

2006). This combination has the advantage of delivering the antigen gene directly to the immunoreactive cells, efficiently targeting both humoral and cellular immune systems (Clark & March, 2006; Haq et al., 2012; Zanghi et al., 2007) and, consequently, enhance the vaccine specificity and the effectiveness of the immune response.

In broad terms, phage vaccines have several advantages relatively to the conventional ones (Clark & March, 2004a; Olofsson et al., 2008). Phage vaccines can be economically manufactured, due to the possibility of rapidly obtaining high phage titers on relatively inexpensive media, with straightforward purification steps. These features allow the attainment of sufficient quantities of vaccines against new pathogens as, for example, during disease outbreaks. Moreover, the lack of pathogenicity of the phage particles in animals and their increased chemical stability are also important when safety issues are concerned. Also, phages enclose natural adjuvant properties that confer them a real advantage in relation to conventional vaccines. Adjuvants are

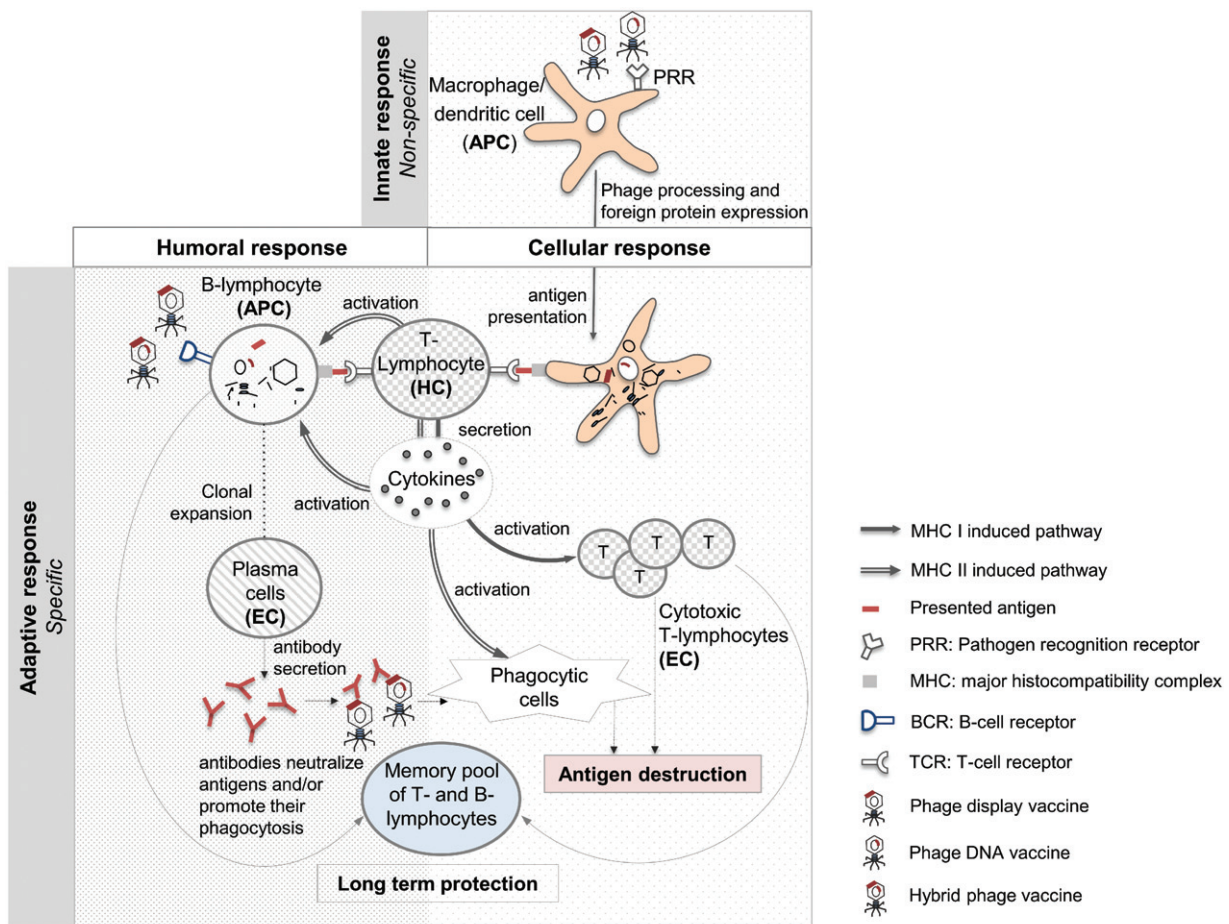


Figure 2. Schematic example of the innate and adaptive (humoral and cellular) immune system response activated by different types of phage vaccines: phage display vaccines, phage DNA vaccines and hybrid phage vaccines. APC: antigen presenting cells; EC: effector cells; HC: helping cells.

compounds that enhance the specific immune response against co-inoculated antigens, particularly by stimulating antibodies production. This feature is related to the presence of unmethylated CpG dinucleotides (cytosine-phosphodiester linked-guanine), naturally found in the phage genome and able to be recognized by the innate immune cells through protein receptors (Toll-like receptors) (Adhya et al., 2014; Krieg et al., 1995; Mason et al., 2005).

Particularly for phage DNA vaccines, apart from the already mentioned manufacturing advantages, the possibility of ensuring the absence of antibiotic resistance genes, the large cloning capacity of the phage vector (e.g. 15–20 kb can be used in standard lambda vector), the potential for oral delivery, and the natural protection offered by the phage capsid to DNA against environmental degradation (contrarily to naked DNA vaccines) are some other relevant advantages (Clark & March, 2004a). Additionally, enclosure of foreign DNA in a phage that acts as a particulate antigen, enables both the induction of antibody production (humoral response) and the activation of the cellular immune response (Gaubin et al., 2003), enhancing the vaccine efficacy.

However, there are still few studies in which phage particles displaying antigenic proteins have been used as vaccines in animal models (Bazan et al., 2012). An example of an effective phage display vaccine was reported by Hayes et al. (2010). In this work, immunodominant regions of the porcine circovirus were successfully displayed in lambda phage capsid, and neutralizing antibodies were produced against this infection in pigs, stimulating both cellular and humoral immune responses.

Wu et al. (2007) used the phage T4 HOC, SOC dual site display system, hence leading to a simultaneous display of two different antigens on the T4 capsid surface. The resultant phages were administered to mice as a vaccine against classical swine fever virus (CSFV). The results of animals' immune response revealed that the antibody titers elicited in mice by the T4 phage-CSFV recombinants were significantly higher than those obtained by immunization with recombinant antigens produced by conventional expression in recombinant *E. coli* cells. In another work, an orally delivered foot-and-mouth disease virus (FMDV) vaccine, in which the capsid protein was displayed on the surface of T4 phage particles, conferred mice 100% protection against a lethal dose of a FMDV-O serotype. Moreover, pigs immunized with this vaccine were protected when animals were cohoused with FMDV-infected pigs (Ren et al., 2008).

Hashemi et al. (2012) concluded that the genetic fusion of a domain of influenza A virus (M2 protein)

with T7 phage-based particles effectively protected mice against this disease, even without adjuvant, as the defense cells' secretion frequency was comparable to those elicited by the peptide itself, emulsified in the conventional adjuvant.

Hashiguchi et al. (2010) described a strong IgG primary response in mice and the induction of long-lasting antibody response with a single immunization with M13 phage injected intraperitoneally, suggesting this phage as a potential vaccine carrier.

Regarding phage DNA vaccines, there are few studies designed for food-producing animals' infections, and most target humans' pathogens (Clark & March, 2004a, 2004b). Ou et al. (2013) tested a lambda phage-mediated DNA vaccine against *Chlamydia abortus* in piglets and proved its capacity of inducing antigen-specific cellular and humoral immune responses providing protective immunity.

Genetically-modified phages

Recent developments on genetic tools are creating new opportunities for the modification of phage genomes to improve their properties for therapeutic and prophylactic applications (Nobrega et al., 2015; Pires et al., 2016). These tools include bacteriophage recombineering of electroporated DNA (BRED), yeast artificial chromosome (YAC) and the CRISPR–Cas system.

BRED (Marinelli et al., 2008) uses bacterial overexpression of plasmid-encoded recombination genes (e.g. the lambda Red system) to enhance the frequency of homologous recombination between phage DNA and DNA substrate after electroporation (Fehér et al., 2012; Marinelli et al., 2012). YAC (Lu et al., 2013) consists on the insertion of the whole phage genome inside a YAC vector by homologous recombination, creating a recombinant YAC that is propagated in the vector host where the phage genome can be modified by yeast recombineering. The modified YAC may then be inserted into bacteria for phage replication. The CRISPR–Cas system is the most recent tool for the genetic modification of phages. This system is involved in the protection of microbial cells from DNA invasion and comprises an array of repeats and spacers. Transcribed spacers guide specific proteins to the target DNA (e.g. phage DNA), and cleave it. The break can then be repaired by homologous recombination if a mutated template is provided (Kiro et al., 2014). Compared with other methods, CRISPR–Cas provides higher percentages of mutated phages, simplifying selection and recovery (Kiro et al., 2014; Martel & Moineau, 2014).

There are several examples of the use of these techniques for genetic manipulation of phage

genomes with therapeutic interest. One of the main goals is the extension of phage host range, which has been achieved, for example, by designing phage T7 for the expression of an endosialidase degradative of the K1 capsule that is present in some *E. coli* strains (Scholl et al., 2005) or by replacing the host binding genes of phage T2 for those of phage PP01 (Yoichi et al., 2005). It is also important to reduce phage toxicity and immunogenicity that may hinder therapeutic efficacy and cause adverse side effects. This can be achieved by developing lysis-deficient and non-replicating phages, avoiding toxin release during bacterial lysis *in vivo* (Hagens et al., 2004; Matsuda et al., 2005). Some manipulations have been performed to enhance phage survival after administration, including the introduction of a single specific mutation on a phage genome that resulted in the generation of long-circulating phages (Vitiello et al., 2005). It is also possible to improve phage activity against biofilms, as demonstrated by Lu & Collins (2007) with the engineering of T7 for the expression of dispersin B, a biofilm-degrading enzyme, which resulted in a 100,000 fold increased efficiency. Genetic modification has also been used to enhance bacterial killing of phages when combined with antibiotics, for example by engineering lysogenic phages to overexpress proteins that target specific nonessential gene networks (Lu & Collins, 2009).

Future perspectives

In an era of increasing emergence of multi-drug resistant pathogens and a scarcity of new antimicrobials, together with the restrictions imposed by European Union to the use of traditional antibiotics in animal husbandry, there is an urgent need to find antimicrobial alternatives.

Phage-based solutions presented herein have potential as therapeutic or prophylactic tools for application in livestock production. In fact, there is compelling evidence from the *in vitro* and *in vivo* studies of the potential of phages against an array of Gram-positive and Gram-negative bacterial pathogens that infect food-producing animals.

Phage particles pose several intrinsic characteristics that make them profitable for the development of products to treat or prevent diseases, as well as for the design of vaccine-delivery platforms. The natural stability, easy production and purification of phages provides cheaper and large-scale capacity for the manufacturing of these products. However, the acceptance of phages for this purpose will depend on its efficacy, safety and

cost compared with other interventions. Therefore, a more detailed biologic and genetic characterization of phages is mandatory to avoid undesirable outcomes. These include a bacterial adaptive response to phage infection by the generation of BIMs or even an increase in bacterial virulence when phages transfer virulent genes such as toxin and antibiotic-resistant genes among bacteria. Moreover, the efficacy of phage therapy requires an appropriate choice of phages, dosage, delivery route and timing of administration, which should be adapted according to the pathogen and animal targeted.

From another point of view, the development of synthetic biology and molecular tools enables the construction of genetically-modified phages with valuable features such as extension of the host range, reduced toxicity and immunogenicity and enhanced survival after administration. Moreover, it also allows to use phage proteins, namely endolysins and exolysins, that present broader antibacterial spectrum, lower immunogenicity and low probability of bacterial resistance and thus can be considered as potentially valuable therapeutics to fight infections. Until now, these enzymes target mainly Gram-positive bacteria but further screening for new ones or engineering these enzymes combined with other proteins, as OM transporters or holins to target Gram-negative bacteria is ongoing, and it is expected to increase the value of phage enzymes as therapeutics for both human and animal health.

Despite all the reported evidences of success on using phage-derived solutions presented herein, the use of these antimicrobials to completely substitute antibiotics is not a straightforward or probable approach, mainly because antibiotics have several uses in the animal production, such as prevention and treatment of diseases and promotion of growth. Therefore, the combination of phage-based alternatives with the traditional ones, when used rationally, may be a path to follow.

Another important issue regarding animal production is the scarce number of studies directed for veterinary purposes. The urgent requisite of decreasing antibiotic use in food-production animals should encourage researchers to design experiments specifically for animal applications.

Furthermore, it is urgent that regulatory bodies establish protocols for phage product regulation, so that the use of phages in veterinary applications does not remain stalled by regulatory issues. Regulatory bodies such as the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA) and

the European Medicines Agency (EMA) have shown some flexibility in approving the use of phages for control of several problematic pathogens.

Presently, there are already several companies developing phage products that can be used in Veterinary Medicine. These comprise BioPhage-PA, a topical phage product for chronic otitis against antibiotic-resistant *P. aeruginosa* that has already completed the phase I/II of clinical trials, and Viridax™, for the treatment of respiratory, systemic and topical infections of *S. aureus* and other Staphylococcal species. Other products were already approved and are being commercialized. These include: feed additives, such as BioTector to control *Salmonella* in poultry; products for direct food applications, such as ListShield™ and Listex P-100™ for *Listeria monocytogenes*, EcoShield™ for *E. coli* O157:H7, Salmonalex™ and SalmoFresh™ for *Salmonella*; and products for agricultural use, such as AgriPhage™ to control *Xanthomonas campestris* or *Pseudomonas syringae* on crops. Omnilytics™ also has some approved products that target *Salmonella* and *E. coli* O157:H7 on cattle hides and poultry prior to slaughter.

Overall, the continuous research of the already existing antibiotic alternatives and the development of safe and efficient new ones, is needed to combat the threat of antibiotic resistant pathogens and to ensure sustainable animal and human welfare.

Disclosure statement

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ORCID

Carla Carvalho  <http://orcid.org/0000-0002-9345-6546>

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