

The applications of bacteriophages and their lysins as biocontrol agents against the foodborne pathogens *Listeria monocytogenes* and *Campylobacter* spp.: an updated look

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Veterinaria Italiana 2018, **54** (4), 293-303. doi: 10.12834/VetIt.311.1215.2

Accepted: 16.06.2014 | Available on line: 31.12.2018

Keywords

Bacteriophage,
Bio-decontaminant,
Campylobacter spp.,
Foodborne pathogen,
Listeria monocytogenes,
Phage-therapy,
Safety.

Summary

Listeria monocytogenes and *Campylobacter* spp. are foodborne pathogens responsible for outbreaks and disease in humans. The emerging problem of bacterial antibiotic resistance and the persistence of pathogens in the environment, especially where foods are processed, are some of the reasons that have led to a re-emerging interest in bacteriophages and their lysins as potential candidates for bio-control. This review focuses on the use of bacteriophages and their lysins as alternative strategies for controlling the foodborne pathogens *L. monocytogenes* and *Campylobacter* spp. In addition, the application of bacteriophages and their lysins in food safety and animal health, as well as phage-resistance development, legislation, and future prospects were discussed.

Aggiornamento sulle applicazioni dei batteriofagi e delle loro lisine nel biocontrollo di *Listeria monocytogenes* e *Campylobacter*

Parole chiave

Batteriofago,
Bio-decontaminante,
Campylobacter spp.,
Fago-terapia,
Patogeno a trasmissione
alimentare,
Listeria monocytogenes,
Sicurezza.

Riassunto

Listeria monocytogenes e *Campylobacter* spp. sono patogeni responsabili di malattie a trasmissione alimentare negli esseri umani. Il problema dell'antibiotico-resistenza e la persistenza dei microrganismi patogeni nell'ambiente, soprattutto nelle produzioni alimentari, sono alcuni dei motivi che hanno portato recentemente alla rivalutazione dei batteriofagi e delle loro lisine come potenziali candidati per il bio-controllo contro i batteri. In questa monografia l'attenzione è stata focalizzata sul potenziale utilizzo di fagi e relative lisine come strategia alternativa per contenere, in particolare, *L. monocytogenes* e *Campylobacter* spp. Sono stati valutati, inoltre, l'efficacia dell'applicazione nella sicurezza alimentare e nella salute animale, lo sviluppo di fago-resistenza, la legislazione e le eventuali prospettive future in relazione al loro potenziale impiego.

Introduction

The term 'bacteriophage' (or phage) refers to viruses that infect bacteria. They are abundant in nature and can be isolated from the same niches where their hosts reside. These small agents were first reported in 1915 by the British bacteriologist Frederick W. Twort (Twort 1915). In 1917, Felix d'Herelle named them 'bacteriophages', literally the eaters of bacteria, and started to use them in

patients with dysentery (d'Herelle 1917). Since then, phage-therapy has developed especially in Eastern Countries, where it was commonly applied with success in animals and in humans (Chanishvili 2012). The clinical use of phages declined slowly with the discovery of antibiotics during the 1940s and 1950s. The interest in bacteriophages as an alternative to antibiotic therapy has re-emerged only recently, and their potential applications are increasingly being

examined for purposes ranging from improving food safety to preventing and treating bacterial diseases, particularly those caused by drug-resistant pathogens (Martinez 2009).

In relation to food safety and public health, foodborne disease outbreaks due to bacterial contaminations can occur even if good hygiene practises are mostly applied (Holah *et al.* 2002). In Europe, 2,480 confirmed human cases of listeriosis were reported in 2017, revealing a statistically significant increasing trend over the period 2008-2017 and a fatality rate of 13.8% (EFSA 2018b). After non-typhoid *Salmonella* spp., *L. monocytogenes* is responsible for the majority of deaths in USA (da Silva and De Martinis 2013) and ready-to-eat (RTE) products have been the most commonly incriminated foods during the last 30 years (D'Alton *et al.* 1997, Fleming *et al.* 1985, Gottlieb *et al.* 2006, Graves *et al.* 2005, Olsen *et al.* 2005). The second leading cause of physician visits, hospitalisation, and death in USA is *Campylobacteriosis* (Scallan *et al.* 2013). The year 2012 registered a 14% increase in the estimated incidence of infection when compared with the period 2006-2008 (CDC 2013). In Europe, *Campylobacteriosis* was the most commonly reported zoonoses in 2017, with 246,158 confirmed human cases (EFSA 2018b).

Bacteria can grow and proliferate in the environment as single or independent cells, or they can organise in aggregates commonly referred to as 'biofilms'. During biofilm formation, bacteria anchor themselves to surfaces by synthesising extracellular polymeric substances that provide them with protection from environmental stress factors and antimicrobial agents (McLandsborough 2013). In particular, *L. monocytogenes* is capable of aggregating on a variety of food processing equipment surfaces, including polystyrene, stainless steel, and Teflon. Viable cells within biofilms are partially protected from salinity and chemicals as antimicrobials and disinfectants/sanitiser (Carpentier and Cherf 2011). The bacteria *C. jejuni* is also known to form biofilm; a correlation between *C. jejuni* biofilm formation and an increased fluoroquinolone resistance development was recently reported (Bae and Jeon 2013).

In terms of antibiotic responses, β -lactam penicillin G and ampicillin are the current drugs of choice for the treatment of listerial infections. Many *L. monocytogenes* isolates have developed a high resistance to these chemicals over the years (Fallah *et al.* 2012, Krawczyk-Balska *et al.* 2012). Regarding *Campylobacter* spp., a high resistance to ciprofloxacin, nalidixic acid, and tetracyclines was observed in isolates from fowl, broiler meat, pigs, and cattle, whereas much lower levels were observed for erythromycin and gentamicin (EFSA 2018a).

Foodborne pathogens significantly enhance

the public health risk because of their high environmental persistence and the constant development of drug resistance patterns. For these reasons, public health systems reserve a high level of attention for tools that can help to control and minimise these emerging hazards.

In this study, the latest findings on bacteriophages specifically active against *L. monocytogenes* and *Campylobacter* spp. are reported, focusing on their applications as bio-decontaminants and bio-therapeutics. In particular, we investigated phages as a valid, safe, and cost-effective strategy for eliminating/reducing the levels of specifically targeted bacterial pathogens in foods, with no deleterious effect on the organoleptic properties and without altering the beneficial microflora.

Phages for biocontrol of *Listeria monocytogenes*

Many *Listeria*-phages have been described (Dorscht *et al.* 2009, Loessner *et al.* 2000, Schmuki *et al.* 2012, Zimmer *et al.* 2003). Only B054, A511, and P100 belong to the *Myoviridae* family (Carlton *et al.* 2005, Klumpp *et al.* 2008, Schmuki *et al.* 2012).

Phages against *L. monocytogenes* have been evaluated for their efficacy as biocontrol agents in a variety of foods (e.g., hot dogs, cheese, and salmon fillet) (Carlton *et al.* 2005, Guenther *et al.* 2009, Soni *et al.* 2009). Among the variants that are determinant for a successful intervention in RTE products, 2 are particularly important: the ratio between phage dose and host load and the food chemical composition (Guenther *et al.* 2009). For this reason there is a need to individually optimise protocols for phage applications with respect to phage characteristics and food matrix (Guenther *et al.* 2009).

Two phage-based formulations have been approved. The first one, ListShield (Intralytix, Baltimore, USA), was regulated in USA as a food additive (USG 2006). ListShield is a mix of 6 different bacteriophages and its activity has been tested specifically on fruits. In particular, this phage cocktail significantly reduced *L. monocytogenes* counts by 2.0-4.6 log units on melons and by 0.4 log units on apples (Leverentz *et al.* 2003). In another study, ListShield yielded a total bacteria reduction of up to 6.8 log units after 7 days storage when applied onto contaminated honeydew melon tissues (Leverentz *et al.* 2004). When used in phage-therapy, ListShield effected a preventive reduced concentration of pathogen numbers in the gastrointestinal tract of mice before being infected with *L. monocytogenes*. Moreover no adverse effects on commensal microbiota composition were observed (Loessner *et al.* 1995). Given the

good results already yielded on fruits, as a phage cocktail formulation, ListShield could be a very good candidate to reduce pathogen contaminations along food chain productions and in foods.

The second formulation approved in USA is LISTEX™P100 (Microcos Food Safety, Wageningen, The Netherlands), which is composed of bacteriophage P100. It was used to control *L. monocytogenes* on surface-ripened red smear soft cheese, yielding a pathogen reduction of at least 3.5 logs (Carlton et al. 2005). Soni and collaborators also demonstrated its activity on fresh channel catfish fillets (*L. monocytogenes* reduction between 1.4 and 2.0 log CFU/g at 4°C, 10°C, and 22°C) (Soni et al. 2009), raw salmon (reductions of 1.8, 2.5, and 3.5 log CFU/g from initial bacterial loads of 2, 3, and 4.5 log CFU/g at 4° and 22°C) (Soni et al. 2010), and on queso fresco cheese (initial bacterial reduction of 2-4 log CFU/cm² at 4°C, but a subsequent bacterial regrowth was reported) (Soni et al. 2012). More recently, Chibeu and colleagues (Chibeu et al. 2013) demonstrated that LISTEX™P100 can enhance RTE meat safety (cooked turkey and roast beef) when used in combination with chemical antimicrobials. Guenther and colleagues (Guenther et al. 2009) reported phage P100 ability to reduce bacterial counts to undetectable levels in chocolate milk and mozzarella cheese brine. A reduction of up to 5 log was also observed on various solid foods as RTE and vegetables.

In this study, phage P100 was used in combination with A511, a lytic phage with a broad host range (the ability to infect almost 95% of *L. monocytogenes* strains of the major serovar groups 1/2a and 4b) (Loessner and Busse 1970). Bacteriophage A511 was also tested alone on soft ripened white mould and red-smear cheeses. This led to a reduction of *L. monocytogenes* cells below the limit of detection (more than 6 log reduction) (Guenther and Loessner 2011).

The presence of *L. monocytogenes* in floor drains is a critical issue in the formation of aerosol because it could lead to *Listeria* dispersal in water processing plants (Berrang et al. 2013). Nevertheless, some researchers reported the use of competitive exclusion lactic acid bacteria in floor drains against *L. monocytogenes* viable cells (Zhao et al. 2006) and biofilm (Zhao et al. 2013). Although there are no scientific reports of similar results obtained with phages, we would like to highlight the potential of phage applications in floor-drains as an additional means to control this pathogen in food productions.

Phages for biocontrol of *Campylobacter* spp.

The prevalence of *Campylobacter*-phages in the environment is estimated to be high (Atterbury

et al. 2005, El Shibiny et al. 2005, Loc Carrillo et al. 2005) and the majority of *C. jejuni* and *C. coli* phages are virulent, with the exception of a few temperate bacteriophages. They are classified into 3 groups (Groups I, II, and III) according to head diameter and genome size (Sails et al. 1998), with long and contractile tails, double-stranded DNA, and icosahedral heads (Connerton et al. 2011). Only 8 genomic sequences have been published (Janez and Loc Carrillo 2013). The risk that phages could carry unknown genes coding for lysogeny or promoting virulence or resistance properties is therefore still a concern and requires further investigations (Carvalho et al. 2012 a, b). Phage studies are mainly focused on preventive/therapeutic applications in animals and as bio-decontaminants on food and contact surfaces, which demonstrates the priority of reducing *Campylobacter* spp. transmission to humans.

Wagenaar and colleagues (Wagenaar et al. 2005) and Loc Carrillo and colleagues (Loc Carrillo et al. 2005) reported the first phage-based treatments against *Campylobacter*-infected livestock. Group III phages administered to chickens challenged with *C. jejuni* determined a significant decrease in bacterial colonisation (Wagenaar et al. 2005), while phages CP8 and CP34 determined a decrease in cell count between 0.5 and 5 log CFU/g of cecal content after being administered to infected broilers (Loc Carrillo et al. 2005). El Shibiny and colleagues (El Shibiny et al. 2009) tested phage CP220 in birds colonised with *C. jejuni* and *C. coli*, producing between 1 and 2 log reductions. More recently, Carvalho and colleagues (Carvalho et al. 2010) reported encouraging results against *C. jejuni* and *C. coli* infections after administering, for the first time, a phage cocktail to chickens by oral gavage and in feed. The phage cocktail administered by both routes was able to reduce the titre of *C. coli* and *C. jejuni* in faeces by approximately 2 log CFU/g.

In another recent study from Kittler and colleagues (Kittler et al. 2013), the authors highlighted the positive effects of administering a phage cocktail to broilers via drinking water from 1 to 4 days prior to slaughter. This led to a reduction of up to 3.2 log CFU in *Campylobacter* spp. loads. Differently from *Listeria*-phages, phage-based products against *Campylobacter* spp. have not yet reached the market, which is potentially due to poor *in vivo* trial results (Loc Carrillo et al. 2005).

Two additional studies used chicken skin tainted with susceptible *Campylobacter* spp. and treated with phages (Atterbury et al. 2003, Goode et al. 2003). Both groups demonstrated 1 log drop in bacterial loads when samples were stored at 4°C. Moreover, Atterbury and colleagues (Atterbury et al. 2003) showed a 2 log drop recovery from frozen-thawed samples, but this result could be due to bacterial

inactivation by freezing more than to a phage effect. Bigwood and colleagues (Bigwood *et al.* 2008) treated raw and cooked beef meats and compared the results of different phage titres ($10\text{-}10^4$ PFU/cm²) against different levels of contamination ($< 100\text{-}10^4$ CFU/cm²). Significant host inactivations (2 log/cm²) were achieved using the highest host cell density and the highest phage titres. Orquera and colleagues (Orquera *et al.* 2012) described how the application of NCTC 12684 and CP81 bacteriophages to raw chicken meat for up to 7 days at 4°C could not produce any relevant reduction in bacterial loads.

Bacteriophage activity against *Listeria monocytogenes* and *Campylobacter* spp. biofilms

Bacterial biofilms consist of microorganisms embedded in a glycocalyx that is predominantly composed of exopolysaccharides (Costerton *et al.* 1994). The glycocalyx provides bacterial protection against environmental stressors, such as desiccation and antimicrobial agents, and may also act as a reservoir for nutrients (Allison 1993).

The ability of some phages to produce glycanase enzymes (polysaccharide depolymerases) has been reported for over 40 years (Adams and Park 1956). The role of glycanase enzymes is primarily related to phage-binding activity to bacterial capsular materials and the degradation of polymers with consequential cell infection. Many phages synthesise these enzymes (Cornelissen *et al.* 2011, Cornelissen *et al.* 2012) but non-synthesising bacteriophages can be genetically modified in order to express polysaccharide-degrading enzyme production,

thus enabling their biofilm dispersion ability. Lu and Collins (Lu and Collins 2007) described the particular activity of a modified T7 bacteriophage that was able to remove 99.97% *Escherichia coli* biofilm cell counts. These results were 2 orders of magnitude better than those observed with a phage that did not produce polysaccharide-degrading enzymes (Lu and Collins 2007). This genetically modified phage has been patented in USA (Lu and Collins 2007).

Few studies have published the effects of phages against *L. monocytogenes* and *Campylobacter* spp. biofilms (Table I).

Briefly, listeriophages LiMN4L, LiMN4p, and LiMN17 were used to test activity against biofilms of *L. monocytogenes* strains isolated from seafood and grown onto stainless steel and stainless steel coated with fish protein surfaces. The phages produced more than a 3 log reduction. The best lysis was achieved when cells were first slightly dislodged (Ganagama-Arachchi *et al.* 2013). Bacteriophage P100 also showed a 3.5-5.4 log/cm² reduction of *Listeria* biofilms grown on stainless steel coupon surfaces (Soni and Nannapaneni 2010a). Montanez-Izquierdo and colleagues (Montanez-Izquierdo *et al.* 2012) confirmed the effectiveness of the P100 biofilm disruption (an average of 5.29 log CFU/cm² reduction) by comparing classical culture methods and the use of epifluorescence microscopy. Roy and colleagues (Roy *et al.* 1993) investigated the ability of listeriophages H387, H387-A, and 2671 to disrupt biofilms grown onto polypropylene surfaces. They observed a reduction of more than 3 log of *L. monocytogenes*.

Regarding phage activity against *Campylobacter* spp. biofilms, very little has been reported. Siringan and colleagues (Siringan *et al.* 2011) verified the

Table I. Phage application to control *Listeria monocytogenes* and *Campylobacter* spp. biofilms.

Host(s)	Phage(s)	Surface(s)	Treatment	Outcome	Reference
<i>Listeria monocytogenes</i> (10401 and 8427)	H387, H387-A, 2671	Stainless-steel, polypropylene	Phage suspensions up to 3.5×10^8 PFU/ml Use of single phages and cocktail	More than 3 log reduction Phage cocktail showed a better efficiency than single phages	Roy <i>et al.</i> , 1993
<i>Listeria monocytogenes</i> (serotype ½ a)	P100	Stainless-steel coupon	Phage suspensions of 10^9 PFU/ml	3.5-5.4 log/cm ² reduction	Soni and Nannapaneni, 2010b
<i>Listeria monocytogenes</i> (CCUG 15526)	P100	Stainless-steel coupon	Phage suspensions of 5, 6, 7 or 8 log PFU/ml	Phage concentrations of 6, 7, and 8 log PFU/ml reduced <i>L. monocytogenes</i> in mean 5.29 log CFU/cm ² , after 24h	Montanez-Izquierdo <i>et al.</i> , 2012
<i>Listeria monocytogenes</i> (sea-food strains 19C09, 19D03 and 19E03)	LiMN4L, LiMN4p, LiMN17	Stainless-steel, stainless-steel coated with fish protein	Phage suspensions of about 9 log ₁₀ PFU/ml on undisturbed and slightly dislodged biofilms Use of single phages and cocktail	More than 3 log reduction with phages being more effective on dislodged biofilms	Ganagama-Arachchi <i>et al.</i> , 2013
<i>Campylobacter jejuni</i> (NCTC 11168 and PT14)	CP8, CP30	Glass	Single phage suspensions of about $10^6\text{-}10^9$ PFU/ml	1 to 3 log ₁₀ CFU/cm ² reduction, 24h post-treatment	Siringan <i>et al.</i> , 2011

dispersal of biofilm matrix from *C. jejuni* NCTC 11168 and PT14 on glass by bacteriophages CP8 and CP30, and reported an average of 2 log CFU/cm² reduction 24 hours post-treatment.

Antimicrobial application of bacteriophage endolysins in food safety

Peptidoglycan hydrolases (PGHs) are a class of enzymes capable of hydrolysing bonds in the peptidoglycan (PG) layer of the bacterial cell wall, resulting in cell death. PGHs produced by phages are called 'endolysins' (or 'lysins'), because they lyse the bacterial cells internally ('lysis from within'). However, suspensions of concentrated endolysins can also be responsible for lysis from outside the cell ('lysis from without'), and this is the principle that underpins their potential application without using viable phages (Abedon 2011). It is important to highlight that their action is principally restricted to Gram-positive bacteria, since Gram-negative prokaryotes have an outer membrane that protects PG from hydrolases (Shen et al. 2012).

Scientists have recently shown a growing interest in lysins because they are safe, biodegradable, non-corrosive molecules with a high bacterial PG affinity (Nelson et al. 2012) and they are easy to produce on a large scale (Zhang et al. 2012). Moreover, this class of enzymes is also believed to be refractory to bacterial resistance development, as we will discuss in the next section.

Few *L. monocytogenes* phage endolysins have been characterised (Oliveira et al. 2013). These include: Ply500 (Loessner et al. 1995), PlyPSA (Zimmer et al. 2003), PlyP35 (Schmelcher et al. 2012), Ply118 (Shen et al. 2012), Ply511 (Shen et al. 2012), PlyP40 (Schmelcher et al. 2012), PlyLM (Schmelcher et al. 2012), and LysZ5 (Zhang et al. 2012). The 'chimera' is an example of a protein-engineering product where the fusion of phage endolysin Ply500 with PlyP35 was able to produce a combined enzyme that is active against a broader spectrum of *Listeria* spp. (Schmelcher et al. 2012).

Few studies conducted between 2000 and 2011 reported a reduction of *L. monocytogenes* cells when treated with lysins PlyPSA (Korndorfer et al. 2006), Ply500 (Schmelcher et al. 2011), Ply118, and Ply511 (Gaeng et al. 2000).

The major limitation of these studies is the use of optical density (OD₆₀₀) to measure the bacterial reduction *in vitro*. In order to achieve more objective results it is necessary to also test lysins activity *in vivo* and to assay bacterial reductions by plate-counting methods. Zhang and collaborators (Zhang et al. 2012) characterised and purified lysZ5 from

L. monocytogenes phage FWLLm3 and tested its activity in reducing *L. monocytogenes* counts in soya milk, with the pathogen concentration reduced by more than 4 log CFU/ml after 3 hours of incubation at 4°C. This was the first report of a *Listeria* phage endolysin tested in foods, and this opens up the possibility of examining the potential use of biocontrol in other RTE foods. Another lysin, PlyLM, a putative N-acetylmuramoyl-L-alanine amidase, was shown to be active against *L. monocytogenes* strains and other bacteria within the genus level, and above all against *L. monocytogenes* biofilm (Simmons et al. 2012).

In particular, the present study demonstrated a 20% biofilm reduction when PlyLM was used alone, yielding a complete digestion of the bacterial monolayer when the endolysin was applied in conjunction with a protease. This is the only study dealing with *L. monocytogenes* biofilm reduction with the use of endolysins. It is important to highlight that the glycocalyx and cell agglomerates within biofilms play an important role as physical barriers between lysins and bacterial cells, and this produces a consequential reduction of their activity.

Bacterial resistance to phages

Bacteria are known to adopt many antiviral mechanisms in order to preserve themselves against phage infection (Bikard and Maraffini 2012, Stern and Sorek 2011). One of the most common is based on the modification of cell-surface molecules (e.g. lipopolysaccharides, pili, and flagella) that are then used as receptors from phages in order to block host recognition and adsorption (Hyman and Abedon 2010, Labrie et al. 2010). These defence mechanisms can be transmitted from resistant to sensitive cells through the transduction of bacterial DNA via phage particles, leading to the development of 'bacteriophage-insensitive mutants' (BIMs) (Emond et al. 1997, Garcia and Molineux 1995, Hudson et al. 2005). Three other phage resistance mechanisms can occur during phage replication within the host cell: the abortive infection, the restriction modification system (Golais et al. 2013), and the CRISPR/cas system (Szczepankowska 2012). Even if much is known about bacterial anti-phage immune mechanisms, it is likely that many still remain to be discovered.

Bacterial resistance to phages does not always work efficiently. Sometimes spontaneous mutations that occur in bacteria lead to phage-resistant strains. This can have deleterious effects on prokaryotes, which does not necessarily confer an evolutive advantage. This could explain the tendency of BIM bacteria to revert to sensitive strains once bacteriophages are no longer a threat in their environment (O'Flynn et al. 2004). In particular, some authors have observed the

ability of phage-resistant *Campylobacter* spp. strains to become sensitive after multiplication in chicken guts without exposure to bacteriophages (Carvalho *et al.* 2010, Scott *et al.* 2007).

None of *L. monocytogenes* phage-resistant strains was isolated in cheese treated with low concentration of phage P100 (Carlton *et al.* 2005). On the other hand, other studies showed the isolation of BIM *L. monocytogenes* isolates from samples taken from a smoked fish processing facility (Vongkamjan *et al.* 2013).

Unlike *L. monocytogenes*, more information is available about *Campylobacter* spp. Resistance to phages CP8 and CP30 was observed in surviving cells within *C. jejuni* NCTC 11168 biofilms after phage treatment, while no resistance was observed in *C. jejuni* PT14 isolates (Siringan *et al.* 2011). Loc Carrillo and colleagues (Loc Carrillo *et al.* 2005) investigated the ability of *C. jejuni* HPC5 to develop resistance to phages CP8 (8% of the strains) and CP34 (11% of the strains) *in vitro*, noting that these isolates were able to revert back to sensitive strains. A similar *in vivo* experiment revealed that only 4% of colonies that recovered after treatment with phage CP34 achieved resistance. Coward and colleagues characterised the interaction between *C. jejuni* and 16 phages used in the United Kingdom as the *Campylobacter* typing scheme (Coward *et al.* 2006). Interestingly, they demonstrated that resistance to this group of phages was associated with motility defects and disruption of capsular polysaccharides (CPS) (Coward *et al.* 2006). In 2007, Scott and colleagues (Scott *et al.* 2007) evaluated the *in vivo* competitive colonisation between phage-resistant and phage-sensitive strains with and without phage pressure in the environment. Their results showed that without a phage predation pressure, the phage-sensitive strains could out-compete the phage-resistant strains. In the presence of phages, the situation was very different, and the phage-resistant strains were able to out-compete phage-sensitive strains. The authors also demonstrated the recovery of phage-resistant mutants and of poor chicken intestine coloniser strains within a *C. jejuni* HPC5 populations of avian gut when treated with phage CP34 (Scott *et al.* 2007).

Sorensen and colleagues (Sorensen *et al.* 2012) exposed *C. jejuni* NCTC 11168 to phage F336 treatment and yielded a large number of phage-resistant strains characterised by the modification of the capsular polysaccharide's (CPS) hypervariable O-methyl phosphoramidate structure (Sorensen *et al.* 2012).

Although phage-resistance development is one of the major concerns for scientists, it can be avoided with a mix of bacteriophages ('phage cocktails'). In fact, the activity of different phages pulled together against the same host significantly reduces the

possibility of bacteria developing resistance against more anti-phage infection systems contemporarily (Leverentz *et al.* 2004). The use of endolysins could also be a good alternative to escaping anti-phage mechanism development. There is no scientific evidence about the existence of lysine-resistant bacterial strains and the few studies that were carried out to isolate them in the environment were all unsuccessful (Fischetti 2005).

The European position on bacteriophages and current relevant international legislation

The European Food Safety Authority (EFSA) had issued 3 scientific opinions about bacteriophages. The first one was released in 2009 by the Panel on Biological Hazards, and deals with 'the use and mode of action of bacteriophages in food productions'. This document described the information available on these microorganisms and their potential role as bio-decontaminants. It recognised the efficacy of some bacteriophages in the elimination of specific pathogens. In this publication, EFSA did not approach the issue of safety associated with the use of bacteriophages, and reported few major concerns in relation to BIMs and efficacy during recontamination (EFSA 2009). In 2012, an application dossier by Micros Food Safety (the Netherlands) for the approval of LISTEX™P100 to reduce *L. monocytogenes* from food surface contamination led to the publication of a second EFSA opinion. The assessment focused on the safety and efficacy of bacteriophage P100 in the treatment of raw fish. Authorities expressed scepticism about the absence of industrial-scale studies, the limited selection of strains used for inoculation, and the lack of results concerning the pathogen reduction in the final fish product. Despite the final conclusion that 'bacteriophages cannot be included on the Qualified Presumption of Safety list of microorganisms intentionally added to food or feed', EFSA agreed that bacteriophage P100 fulfils the safety requirements (EFSA 2012).

The last EFSA opinion on Listex™P100 was issued in 2016. Safety and efficacy of the phage against *L. monocytogenes* was recognised for 3 ready-to-eat product categories (meat and poultry, fish and shellfish, dairy products). Experimental studies indicated that *L. monocytogenes* strains resistant to Listex™P100 could develop, but cleaning of surfaces where the phages are applied together with disposal of unsold treated products could reduce this risk. Moreover, it was speculated that phage P100 resistant strains can be accompanied by more sensitivity to some classes of antimicrobials and that phage persistence in the environment is low (EFSA 2016).

The United States Food and Drug Administration (FDA) and United States Department of Agriculture were more tolerant about LISTEX™P100, recognising and granting the product Generally Recognized as Safe (GRAS) status in 2006 (FDA 2006, USDA 2006, USG 2006). In 2011, phage P100 was classified as a 'processing aid' by USDA and the Food Safety and Inspection Services (1USDA 2011). Today, LISTEX™P100 is used in USA, Canada, and Switzerland. In the EU, the Dutch Ministry of Health issued a formal statement confirming that LISTEX™P100 can be used as a processing aid. Unlike *L. monocytogenes*, no official phage-based products have been approved against *Campylobacter* spp.

Eastern European countries and the Soviet Union are mainly involved in the application of phage-therapy (Kurlenda and Grinholc 2012). Despite extensive phage use in many reported clinical cases (Parfitt 2005, Slopek et al. 1987, Sulakvelidze et al. 2001), today there are no official regulations standardising its use. In fact, a general scepticism still prevails, and the validity of most relevant studies has been questioned, in part due to a relaxed scientific rigour. According to Verbeken and colleagues (Verbeken et al. 2007), novel therapies must comply with paragraph 32 of the Declaration of Helsinki and be practiced under the supervision of an Ethics Committee. In 2005, the Polish Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences performed experiments under 3 conditions: the approval by an institutional review board, the written informed consent of patients, and health-insurance (Gorski et al. 2009). The Burn Wound Centre in Belgium also performed a clinical trial after approval from an Ethics Committee. The results revealed that phages are safe for patients and do not cause any adverse side-effects to eukaryotic cells or natural flora (Bruttin and Brussow 2005, Mai et al. 2010).

Merabishvili and colleagues (Merabishvili et al. 2009) also addressed the important issue of developing phage-based compounds under quality control. In their study, they described a small-scale laboratory-based production of a phage cocktail designed for the treatment of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections in burn wound patients.

If the process leading to the approval of phage-use among different Countries worldwide seems to be controversial, the situation for phage endolysins could be considered less complicated. In fact, unlike bacteriophages, which are referred to as

natural product, lysins are molecules purified from a recombinant expression system, which could represent a less problematic issue for approval.

Future perspectives

Two of the main pathogens responsible of serious foodborne outbreaks are *L. monocytogenes* and *Campylobacter* spp. Their insidious persistence in animals is well-known, as is the increasing development of antibiotic-resistance patterns and their deleterious effects. These are some of the reasons for an increasing interest in bacteriophages and their potential innovative applications. As natural killers of bacteria, phages are abundant in nature. Moreover, their isolation/replication techniques are relatively easy to perform and cost-effective when compared with the preparation of new antibiotics.

Nevertheless, few variants can negatively influence the phage/lysin activity by limiting their delivery to the sites of infection e.g. solid food matrix, biofilm structure (glycocalyx), and anti-phage detergents.

In order to be applied in food productions and therapy, phages should present the following characteristics: strict lytic cycle, broad host range, lack of transduction of bacterial DNA, absence of pathogenic genes or allergenic proteins, sequenced genomes, and long-term stability. These microorganisms are extremely versatile and can be engineered in order to be more efficient in attacking their hosts (Lu and Collins 2007).

Other applications in the future may include the production of 'bio-food packaging materials': these novel technologies could be based on encapsulating phage inside electrospun fibres (Korehei and Kadla 2013) or on immobilising phages onto biological membranes like cellulose (Anany et al. 2011). The inclusion of bacteriophages inside food packaging could be a valid strategy for long-lasting pathogen control during shelf life. 'Ghost particles' could be used in order to gain advantages from the 'lysis from without', with the application of 'empty' phages and without genome implication.

Phage-resistant development in host bacteria needs to be monitored. Further investigations based on bacteriophage DNA sequencing along with more in-depth research demonstrating phage efficacy at industrial level (trials/challenge tests) are required to better understand phage biology and to assess their potential approval for animal health and in food/feed productions.

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