Supplementary tables

SUPPLEMENTARY TABLES

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Application of bacteriophages in post-harvest control of human pathogenic and food spoiling bacteria. Crit. Rev. Biotechnol.

Supplementary Table 1. Effects of bacteriophage treatments against Gram-positive foodborne pathogens.

Target bacterium	Reported effects	Reference(s)
Listeria monocytogenes	Phage mixtures LM-103 and LMP-102 applied on honeydew melon slices reduced *L. monocytogenes* populations by 2.0 to 4.6 log cycles, but not on apple slices (due to phage inactivation).	Leverentz et al*.,* 2003
	Enhanced effect in combination with nisin	
	LMP-102 approved and commercialized as ListShield™ for application on ready-to-eat foods and for decontamination of food contact surfaces	US FDA, 21 CFR §172.785; EPA registration number 74234-1
	Myovirus P100 (Listex™ P100) is very active in foods such as ready to eat meats and poultry, fresh cheese, smoked fish, fruits, vegetables, either alone or in combination with other antimicrobials	Carlton et al*.,* 2005; Guenther et al., 2009; Soni and Nannapaneni, 2010a; Soni et al., 2010, 2012; Chibeu et al., 2013; Oliveira et al., 2014
	Phages A511 and A511-like are very active in different ready-to-eat foods	Guenther et al., 2009; Bigot et al., 2011
	Inactivation of *L. monocytogenes* forming	Roy et al., 1993; Hibma et

	biofilms on various substrates (stainless steel, polystyrene) by P100 and other phages	al., 1997; Soni and Nanapaneni, 2010b; Montanez-Izquierdo et al., 2012; Ganegama Arachchi et al., 2013
	Cloned phage endolysin LysZ5 has broad antilisterial spectrum, being active in soya milk	Zhang et al., 2012
Staphylococcus aureus	Inactivation of *S. aureus* by phages ΦA72 and ΦH5 in dairy substrates (milk, fresh and hard-type cheeses)	García et al., 2007; Obeso et al., 2010; Bueno et al., 2012
	Phages ϕ88 and ϕ35 survived high hydrostatic pressure treatment at 400 MPa and afforded protection against proliferation of *S. aureus* in pasteurized milk during storage	Tabla et al., 2012
	Phage endolysin LysH5 has lytic spectrum restricted to *S. aureus* strains	Obeso et al., 2008
	Endolysin LysH5 and nisin act synergistically in milk	García et al., 2010

Supplementary Table 2. Effects of bacteriophage treatments on Gram-negative foodborne pathogens.

Target bacterium	Reported effects	Reference(s)
Salmonella enterica	Serotype-specific *Myoviridae* and *Siphoviridae* phages are effective in decontamination of sprout seeds	Pao et al., 2004; Kocharunchitt et al*.,* 2009
	Decontamination of poultry carcasses by single phages and phage cocktails	Chighladze et al., 2001; Goode et al., 2003; Higgins et al., 2005; Atterbury et al., 2006
	Decontamination of skin sections from pig carcasses by the bacteriophage cocktail PC1	Hooton et al., 2011
	A cocktail of three different bacteriophages achieved significant reductions of *S.* Typhimurium and *S.* Enteritidis on pig skin, chicken breasts and packaged lettuce, but not on fresh eggs	Spricigo et al., 2013
	Bacteriophage wksl3 has strong lytic activity on *S*. Enteritidis and *S*. Typhimurium in chicken skin	Kang et al., 2013
	Inactivation of *Salmonella* in ready to eat foods of animal origin by phages: chicken sausages (phage Felix O1), cooked beefs (phage P7), and Cheddar cheese (phage SJ2)	Whichard et al., 2003; Bigwood et al., 2008; Modi et al., 2001
	SalmoFresh™ commercial phage preparation recommended for inactivation of the most common/highly pathogenic serotypes in red meat and poultry	Wollston et al., 2013
	SalmoFresh™ and SalmoLyse™ phage preparations inactivate *Salmonella* on hard surfaces (glass and stainless steel)	Wollston et al., 2013

Escherichia coli	ECP-100 cocktail of three *Myoviridae* phages inactivated *E. coli* O157:H7 on tomato, spinach, broccoli, lettuce and ground beef	Abuladze et al*.,* 2008; Sharma et al., 2009; Sharma, 2013
	ECP-100 commercialized as EcoShield™ was active in contaminated beef and on leafy greens packed or not under MAP	Carter et al., 2012; Boyacioglu et al., 2013; Ferguson et al., 2013
	Phage cocktail BEC8 acts synergistically with *trans*-cinnamaldehyde on *E. coli* O157:H7 in leafy greens	Viazis et al., 2011
	ECP-100 was effective in decontamination of *E. coli* O157:H7 adhered on glass coverslips and gypsum boards	Abuladze et al., 2008
	BEC8 was effective in decontamination of *E. coli* O157:H7 adhered on sterile stainless steel chips, ceramic tile chips, and high density polyethylene chips	Viaziz et al., 2011
	Spray application with phage cocktail reduced *E. coli* O157:H7 biofilms on spinach harvester blade	Patel et al., 2011
	Inactivation of enteropathogenic and Shiga toxigenic *E. coli* strains on meats, and also during milk fermentation	Tomat et al., 2013a
***Shigella* spp.**	Species-specific bacteriophages reduced *Shigella* counts in ready-to-eat spiced chicken	Zhang et al., 2013
Campylobacter jejuni	Reductions of *Campylobacter* viable counts on chicken skin	Atterbury et al., 2003; Goode et al., 2003
	Inactivation of *Campylobacter* on both raw and cooked beefs	Bigwood et al., 2008
	Bacterial inactivation and biofilm dispersal on glass surface	Siringan et al., 2011
Cronobacter	Inactivation of the bacterium in artificially	Kim et al., 2007; Zuber et

sakazakii	contaminated infant milk formula by added phages	al., 2008
***Vibrio* spp.**	Inhibition of *Vibrio vulnificus* by a cocktail of bacteriophages singly or in combination with oyster extract	Pelon et al., 2005
	Inhibition of *Vibrio parahaemolyticus* in oysters and in oyster flesh by a *Siphoviridae* phage	Jun et al., 2014

Supplementary Table 3. Effects of bacteriophage treatments on spoilage bacteria and other bacteria relevant to foods.

Target bacterium	Reported effects	Reference(s)
***Pseudomonas* spp.**	Inactivation of *Pseudomonas fragi* in refrigerated milk	Ellis et al., 1973
	Inactivation of *Pseudomonas* spp. on retail beef steaks	Greer, 2005
	Inactivation of *P. fluorescens* biofilms on stainless steel (phage ΦIBB-PF7A)	Sillankorva et al*.,* 2008
Clostridium tyrobutyricum	Inactivation in milk by phage ΦCTP1 endolysin	Mayer et al., 2010
Brochothrix thermosphacta	Inactivation of the bacterium and suppression of the associated off-odors on pork adipose tissue. Phage-resistant bacteria reported	Greer and Dilts, 2002
Leuconostoc gelidum	Inhibition of this spoilage bacterium on refrigerated pork adipose tissue packed or not under vacuum	Greer et al., 2007
Lactobacillus brevis	Inhibition of spoilage strains in commercial beer	Deasy et al., 2011

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Supplementary Text 2

SUPPLEMENTARY TEXT

Rubén Pérez Pulido, Mª José Grande Burgos, Antonio Gálvez*, and Rosario Lucas López. Application of bacteriophages in post-harvest control of human pathogenic and food spoiling bacteria. Crit. Rev. Biotechnol.

THE BIOLOGY OF BACTERIOPHAGES

Bacteriophages are obligate parasites that are widely distributed in Nature and play an important role in controlling the population densities of bacteria in many different environments (Chibani-Chennoufi et al., 2004). Bacteriophages are the most abundant form of life on the planet with estimated 10^{31} phage particles in the biosphere (Rohwer and Edwards, 2002). According to their life cycles, bacteriophages can be classified into lytic (e.g. those causing cell lysis) and temperate or lysogenic phages (e.g. those capable of integrating their genetic material into the bacterial chromosome) and persist as prophages for generations without causing cell damage. Lysogenic phages will only reduce bacterial populations when induced to engage into a lytic cycle. However, phage induction can be achieved under certain stress conditions (such as exposure to UV light or heat) that can occur during food processing. Some defective prophages can also be induced under stress conditions, resulting in host cell lysis and release of phage tail-like structures having antimicrobial activity. These were thought to be bacteriocins at one time, as exemplified by colicin 15 from *E. coli* (Sandoval et al, 1965), R- and F-type pyocins from *Pseudomonas aeruginosa* (Michel-Briand and Baysse, 2002) and monocins from *L. monocytogenes* (Zink et al., 1994, 1995).

A phenomenon associated with certain prophages is phage conversion, by which a bacterium can acquire relevant genes from the phage genome, such as those encoding

for toxins or antibiotic resistance (Brabban et al., 2005). Acquisition of such genes can enhance bacterial virulence, as exemplified by *Vibrio cholerae* or *E. coli* serotypes producing Shiga-like toxins (Hagens and Loessner, 2010) or by the diversity of virulence genes associated with prophages detected in *Salmonella* genomes (Moreno Switt et al., 2012). This can be one reason not to propose application of lysogenic phages for biopreservation (Brüssow et al., 2004).

Lytic bacteriophages propagate within bacterial cells and cause host cell lysis as part of their multiplicative cycle, thus offering a strong potential as biocontrol agents. The lytic cycle can amplify the phage population, which may result in an increase in the number of infective phage particles or plaque forming units (PFU) in the treated samples or partially compensate for the inactivation of phage particles that may occur by interaction with food components. In this respect, phages with high burst size should be preferable for biopreservation. In addition to the lytic cycle, phages may also induce what is known as lysis-from-without or lysis from outside (LO). This happens through adsorption of bacteriophages to cell surfaces at a high multiplicity of infection (MOI, the ratio of phage particles to target bacterial cells) without completing the infection cycle (Delbrück, 1940; Abedon, 2011). LO requires that a sufficient number of phage particles be adsorbed onto the cell, resulting in cell wall damage and subsequent cell lysis due to stress placed on structural weak points in the cell envelope (Abedon, 2011; Sharma, 2013). While lytic cycles depend on the bacterial cell metabolic activity and therefore are influenced by growth temperature, the initial adsorption and lysis in the LO process can occur at low temperature. Accordingly, biocontrol based on LO could be suitable for foods stored under refrigeration (Sharma, 2013).

Bacteriophages are highly host specific, mainly due to the receptor recognition step in the interaction of phage particles with the host. For application as biocontrol

agents, selected phages with broader host specificity or combinations of different phages should be recommended. Bacteriophages, for use as biocontrol agents, should meet certain properties: they must be strictly virulent, with a broad host-range, unable to perform generalized transduction, and unable to enhance the virulence or pathogenicity of the host or induce its lysogenic conversion (Hagens and Loessner, 2007, 2010). DNA sequencing and in silico analysis of phage genomes is important in order to establish that they not carry genetic determinants coding for any known or suspected toxins, antibiotic resistance genes, or other factors involved in regulation of virulence and/or pathogenicity. Preferably, the phages should be susceptible to propagation on a non-pathogenic host, avoiding large-scale cultivation of pathogens in the process of phage preparation and also to prevent any interference of phage preparation impurities with downstream pathogen detection systems (Hagens and Loessner, 2007, 2010).

Factors influencing the efficacy of lytic bacteriophages

Bacteriophage-based strategies for food biopreservation have attracted great interest in recent years. The main approaches proposed application of bacteriophages with the aim of reducing contamination of foods with foodborne bacterial pathogens include: (i) treating domesticated livestock with phages in order to decrease pathogen colonisation; (ii) treatments for decontaminating inanimate surfaces in food-processing facilities and other food establishments in order to decrease contamination of foods (biosanitation); and (iii) post-harvest treatments involving direct applications of phages onto the harvested or processed foods in order to decrease the levels of pathogens or spoilage bacteria and improve the food safety and quality (Sulakvelidze, 2013).

The efficacy of bacteriophages in food systems may depend on several factors influencing phage stability as well as the ability of phages to bind and infect their target

bacteria, such as the food pH, temperature, ion content and osmolarity, interaction with food components (fat, proteins, carbohydrates...), diffusion rate (e.g. surface microstructure, available fluid) or the concentration and accessibility of target bacteria (Jonczyk et al., 2011; Ly-Chatain, 2014). Therefore, phage application must be optimized for each particular food system. The MOI is an important parameter to take into consideration. Although many studies have reported some killing of target bacteria at low MOI values (usually between 10^3 and 10^4), application of phages at high MOI values (10^5 to 10^7) is recommended in order to improve the effectiveness of treatments and to avoid bacterial regrowth. Thus, for an expected bacterial load of ca. 10^2 CFU/ml or gram of food, final phage concentrations of 10^7 to 10^9 PFU/ml or gram could be recommended. Therefore, phage preparations containing at least 10^{11} PFU/ml could be suitable for application in foods at high MOI.

Phage stability may have a great impact on the efficacy of biocontrol treatments. Inactivation of bacteriophages in foods and food-processing facilities is a multifactorial process, although pH and temperature seem to be critical parameters. Bacteriophages are generally stable between pH 5 and 8, although this pH interval can be somewhat wider (between 4 and 9) at lower temperatures (EFSA, 2009; Jończyk et al., 2011). In milk whey, phage survival was reported at pH 3.5 to 6.8, but rapid inactivation was observed at pH <3 (Smith et al., 1987). In contrast, survival of phages in sauerkraut (pH <3.5) was reported after 60 and even 100 days (Lu et al., 2003). Phage thermotolerance seems to be in correlation with the environment/host system from which they are derived (EFSA, 2009). Phages from psychrotrophic bacteria seem to be less heat tolerant, compared for instance to phages from thermophylic LAB (Hudson et al., 2005). A higher thermotolerance (80 to 95 ºC for 5 min) has also been described in lactococcal phages (Atamer et al., 2008). By contrast, low temperature improves phage

stability considerably, as shown for tailed phages (some of which retained a titre of 10^3 after years at 4 ºC) by Ackermann et al. (2004). Survival of bacteriophages specific for *Salmonella* and *Campylobacter jejuni* on chicken skin stored at 4 ºC has been reported (Goode et al., 2003; Atterbury et al., 2003a). Phages specific for *Salmonella* inoculated onto untreated chicken showed a reduction of 3.0 $\log_{10}$ PFU/cm^2 at time zero to $\log_{10}$ 2.1 $\log_{10}$ PFU/cm^2 at 48 H (Goode et al. 2003). Recovery of phages specific for *Campylobacter* remained constant at 42 to 44% of the initial inoculated titer over a 6-day period of storage at 4 ºC, but then decreased to 17% at day 10 (Atterbury et al., 2003a). Bacteriophages surviving at low temperatures can provide protection against bacterial proliferation once the food products are warmed. Osmotic shock and pressure can generate bacteriophage ghost particles which may not initiate a lytic cycle, but still may be able to attach and cause lysis from without.

Bacteriophages may be inactivated by ultra violet light, and also by fluorescent light (Iriarte et al., 2007). Nevertheless, DNA damage caused by ultra violet light could still be repaired after infection by bacterial DNA repair mechanisms. Bacteriophages may exhibit large differences in sensitivity to high hydrostatic pressure (HHP). Aertsen et al. (2005) determined the inactivation of two *E. coli* Shiga toxin (Stx)-converting *Siphoviridae* bacteriophages upon pressurization in phage buffer for 15 min at 20 °C. While one phage was readily inactivated several $\log_{10}$ cycles at 100 MPa, the other one showed little loss of activity at pressures up to 400 MPa. In a recent study, the *Siphoviridae* phages phiIPLA35 and phiIPLA88 specific for *Staphylococcus aureus* were treated by HHP (5 min, 10 ºC) in pasteurized milk (Tabla et al., 2012). Phage titres were not affected by treatment at 400 MPa, although pressurization at 500 MPa resulted in a 92% phage inactivation. Disinfectants, including peracetic acid, ethanol and sodium hypochlorite may cause rapid inactivation of phage particles (Binetti and Reinheimer,

2000; Suarez and Reinheimer, 2002). However, phages may exhibit a wide range of resistance to chlorine, as shown in one study with coliphages (Kennedy and Bitton, 1987).

The efficacy of bacteriophages as biocontrol agents may be compromised by the development of bacterial resistance after continued use of phage preparations. First, it is possible that phage-treated environments become sooner or later colonized by naturally resistant strains. Second, phage exposure may select for bacteriophage insensitive mutants (BIMs). This is a natural phenomenon occurring in Nature in the interplay between bacterial populations and bacteriophages. Phage resistance may result from interference at different steps of the phage multiplicative cycle, especially during phage adsorption, but also at the steps of phage DNA entry, stability, and replication (Hyman et al., 2010; Labrie et al., 2010; Bikard and Marraffini, 2012). Phage adsorption can be prevented by several mechanisms, such as modification of phage receptors, blocking of phage receptors by production of specific proteins, or by production of an extracellular matrix. Production of bacterial capsules can block phage adsorption, but certain bacteriophages do use bacterial capsules as receptors. Capsular-negative mutants are insensitive to K antigen-specific phages. Capsular phase variation can also confer phage resistance, as shown for the O-methylphosphoramidate phage receptor in *C. jejuni* (Sørensen et al., 2012). Interference with phage adsorption can also result from phenotypic resistance, where a fraction of the bacterial population may exhibit a reduced phage adsorption as result of several factors such as starvation, changes in gene expression after exposure to products of phage lysed bacteria in the environment, or blocking of bacterial receptors by phage proteins released by lysed cells (Bull et al., 2014).

Following phage adsorption, DNA entry can be prevented by superinfection exclusion system proteins, which are often found in prophages (Labrie et al., 2010). For example, the coliphage T4 superinfection exclusion systems prevent subsequent infection by other T-even-like phages. One inside the host cell, phage DNA can be degraded by bacterial restriction modification systems, or by CRISPR (clustered, regularly interspaced, short, palindromic repeats)—Cas (CRISPR-associated) systems (Sorek et al., 2008; Labrie et al., 2010; Gasiunas et al., 2014; Sampson and Weiss, 2014). Interestingly, a restriction-modification system has been shown to be responsible for temperature-dependent phage resistance in *L. monocytogenes* epidemic clone II strains being completely resistant to phage when grown at low temperatures of ≤30°C (Kim et al., 2012). In CRISPR-Cas systems, foreign DNA is neutralized by the Cas nucleases or helicases (Sorek et al., 2008; Gasiunas et al., 2014; Sampson and Weiss, 2014). A Cas-independent CRISPR system has been described recently in *Listeria*, associated to a polynucleotide phosphorylase (PNPase) enzyme (Sesto et al., 2014). Bacteria can also counteract bacteriophages by abortive infection systems, targeting crucial steps of phage multiplication such as replication, transcription or translation (Labrie et al., 2010). Testing of surviving bacterial isolates after phage treatments for their sensitivity to the phage preparation used, and updating the phage preparation are recommended as a strategy to deal with phage resistance.

Strategies for application of lytic bacteriophages

Phages can be applied on foods or food surfaces by dipping or spraying with phage suspensions (Anany et al., 2011; Hagens and Loessner, 2010). There is also a growing interest in the development of antimicrobial packaging to actively control the

growth of microorganisms in foods (Larson and Klibanov, 2013; Kerry, 2014), and the large body of knowledge available on immobilization technologies could well be applied to bacteriophages. So far, most studies carried out with bacteriophages have focused on microencapsulation of phage preparations for oral delivery purposes. Nevertheless, the polymeric materials used for microencapsulation (such as alginate and others) are also being applied for immobilization of antimicrobial substances and application of edible coatings on foods (Quirós-Sauceda et al., 2014). Tentatively, a similar approach could be applied with bacteriophages.

Immobilization of bacteriophages on activated coatings may offer several advantages, including phage stabilization, a prolonged and gradual release of phage particles, and protection against cross contamination. In one study, Murthy and Rainer (2008) patented the development of stabilized bacteriophage formulations in which phage particles were adsorbed onto a solid matrix (skim milk powder, soya protein, albumin powder, single cell proteins, trehalose, mannitol, sugar and sugar alcohol) and then dried by heating under vacuum. Bacteriophages stabilized by adsorption to a matrix could then be encapsulated into different types of solid supports (gelatin, wax, methacrylates or shellac). Furthermore, Salalha et al. (2006) described the encapsulation of bacteriophages T4, T7 and lambda in electrospun polymer nanofibers. The immobilized phages remained active and were able to infect their target bacterial host after dissolution of the polymer fibers. In another study, cocktails of phages active against *Listeria* or *E. coli* immobilized on modified cellulose membranes were shown to be active under different storage temperatures and packaging conditions (Anany et al., 2011). Furthermore, a recent patent described a method for applying bacteriophage to a food in combination with a fatty or waxy coating layer (Terhaar and Hanna, 2014).

Bacteriophages could also be applied in combination with other antimicrobials, as part of hurdle technology. The concept of hurdle technology began to apply in the food industry in a rational way after the observation that survival of microorganisms greatly decreased when they were confronted with multiple antimicrobial factors (Leistner and Gorris, 1995; Leistner, 2000). After exposure of a bacterial population to a single antimicrobial factor there is often a heterogeneous response, depending on the intensity of treatment as well as many other factors. By contrast, when cells are exposed to a combination of antimicrobial factors, the repair of multiple damages may require much higher energy costs, leading to energy exhaustion and cell death. Exposure to multiple factors may also reduce the risk of developing resistance. In spite of the potential applications, the numbers of studies on bacteriophages in combination with other hurdles are very limited.

Natural antimicrobials such as bacteriocins and others could be applied in combination with bacteriophages for food biopreservation. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack et al., 1995). The bacteriocin nisin is licensed as a biopreservative in many different countries. A mixture of nisin and listeriophage LH7 displayed a combined effect and reduced levels of cells substantially without regrowth in broth, but for unknown reason -possibly because of the complexity of the natural food system employed- the same effect was not observed in vacuum-packaged fresh beef (Dykes and Moorhead, 2002). Nevertheless, a combination of nisin and lytic bacteriophages was reported to be effective for control of *L. monocytogenes* in fresh-cut fruits (Leverentz et al., 2003). Similarly, subinhibitory concentrations of nisin and the bacteriophages Φ35 and Φ88 had a synergistic effect on *S. aureus* Sa9. However, after prolonged incubation it was found that the bacterium was able to adapt to nisin, and the nisin-adapted strain became partially resistant to both phages

(Martínez et al., 2008). In contrast, bacteriophage insensitive mutants were not nisin resistant.

Application of lytic bacteriophages for inactivation of bacteria adhered to food contact surfaces or in the form of biofilms also seems a promising approach. Bacterial cells may remain adhered to surfaces and, under proper conditions, they can multiply and develop biofilms. These are defined as microbial communities embedded in a polysaccharide matrix, formed at liquid/solid interphases (Watnik and Kolter, 2000). Biofilms have become problematic in the food industry, since they may limit the efficacy of disinfection operations and provide a continued source of bacterial contamination (Srey et al., 2013). Lytic bacteriophages can remain adsorbed to biofilms, resulting in an amplified infection that effectively kills bacteria. Some bacteriophages may also encode for enzymes that destroy bacterial exopolysaccharides important for biofilm formation or they can be genetically-engineered to do so. Bacteriophages encoding polysaccharide-degrading enzymes (also called enzymatic bacteriophages) could find application not only for inactivation of biofilm bacteria but also for removal of biofilms (Lu and Collins, 2007; Drulis-Kawa et al., 2012).

Bacteriophage endolysins

Phage lysins or endolysins are peptidoglycan hydrolases employed by the majority of bacteriophages to enzymatically degrade the peptidoglycan layer of the host bacterium `from within' at the end of their lytic multiplication cycle (Young et al. 2000). They can be divided into three general types: glycosidases, amidases and endopeptidases (Lopez and Garcia, 2004), and may contain one or more enzymatic domains targeting the glycan moiety or the peptide bonds in bacterial peptidoglycan, as well as specific cell wall recognition domains (reviewed by Oliveira et al., 2012 and

Drulis-Kawa et al., 2012). Some phage endolysin can be highly host-specific, as exemplified by the *Listeria* phage PSA endolysin (Korndörfer et al., 2006) or the *Staphylococcus* bacteriophage endolysin LysH5 (Obeso et al., 2008), and could be applied in foods against defined pathogenic bacteria without affecting other microbial populations. Others may have much broader specificity than their corresponding infective phage particles. For example, the LysB4 lysin from *Bacillus cereus* phage B4 is active on Gram-positive bacteria such as *B. cereus*, *Bacillus subtilis* and *L. monocytogenes* and even on Gram-negative bacteria when tested in the presence of other antimicrobials (such as EDTA) that disrupt the bacterial outer cell membrane permeability barrier (Son et al., 2012). Endolysins (also known as "enzybiotics") have attracted great attention for food biopreservation (Fischetti, 2010; Schmelcher et al., 2012a; Rodríguez-Rubio et al., 2013). In addition to their rapid lytic activity (allowing bacterial lysis within minutes), it has been argued that there is a low chance of developing bacterial resistance because bacteriophage endolysins have evolved to target essential bonds in the peptidoglycan through the coevolution of bacteriophages and their hosts (Loessner, 2005). Also, some of them are thermally resistant, and can be applied on heat processed foods. Phage endolysins have also attracted interest to combat bacteria forming biofilms. An additional advantage of using phage lysis could be that an approval for phage lysins may be less challenging that an approval for a whole virus-based food additive.

Since endolysins are enzymes, their stability and hydrolytic activities in food systems may be affected by several factors such as temperature, divalent cations, pH and ionic strength, among others. Most phage and bacterial lysins studied require neutral or slightly acidic conditions, with activity rapidly decreasing above pH 7.5 or 8 (Vasala et al., 1995; Pritchard et al., 2004; Yoong et al., 2004; Donovan et al., 2006;

Sugahara et al., 2007). However, one study showed that *L. monocytogenes* phage endolysins HPL511, HPL118, HPL500, and HPLP35 exhibited highest activity at elevated pH values at around pH 8–9 (Schmelcher et al., 2012b). Lytic activity was abolished by EDTA and could be restored by supplementation with various divalent metal cations, indicating their role in catalytic function. The endolysins HPL118, HPL511, and HPLP35 were highly stable and retained considerable activity after heating at 90 ºC for 30 min. It was suggested that these thermostable endolysins may find application in food products that undergo heat treatment such as pasteurized milk products (Schmelcher et al., 2012b). According to their optimum pH, those endolysins would be considered more suitable for foods having pH values close to neutrality.

In Gram-negative bacteria, one study revealed that L-alanoyl-D-glutamate peptidase from *E. coli* phage T5 had highest activity at pH 8.5 (Mikoulinskaia et al., 2009). Another study showed that recombinant Lys394 endolysin (from the broad-range *Salmonella* bacteriophage S-394) has maximum activity at pH of 8.5 and low ionic strength (Legotsky et al., 2014). Another *Salmonella* phage endolysin, Lys68, remains active over a pH range from 4.0 to 10.0, with a pH optimum around 7.0 (Oliveira et al., 2014). Application of endolysins against Gram-negative species seems to be limited because the outer membrane prevents access of endolysins to peptidoglycan layer from outside. Nevertheless, endolysin activity can be enhanced by application of outer membrane-permeabilizing agents such as polycations (like poly-L-arginine; Legotsky et al., 2014), EDTA, or organic acids (like citric and malic acid; Oliveira et al, 2014) or even by high hydrostatic pressure treatments (Briers et al., 2008). In addition, a number of phage endolysins have been reported to carry positively charged and/or hydrophobic regions that supposedly interact with and permeabilize bacterial outer membranes (Schmelcher et al. 2012a). Examples are phage endolysins Lys1521 from *Bacillus*

amyloliquefaciens and LysAB2 from *Acinetobacter baumannii*, both of them being active on Gram-positive and Gram-negative bacteria (Morita et al. 2001; Lai et al. 2011).

On Gram-positive bacteria, phage endolysins have been reported to show synergistic effects with antibacterial peptides useful as food preservatives like nisin (García et al., 2010) and lysostaphin (Becker et al., 2008). Application of phage endolysins as part of hurdle technology in food preservation could take advantage from previous work done with lysozyme from higher organisms (considering that hen egg white lysozyme and T4 bacteriophage lysozyme have the same catalytic function in spite of having non-homologous amino acid sequences). Thus, phage endolysins could be applied in combination with a wide variety of other hurdles such as bacteriocins, food preservatives, essential oils, phenolic compounds, or with physico-chemical treatments (such as heat, pulsed-electric fields, high hydrostatic pressure, UV irradiation, and others), provided there is no interference with endolysin activity.

Phage endolysins (singly or in combination with other antimicrobials) could also be applied in edible coatings (for example in combination with chitosan) or in activated films as already reported for lysozyme (Rao et al., 2008; Roman et al., 2014). Nevertheless, given the wide diversity of phage endolysins and the variety of food conditions involved, extensive testing would be required for each particular food application of endolysins as biopreservatives. In addition to pH, other factors such as interaction with food components or inactivation by proteolytic enzymes, the accompanying food microbiota and the sensitivity of the target bacteria need to be taken into consideration, similarly to what has been proposed for other proteinaceous antimicrobials like bacteriocins (Gálvez et al., 2007). The production costs of endolysins could be a significant barrier to their application. This could be overcome by

optimization of expression systems and industrial production processes, similarly to other enzymes commercially available for different industrial applications. Heterologous expression of endolysins (by for example yeasts or lactic acid bacteria) could be a cheap alternative for incorporation of these antimicrobial enzymes in food systems.

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