Bacteriophage for Biocontrol of Foodborne Pathogens: Calculations and Considerations

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Abstract: The use of phage or phage products in food production has recently become an option for the food industry as a novel method for biocontrol of unwanted pathogens, enhancing the safety of especially fresh and ready-to-eat food products. While it can be expected that many more phage products currently under development might become available in the future, several questions may be raised concerning the use of such products, regarding both immediate and long-term efficacy, consumer safety, and application methods. The available evidence suggests that, with a few caveats, safety concerns have been satisfactorily addressed. Answers concerning efficacy are more complex, depending on particular applications or the target pathogens. To ensure long-term efficacy beyond what can be tested on a laboratory scale, food safety concepts employing phages will have to be well-thought out and may involve rotation schemes as used with bacterial starter cultures, the use of

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phage cocktails, or application of phages combined with other antimicrobials. This review will discuss these issues on the basis of the available literature as well as providing an outlook on the potential of phages in future applications.

Key Words: Listeria, Salmonella, Campylobacter, E. coli, *foodborne disease*

INTRODUCTION

Bacteriophages are bacterial viruses that only infect and multiply within their specific hosts. Host specificity is generally found at strain level, species level, or, more rarely, at genus level. This specificity allows for directed targeting of dangerous bacteria using phages. The concept of fighting pathogens with their bacteriophages or using phages directly in foods has been around for many years; many reviews on this subject have been published [e.g., 1, 2, 3, 4, 5]. The simplicity of this principle, however, may be deceptive because the effectiveness of using of phage for bacterial control depends on the likelihood that phage and bacteria are in the same place on one hand, and on the host being susceptible to the particular phage on the other.

Phage therapy of infectious diseases was proposed and implemented shortly after the discovery of phages by Twort [6] and D'Herelle [7]. While early efforts were partly successful, it was the advent of antibiotics that put a stop to more research in many countries. In those countries where phage therapy research and application was continued, it was and still is used widely. While critics point out that these studies do not follow rigorous Western standards of evaluation such as the inclusion of double blind trials, it is also obvious that oftentimes treatment was successful [8] (see Article 7 on phage therapy of humans). While perhaps not the answer to all the problems posed by infectious bacterial diseases, phage therapy is more than promising and the lack of phage

products currently available to physicians can only be explained by the enormous costs involved in getting new medicines to the market.

The use of phages to remove pathogens from food is equally appealing. This is especially true for bacterial pathogens such as *Listeria monocytogenes*, which, upon entry into the host body, behave as intracellular pathogens and can therefore not be reached by the immune system or by phage administered to a person suffering from Listeriosis. Generally, the applicable food laws provide a less complicated avenue for the use of phages, as testified by FDA approval of several phage products for use in food manufacturing [www.fda.gov/]. The need for control of pathogens during the manufacture of food is reflected by the incidence of foodborne bacterial infections. The number of cases of listeriosis, for example, has stabilized or is on the rise in many countries, especially in Europe, after having undergone a steep decline in the first part of the last 20 years [9]. Similar trends can be observed for other foodborne infections, and new orally transmitted bacterial diseases are emerging [10].

With respect to the regulatory issues associated with the use of phages for treatment of bacteria in foods, a mixed *Listeria* phage preparation (www.intralytix.com) received approval to be used as a food additive in the production of ready-to-eat meat and poultry products, and another phage preparation comprising a virulent single *Listeria* phage (www.ebifoodsafety.com) even received the highly desirable GRAS (generally recognized as safe) status for its use in all food products. Phage preparations active against *E. coli* and *Salmonella* are also offered (www.omnilytics.com); some have approval for being sprayed, showered, or nebulized on cattle and chickens respectively, prior to slaughter of the animals (see Article 5 on phage therapy of animals). Moreover, phage preparations active against tomato and pepper pathovars of *Pseudomonas putida (*www.omnilytics.com), developed for treatment of plants against bacterial spot diseases, have been approved for use by the US Environmental Protection Agency (EPA) (see

Article 4 on phage therapy of plants). These recent developments highlight the fact that, besides the use of phage for direct addition to food, much effort has also gone into phage-based control of pathogens that can colonize plants or animals used in food production. It can be expected that many more phage products will appear on the market in the near to mid-term future.

The development of phage therapy reagents from the laboratory bench to application in the food industry are recent and still on-going, and many questions are raised whenever the subject is discussed. Among the most common questions are those concerning phage safety and efficiency of treatment, but also questions on phage resistance as well as practical questions concerning production, purification, and specific application procedures. In this review we endeavor not only to summarize the research that has been published in this field but also, where possible, to shed light on some of the issues that concern the use of phages as antibacterials in food.

CONSIDERATIONS REGARDING THE APPLICATION OF PHAGES

Historically, most of the work on phage infection kinetics has been done in liquids, and usually with dense pure cultures of highly permissive host bacteria. Such kinetics can be applied for modeling approaches in the fermentation business and the dairy industry, where phages infecting starter cultures can effectively ruin entire fermentation batches, such as yoghurt or cheese. The most important parameters are the absolute concentrations of phages and host bacteria as well as the phage affinity for those bacteria (adsorption constant). For example, when initial bacterial numbers are relatively constant at the beginning of a batch production cycle, it is changes in the concentration of phages present that will determine the kinetics of the infection process and therefore the frequency of bacterial survival. Due to the amplification of phage numbers that results from phage infection of bacteria, in liquids with high numbers of host cells present (critical host cell concentration threshold is approximately 10^5 cells per ml), even a very small initial number of phages can cause complete lysis of the bacterial culture in a relatively short time frame.

Low Bacterial Densities

With respect to phages used against pathogens in food matrices, one faces a completely different set of premises. This occurs because a significant proportion of foods to be treated will be solid rather than liquid, and, with modern hygiene regimens in place, any bacterial contamination is likely to occur at very low numbers. Under these circumstances, it is critical to understand that a sufficiently high number of phages is required to hit and infect the few bacterial target cells present. In other words, low numbers of bacteria are unlikely to be affected by low numbers of phages because phages and bacteria are unlikely to meet. In a more biochemical sense, the concentration of one of the reaction partners (phage) must be sufficiently high to enable contact and subsequent reaction (infection and killing), even when the other reaction partner is present at a very low concentration only (numbers of bacteria). In fact, once a critical concentration threshold of phage numbers is reached to enable it to cover the entire available space within any given matrix, the concentration of the bacterial host is not important, i.e., it does not matter whether only 1 or 10^6 cells per ml are present, they will all be infected.

In this context, it helps towards visualizing the extremely small dimensions of phages and bacteria in comparison to the very large volumes they encounter when free-floating in, e.g., one milliliter of a liquid. A milliliter is 1 x 1 x 1 centimeters, or 1 x 1 x 1 meters^{-2}. For reasons which will be become obvious in a moment, a milliliter also can be expressed in units of length that are one millionth of a meter long, i.e., 10^{-6} meters. Since 10^{-6} meters is 1/10,000th of a meter^{-2}, one ml can be expressed as equal to 10,000 x 10,000 x 10,000 meters^{-6}. An average bacterial cell, by contrast, measures approximately 1 x 3 meters^{-6} (whereas an average phage particle is around 0.05 x 0.2 meters^{-6}). However, if the bacterium were to be transformed approximately one-million-fold, i.e., to the size of a human (0.5 x 2 meters), then it would exist, correspondingly, in a volume of

10,000 x 10,000 x 10,000 in meters. That is, a body of water which is 100 km^2 on its surface and 10 km deep, i.e., 1000 km^3!

By comparison, Loch Ness of Scotland has a volume of "only" ~7 km^3. Thus, envisage the likelihood of a density-neutral, approximately apple-sized phage encountering a human who is scuba diving (perhaps very deeply) in Loch Ness, and you will still be underestimating by ~100-fold how long it could take an individual phage to find an individual bacterium within an ml of fluid. The analogy is not perfect, however, since, as nanotechnologists will testify, the extreme microscopic is a very different world from the macroscopic, and indeed thermal motion exerts a much greater impact on the movement of phage-like entities than on apple-like entities, plus, while complete mixing within a single ml is trivial, complete mixing of Loch Ness is not.

Both of these latter processes, thermal motion-driven particle diffusion and mixing due to either fluid flow or active swimming (bacterial motility, for example), will result in greater likelihoods of encounter between phage and bacterium than between man and fruit, if driven by random processes alone [11]. Nevertheless, using standard assumptions of mass-action interactions and moderate phage adsorption constants, e.g., ~10^{-9} ml min^{-1}, on average it will take on the order of 1,000 years for 1 phage and 1 bacterium to meet within 1 ml of fluid (see the appendix of [5] for a broader discussion of this phenomenon). More practically, if the concentration of bacteria and phages is approximately 10^6 units per ml or less, then the chance that a phage and bacterium will meet through diffusion alone, in a limited time, though larger, is still impractically small. For example, using the same calculations as above, with 10^6 phages per ml, and an adsorption constant of 10^{-9} ml min^{-1}, it will take approximately half a day for half the bacteria present to be adsorbed. As a conclusion, the number of phage used in any application where the number of target cells is the limiting factor must be sufficiently high (threshold of approx. 1 x 10^8 PFU/ml) to ensure

sufficiently rapid contact of the two partners. See Bigwood et al. [12] for very recent experimental verification of this claim.

Complication of bacterial replication

In a food-related application, the issue of whether the bacteria are able to replicate in the particular environment also influences efficacy of the phage treatment. If the doubling time of the bacteria is shorter than the time necessary to achieve an infection and kill a bacterium, then the number of bacteria will initially increase in spite of phage presence and the bacteria will remain present if they do not reach a critical number allowing exponential phage replication. The same principles are true for application on solid surfaces, such as for bacteria found in association with food matrix, but there movement by diffusion of the phages will be more limited. Therefore, a critical number of phages is necessary to achieve both infection and a fast and significant drop in bacterial viable counts.

Additional Considerations and Summary

The exact concentration of phages that needs to be used for a given application will depend on several factors: surface micro-structure which affects phage diffusion rates, phage ability to diffuse at all (since phages may become bound to otherwise inert surfaces), and accessibility of target bacteria (that is, bacteria may be able to enter into the food matrix or otherwise become inaccessible to phages in the course of bacterial growth or food processing); the amount of fluid that is available (which impacts on one hand the volume through which phages must diffuse in order to encounter bacteria, if significant volumes are present, and on the other hand the ability of phages to diffuse at all if little volume is present); and the target reduction levels sought, with higher reductions necessitating more phages. Exceptions to low-level contaminations in foods can be found in phage treatment of "food animals" which are infected/colonized with zoonotic pathogens. These organisms can reach very high numbers, which of course changes the spatial

limitations on phage population expansion (and thereby likelihood of phage-bacterium encounter). Here, treatment with phages may lead to significant phage replication such that progeny phages will also be able to infect the target cells and contribute to sought decreases in bacterial numbers (see Article 3 on phage therapy pharmacology).

SAFETY OF BACTERIOPHAGES

Phages are Non-Toxic

Phages are highly specific and can infect only a very limited range of host bacteria. All available evidence indicates that their oral consumption (even at high levels) is entirely harmless to humans. Safety studies have been performed for example with the *Listeria*-phage P100, in which rats were fed high doses of phages with no measurable effects compared to the control group [13]. A study with *E. coli* phages both in mice and in human volunteers also showed no significant effects on the test subjects. Perhaps the most remarkable aspect of this latter study was the fact that although these phages were able to infect commensal *E. coli* strains *in vitro*, they seemed to have little effect on the *E. coli* occurring in the gut ecological systems of the animals or human volunteers. In the mouse model, only *E. coli* cells implicated in an artificial infection model were affected by the phages [14, 15]. It was speculated that the commensal *E. coli* population lives in niches not easily accessible to phages. Since the human intestinal tract generally hosts a plethora of phages (which nonetheless does not result in dysbiosis of the gut), this speculation makes sense and is likely true for also for the other bacteria and their bacteriophages living in the gut.

Overwhelming additional (albeit more circumstantial) evidence exists corroborating the results observed in specific safety studies. For example, even though phage therapy was not used extensively in the Western world over the past more than 50 years (except for France), thousands of people have received phage therapy in other countries, especially the former Soviet Union and Poland (see Article 7 on phage therapy of humans). Although the phages used were mostly

administered orally or superficially, they were also injected intramuscularly, intravenously, and even into the pericardium and carotid artery [16]. Most noteworthily, none of the reports mentioned significant phage-related undesirable side effects.

Phages are Ubiquitous in Foods

Our environment features a massive abundance (both in numbers and variety) of phage particles, with aquatic environments currently holding the record - up to 10^9 phages per milliliter have been reported for certain freshwater environments, and up to 10^7 phage-like-particles per millilitre were found in marine surface systems. Similar numbers have been reported for terrestrial ecosystems such as topsoil. In fact, with estimates of 10^{31} (or more) in total, phages represent the by far most abundant form of self-replicating units in the biosphere [17].

Apart from environmental sources, humans are constantly exposed to contact with phage by way of their food. Bacteriophages are associated with bacteria and any foodstuff that has not undergone extensive processing will contain phages, with fermented food of course having especially high numbers of those phages infecting the fermentation flora. Fresh vegetables are also a rich source of bacteriophages. Several studies have been undertaken to enumerate phages in food but it should be kept in mind that only specific phages were investigated, always employing only a specific and limited sets of host bacteria. Because of this bias, total phage populations are not properly reflected; even some of the species-specific phages under investigation may have been missed due to a lack of susceptible host bacteria used in the study. A few examples: Fermenting cabbage (Sauerkraut) is a good source of phages [18, 19], with one study describing 26 different phages isolated from commercial Sauerkraut fermentation plants [20]. Swiss Emmental cheese yielded phages infecting *Propionibacterium freudenreichii* at levels of up to 7×10^5 PFU/g [21]. In Argentina, phages infecting thermophilic lactic acid bacteria have been isolated from dairy plant

samples at numbers of up to 10^9 PFU/ml, though these were from batches that failed to achieve the desired fermentation levels [22].

Perhaps more importantly, phages can also be isolated from non-fermented foods. *E. coli* phages have been recovered from fresh chicken, pork, ground beef, mushrooms, lettuce, raw vegetables, chicken pie, and delicatessen food, with counts as high as 10^4 phages per gram [23]. Also, *Campylobacter* phages have been isolated at levels of 4 x 10^6 PFU from chicken [24], and *Brochothrix thermosphacta* phages from beef [25]. It is clear that we consume vast amounts of phages every day, even if we limit our diets to eating only foods which are unspoiled and fresh. The obvious conclusion that can be drawn from all of this evidence is that phages can safely be consumed and therefore deserve the GRAS status (http://www.cfsan.fda.gov/~rdb/opa-g218.html).

CHOICE OF PHAGE FOR FOOD APPLICATIONS

Reasons to Avoid Lysogeny

The apparent safety of phages in general does not imply that all phages which can infect a given host are suitable for use in biocontrol of pathogenic bacteria. This is because unlike virulent (strictly lytic) phages which invariably proceed directly to production of progeny phages after infection, temperate phages do not always kill their hosts, in many cases can integrate their genome into the bacterial chromosome (lysogenization), and can thereby alter the phenotype of infected hosts. Although, most phage genes should be silent in the resulting lysogenic bacteria, phages may include moron genes which can be independently transcribed and may be able to phenotypically alter the bacterium (lysogenic conversion), sometimes increasing the pathogenicity or virulence of their hosts.

An excellent example for pathogenicity associated lysogenic conversion is *Vibrio cholerae*, where the cholera toxin, CTX, is encoded on the integrative phage CTXΦ by the *ctxA* and *ctxB* genes [26]. Other foodborne pathogens known for a phage-dependent virulence phenotype are

Shiga-like toxin (STX) producing *E. coli* where in many cases, the implicated *stx1* and *stx2* genes are encoded on temperate phages integrated into the host genomes [27]. With some *E. coli* phages, products of the virulence-associated genes are not transcribed until the prophage is excised and enters the lytic cycle ,but nonetheless their expression can aggravate disease symptoms when the phages are liberated in part of the population [28]. There are many more examples of phages influencing the pathogenicity phenotype of orally transmitted, food-associated bacteria.

Altogether, the possibility for lysogenic conversion diminishes the usefulness of temperate phages for biocontrol of pathogens, even if no virulence phenotype can be observed in the laboratory. Temperate phages are also unsuitable because they generally have narrower host ranges than virulent ones. Many bacteria are natural lysogens, and repressor-mediated immunity systems will prevent closely related phages from completing a successful infection (i.e., phages featuring homologous repressor proteins and operator sequence binding sites). This homoimmunity is a barrier to biocontrol and could possibly hamper efficient large-scale production of these phages as many phage resistant lysogens would grow during fermentation.

Avoidance of Generalized Transduction

Besides lysogeny, a second phenomenon should be kept in mind when selecting candidate phages. Generalized transduction is a process where host DNA is packaged into phage heads, rather than phage DNA. The resulting particles can still recognize target bacteria, attach, and introduce this non-viral DNA which may then recombine with the genome and potentially introduce new genes into the recipient bacterium [29]. If the host employed for the production of phage stocks is non-pathogenic or even GRAS, then the phenomenon is unproblematic. However, if such phages are propagated on pathogenic bacteria, then transducing particles may contain information that possibly transforms recipient cells into pathogens. Distribution of a virulence-associated genome region via transduced DNA has been implied for several pathogens [30]. When a pathogenic

propagation host cannot be circumvented, only phages not able to transduce should be used. At least in *Listeria* and *Clostridium*, but probably as a more general rule of thumb, phages having defined, fixed genome ends are unable to transduce, whereas phages with terminally redundant and circularly permutated genomes are capable of transduction [31, 32, 33, 34].

Desirable Properties of Food-Applied Phages

Considering the above, phages suitable for biocontrol of pathogens in food should have the following properties (see also Article 1 on phage isolation and purification):

- Broad host range (infecting members of the target species and/or genus)
- Strictly lytic (virulent)
- Propagated on non-pathogenic host
- Complete genome sequences known
- Lack of transduction of non-viral (i.e., bacterial) DNA
- Absence of any genes encoding pathogenicity associated or potentially allergenic proteins
- Oral feeding studies show no adverse effects
- GRAS approval for use in foods
- Sufficiently stable over storage and application
- Amendable to scale up for commercial production

The basic biological characteristics can be inferred from relatively straightforward experiments and bioinformatic analysis of the phage genome sequence. While our knowledge of gene-functions is as yet limited, this approach by far exceeds the usual small scale safety assessment that is performed when novel fermentation organisms are developed and used for production of food or industrial/biotechnological fermentation processes.

BACTERIAL TARGETS FOR PHAGES IN FOODS

A considerable number of bacterial diseases are primarily foodborne. While in many cases phage-based biocontrol of these pathogens in foods may be possible, only those organisms which have thus far been investigated will be discussed here. Many of the insights and conclusions derived from these studies will apply to other phage biocontrol measures as well.

Animal Reservoirs

Several foodborne pathogens have their reservoir in animals consumed by humans (see Article 5 on phage therapy of animals). In-vivo biocontrol of these bacteria in the live animals might be considered as phage therapy, but there is a subtle and important difference. Therapy applies to infections and, in most cases, the animals are not infected but rather colonized commensally by the human pathogens. The distinction is important because in an infection, the animal immune system would be fighting to clear the causative agent along with any therapeutic measures taken. Alternatively, such pathogens could be controlled in post harvest applications. The pros and cons of these divergent approaches will be discussed later.

Target Bacteria for Pre-Harvest Biocontrol

For both pre- and post-harvest application of phages to control unwanted bacteria we prefer, for reasons just espoused, the term "biocontrol" to that of "phage therapy". Table 1 thus presents a synopsis of studies on post-harvest phage *biocontrol* of undesirable bacteria. While some of the underlying issues vary because of characteristics specific to the target organism, other considerations relating to phage treatment of foodstuffs in general are shared. In the remainder of this section we focus specifically on introducing relevant target bacteria.

Campylobacter. *Campylobacter* is a Gram-negative bacterium that is well adapted to colonizing the avian gut. The birds have no symptoms of illness and that means the target in an *in vivo* (i.e., in bird) approach of phage biocontrol is combating a bacterium in its natural ecological niche. Both *C. jejuni* and *C. coli* are, however, significant causes for food-borne enteritidis in

humans, with an infective dose requiring no more than 400-500 cells. Although the exact way of how *Campylobacter* causes disease inside the human host is still mostly unclear, some strains appear to produce an exotoxin similar to cholera-toxin, which leads to watery diarrhea [35].

Salmonella. *Salmonella* is well known for causing diarrhea and remains one of the principal causes of food-borne illness world-wide. Like *Campylobacter*, *Salmonella* strains are often commensals in chicken and turkey. Therefore, eggs may carry the bacterium and meat is regularly contaminated during slaughter of colonized animals. As with *Campylobacter,* this opens up two different approaches for phage intervention, at pre-slaughter level and after slaughter. Moreover, birds are not the only reservoir for these bacteria, which can be found colonizing other animals used as food, such as pigs [36]. This means that foodstuffs other than chicken meat and eggs are also at danger of being contaminated with *Salmonella*. Treatment after slaughter and on different food products may also be considered.

E. coli. The different pathovars of *E. coli* have a number of reservoirs, among which are humans themselves, from which they can enter the food-production process. The enterohemorrhagic strains (EHEC) such as O157:H7 are associated mostly with ruminants, where they colonize the intestines. EHEC strains have been the focus of attention because of the severity of the disease they cause, which is characterized by heavy bloody diarrhea, possibly followed by the Hemolytic Uremic Syndrome (HUS). The fatality rate of EHEC infection compared to other *E. coli* infections is high, even among healthy individuals [37]. Looking at the zoonotic character of the causative organism, an approach to reduce or eliminate these bacteria on the farm prior to slaughter, as with *Campylobacter* and *Salmonella* could be an elegant solution (see Article 5 on phage therapy of animals). An alternative to the treatment *in vivo* is application of phages on food items to reduce the risk of contamination.

Listeria. *Listeria monocytogenes* is the causative agent of the foodborne listeriosis. Though well known, the actual disease incidence, while on the rise in many parts of Europe, remains relatively low. *Listeria* is an opportunistic pathogen, affecting mainly the very young and old, immuno-compromised patients as well as pregnant women. However, it is characterized by a high mortality rate, which together with the ubiquitous distribution in many environments, hardiness, and ability to multiply at refrigeration temperatures makes it an important food-associated pathogen. Several foods are especially at risk of contamination with *L. monocytogenes* such as fresh and ready-to-eat foods. Others have a lower risk of being contaminated but allow the organism, when present, to grow to very high numbers. As a consequence, testing for *Listeria* is mandatory in many foodstuffs. Unlike the previously described pathogens, no clear reservoir for the bacteria can be pointed out. Rather, contaminations may occur via ingredients, factory workers, contaminated faulty production equipment, or the factory environment.

L. monocytogenes is usually killed during pasteurization or other heat treatments. This means, however, that foodstuffs which are not heated before consumption pose the highest risk [38]. Contamination of these higher risk food stuffs can occur up to and including the last processing step, and phage-based intervention on the food item during these critical steps, likely to carry the risk of contamination, may help avoid these contaminations.

Additional Nuisance Bacteria

As noted, there exist a number of foodborne bacteria which could serve as targets for phage-mediated biocontrol. An additional possible example includes *Enterobacter sakazakii*. *E. sakazakii* has caused outbreaks of disease, mostly in newborns and infants. Disease results in high mortality and extended illness and infection is thought to occur following exposure to contaminated, reconstituted infant formula [39].

Biocontrol by phages in foods need not be limited to bacterial pathogens. Thus, unlike the other organisms investigated in biocontrol strategies, Greer and Dilts [1] studied phage prevention of the growth of spoilage organisms, and the ability of phages to extend shelf-life of the treated product.

Generalizations

All of the above organisms have in common that their initial numbers during contamination of any food are likely to be very low. Any realistic experimental setup would therefore mean employing low levels of bacteria. Most antimicrobials work by suppressing growth rather than killing the target bacterium and testing schemes for growth-suppression call for high levels of target bacteria at least in the guidelines for testing efficacy of anti-listerial measures as proposed by the US meat industry [40]. However, the same article clearly states that when investigating an antimicrobial that has the ability to kill the target organism as a post-lethality treatment, that is, following a bacterium-killing processing step such as cooking, then lower or even very low artificial contaminations are preferable. Another important factor in the experimental setup is the temperature which is chosen. Abusive (high) temperatures providing optimum growth conditions for the undesired contaminants may occur either during storage at home or even at the retailers. Therefore, efficacy testing at higher than normal storage temperature has its merits, but perhaps in parallel with testing under recommended storage conditions.

EXPERIMENTAL PROOF OF CONCEPT

Clearly there are many more possible targets for phages, such as all those bacteria which are inherent to more specialized foods or which contaminate fermentation processes. These may not necessarily all be pathogens, but instead bacteria that can cause spoilage or other undesirable effects during fermentation or production of food and feed. Given that further exploration of biocontrol of foodborne bacteria using phages is unlikely to be emphasized without proof of concept, in this

section we provide an overview of studies which have provided evidence that phages can indeed significantly reduce levels of bacterial contamination in foods.

Control of *Campylobacter*, *E. coli*, and *Salmonella*, mostly on Animal Products

In a study addressing the effect of virulent phages against both *Campylobacter* and *Salmonella* on artificially contaminated chicken skin, the authors achieved a 95% reduction in *C. jejuni* counts and complete eradication of *Salmonella* [41]. However, since *Campylobacter* can not grow and multiply under the environmental conditions found on produced meat, thereby presumably interfering with biocontrol efficacy at this point, more research has been devoted to pre-harvest applications for *Campylobacter* control.

Isolates of the broad host range *Salmonella* phage Felix-O1 have repeatedly been tested for biocontrol of *Salmonella* in foods such as Frankfurters [42]. Although the experiments were performed at high temperatures and with a high initial inoculum (conditions not likely to be encountered during production and storage of such food), an approximately 2 $\log_{10}$ reduction of bacteria was observed. In a different study, a phage termed SJ2 was incorporated into the starter culture along with *Salmonella* during cheddar cheese production, which resulted in the absence of viable *Salmonella* cells after several months of storage, whereas high numbers of target bacteria were readily isolated from non-treated control cheeses [43].

The US EPA has recently approved the use of an anti-*Salmonella* phage product to be sprayed or used as a wash on chicken prior to slaughter. The argument for such an approach is that contamination of the meat with intestinal contents can largely be avoided during slaughter, but dust caught in the animal plumage, which for some part will contain feces and the associated bacteria, is the responsible carrier for many carcass contaminations. Another commercial product containing phage to be applied as a spray or wash on cattle hides prior to slaughter targeted against *E. coli*

O157:H7 has also received EPA approval. Unfortunately, however, no further efficacy data are available.

Beef artificially contaminated with *E. coli* O157:H7 at levels of 10^3 CFU/g has been experimentally treated with a cocktail of three different phages and stored at 37°C. No viable cells were found remaining in seven out of nine samples and, in the remaining two samples, cell counts were found to be below 10 cfu/g [44]. Another recent study used a phage cocktail against *E. coli* O157:H7 in a variety of foods and on hard surfaces [45]. Different phage dosages were tested on ground beef, tomato, spinach, and broccoli, and results ranged from 94 to 100% elimination of the target bacteria at higher phage doses. The authors claim that the phages used are unable to transduce based on their virulent nature. Virulent phages are of course unable to elicit lysogenic conversion, but it should be considered that lack of integration per se does not exclude transduction, where host DNA is inadvertently packaged instead of progeny phage DNA and where the infectious particles thus formed can subsequently introduce host DNA from one cell into another [31].

Control on Plant Products

The potential of phages to reduce *Salmonella* on contaminated fruit has been investigated [46]. While a high level reduction was observed on honeydew melons, the phages had little effect on the bacteria on apple slices. This correlated with rapid reduction of infective phage particles that could be recovered from the surface of the apples, which the authors attributed to the low pH. It might also be due to other plant-derived components, which have been reported to more rapidly inactivate phage (Guenther et al., submitted). In principle, differentiation of these two explanations could involve determinations of phage-decay rates in the presence of apple extract versus pH-adjusted apple extract. In general, these observations are suggestive of the utility of testing phages for durability within the intended-use environment prior to investing a great deal in further phage characterization. Indeed, one could envisage phage enrichment schemes involving the amplification

of heterogeneous phage mixtures in the presence of both specific target bacteria and extracts of specific target food (see Article 1 for more on principles of phage isolation).

Control of Additional Bacterial Targets

Reports on other bacterial targets are less frequent. Examples include the attempt to prevent or slow down spoilage of beef by using phages targeting the primary spoilage microflora of fresh meats, mostly consisting of *Pseudomonas* spp. [47]. However, only small effects were observed and the authors concluded that the host range of the pool of phage employed was too narrow. A seven-phage cocktail was used in the study, but host range studies had previously indicated that a significant percentage of food isolates were not affected by any of the employed phages. In such a multi-phage application where single phages each have a very limited host-range, it must also be noted that the dosage of each individual phage must be above the critical threshold concentration where phage-host encounters become likely. Another report describes the successful control of *Enterobacter sakazakii* in reconstituted infant formula by two newly isolated phages [48]. In these experiments, a dose and temperature dependent response to phage infection was observed, again showing that higher doses were more effective.

Focusing on *Listeria*

Several studies have described the use of *Listeria* phages as a means to control *Listeria* in foods. Cocktails of different phages, alone and in combination with a bacteriocin were tested on honeydew melon and apple slices [49, 50]. As seen in similar experiments with *Salmonella* phages [46], a reduced activity correlating with phage instability was observed on apples. A reduction of 2 to 4.6 logs of the bacteria was observed using phages alone, which performed better than nisin alone, and a cumulative effect was observed when the bacteriocin was also added. One of the two cocktails described in the report has since received FDA approval for use as additive in the manufacture of some meat products.

Another report describes the use of P100, a single, broad host range, virulent *Listeria* phage [13]. The efficacy of the phage in combating artificial contamination in the manufacture of smeared cheeses was evaluated, showing that depending on dose and treatment regimen the contamination could be reduced below levels of detection for the entire ripening time [13]. The failure of cells to re-grow was observed only in the highest dose treatment, indicating that the killing effect only at the highest used doses was complete. The report also contains data pertaining to the safety of this bacteriophage, both high-dose oral rat feeding data - showing no measurable differences between rats having been fed high doses of phage and those in the control group - as well as *in silico* genome analysis showing an absence of virulence genes, toxin proteins, and matches between putative encoded proteins and known allergens from specialized databases. An even more comprehensive report with detailed efficacy data using P100 and A511 (another virulent *Listeria* phage) on a large variety of ready-to-eat foods has recently been conducted [51]. Following optimization of food-specific application protocols, reduction of more than 99% could be obtained in most cases.

On the contrary, one study found that addition of phages had no significant effect on the presence of *Listeria* on artificially contaminated beef [52]. Analyzing the data, the reason for the failure may have been the fact that insufficient phage concentrations were used. The authors dipped the contaminated beef in a solution containing 10^3 PFU/ml of phage LH7. After removal, one could expect the surface to retain approximately 5-10µl/cm^2 of the phage solution. This would result in a phage concentration of only 0.5-1 phage per square centimeter. Considering that contamination levels in the experiment were 1000 bacterial cells/cm^2, the chance of a single phage to infect a host cells was extremely low, thus the effect was found insignificant (see considerations discussed above) (see also Article 3 on phage therapy pharmacology).

PRE- HARVEST VS. POST-HARVEST INTERVENTION

Complete eradication of bacteria adapted to the animal gastrointestinal tract, featuring a complex and highly dynamic ecosystem, may be beyond what bacteriophages can offer. Nonetheless, risk analysis modeling indicated that a 2 $\log_{10}$ reduction in the feces of the slaughtered animal could reduce risks to consumers by 75%, and 1 log reduction levels could still reduce risks by 45% [53]. Treatment of the meat after slaughter is an alternative, but clearly different approach.

Intervention strategies at the farm level using *Campylobacter, Salmonella*, *and E.coli* bacteriophages have been considered for biocontrol purposes. Unlike in the eradication of pathogens on finished or semi-finished food products, the numbers of target bacteria in the animal intestines are usually very high. *Campylobacter* counts in broiler chicken feces often reach numbers of 10^7 CFU/g [54]. Once the first chicken is colonized, the rest of the flock will follow and colonization in the flock tends to be universal, because of the coprophagic nature of chickens [55].

On-farm, *in vivo* biocontrol of zoonotic pathogens may be attractive because of its elegance, especially since a significant degree of phage replication (progeny phage may contribute to the biocontrol efficacy) may allow lower dose applications. It is obvious from experimental data, however, that complete eradication of the target organisms will be extremely difficult or impossible to achieve. Nonetheless, reduction prior to slaughter will contribute to consumer safety. Most experiments have been undertaken with specific target bacteria which were susceptible to the phage or phages used. Colonization of animals in herds or flocks spreads exponentially, and the sources of primary contamination are varied and previous occupants of pens and holding facilities may contribute as a source. This means that phages used should not only have a sufficiently broad host range against a wide variety of strains in collections, but rotation of different phages or the use of cocktails may be necessary to avoid selection of specialized phage-resistant strains in the long run. In environmental application in or around food processing facilities, a similar problem might occur, but not in the phage treatment of food, where all phages (including infected and possibly resistant

bacteria) are removed from the contamination source, thereby preventing establishment of a phage-resistant house flora.

It is obvious that both pre- and post-harvest applications have their merits and drawbacks, and ultimately both strategies may be adapted, at least for some target organisms. Treatment of finished food products, in particular, will always involve large numbers of phages. These phages will furthermore have to be purified to remove cell wall-associated endotoxin or other undesired cellular debris. Whether phages to be used in livestock need to be purified to the same extent or if feed law requires even more extensive purification has not been determined. Indeed, these questions have more of an economic aspect than a scientific one and economics will play a large role in deciding which approaches will be taken. However, these considerations in no way detract from the validity of scientific experiments which indicate the technical possibilities of phage biocontrol.

BACTERIAL RESISTANCE TO PHAGES

The important subject of resistance is raised whenever phage therapy or treatment of foodstuffs is discussed. In order to not become "extinct", bacteria have evolved to be able to escape and balance phage predation at some level. Different resistance mechanisms exist through which bacteria can protect themselves against bacteriophage attack.

Restriction-Modification and Abortive Infection

Restriction enzyme systems, for example, recognize and cut foreign DNA which does not contain correctly modified bases. Phages, on the other hand, can protect themselves against these endonucleases by modifying their own DNA, or by altering or avoiding the specific sequence motifs recognized by the restriction enzymes. Also, target cells will have a second enzyme that modifies the cellular DNA in order to protect itself from the nuclease activity. During phage infection there consequently is a possibility that the phage DNA will be modified and protected

prior to restriction. It then will no longer be recognized as foreign, and the infection can run its course leading to release of progeny phages that all have modified DNA [56].

Some bacteria have abortive infection mechanisms which result in infection death without producing progeny phage. Such systems are disastrous for phages from an ecological point of view. However, they do not affect the intended outcome of a food-treatment scenarios since infected bacteria still die, at least so long as successful biocontrol does not require phage production of new phages. Although these mechanisms are widespread among bacteria, screening for phages using a large number of bacterial isolates from the environment and food sources will result in the identification and selection of phages able to overcome these barriers.

Bacterial Mutation to Resistance

Spontaneous mutations by bacteria can sometimes also result in phage-insensitivity. A famous experiment demonstrated that such mutations occur at the same rate in a bacterial population whether phages are present or not [57]. However, mutations are mostly detrimental, and any effects related to phage resistance may disappear when the phage pressure is relieved, either by reversion to the wild-type (i.e., back mutation) or because remaining wild-type cells replace the mutant cells because of their greater fitness. These phenomena have also been observed in phage treatment of foods. Apparently unstable mutation featuring aberrant cell shapes, followed by reversion after a few generations, has been reported [44]. In this study, a mutation rate of 10^{-6} was calculated to occur during phage treatment. In this particular application where phage is applied on the food to eradicate pathogens, such a mutation rate will not have any measurable effect on overall efficacy, simply because the number of bacterial cells per weight unit is too low to give rise to significant numbers of phage resistant mutant bacteria.

Considering a realistic situation with low-level contaminations, any intervention strategy which reduces the bacterial burden by more than two orders of magnitude will significantly

enhance food safety. However, a decrease of 2 $\log_{10}$ does also mean that statistically one percent of the cells will be missed, which will survive and may eventually grow to higher numbers (though if starting with low numbers of cells along with only modest additional bacterial growth, this regrowth should still give rise to only 1% as many bacteria as would have been present given growth without the initial reduction, a situation that is quite different from bacterial growth to the stationary-phase densities which can be observed in the laboratory). It thus is evident that a theoretically possible mutant survival frequency of 10^{-6} will be irrelevant in those cases where food is treated with phage and subsequently leaves the production facility, that is if a 10^{-2} survival rate of non-mutant bacteria is expected.

If phage-resistant bacterial mutants do not escape into an environmental niche where phage selective pressure is high, then such mutants cannot be expected to present any danger to long-term efficacy of phage-based intervention. However, in other types of application, mutation may be a significant feature. For instance, if phages were to be used as an environmental cleaning agent in areas suspected of holding niches for the target bacteria, mutation could allow the bacteria to adapt and still exist in those niches. Subsequent treatment of the foodstuff contaminated via this niche using the same phage would then fail. A similar situation arises when trying to eradicate bacteria among farm animals.

NUTS AND BOLTS OF PHAGE APPLICATION TO FOODS

Laboratory experiments clearly show that phages can help control foodborne pathogens. While phage biologists main target has to be selecting appropriate phages with sufficient host range and with safety of the preparation in mind, some of the thoughts should consider actual applications. This begins with the laboratory challenge studies undertaken: is the tested food stuff at risk from contamination? Is the contamination scenario realistic? Is the method of applying the phages possible in reality? While the method of application (dipping, spreading or spraying) may

have little effect on efficacy, the amount of fluid in which the phages are applied is important (S. Hagens, unpublished results). The moment of phage application in relation to contamination may also play a role. If the moment of phage addition is seconds after contamination (i.e. as in a possible treatment after slicing of cooked RTE meat products), then experimental conditions should mimic this situation. As a general rule, the most indicative studies will be those where realistic contamination and treatment scenarios are used.

Actual introduction to processing facilities may pose additional challenges. An application method that can be incorporated into the normal processes is ideal. If that is not possible, then the application should be the most convenient, most economical, and least invasive to the process itself. Identification of the contamination source(s) may be very helpful as well. If a raw material is regularly contaminated and none of the processing steps is lethal to the target bacterium in question, then the most logical step would be treatment of the raw material as it enters the production facility. This has two advantages, firstly the surface-to-weight ratio of the incoming product is likely to be smaller than that of the final product and thus the overall phage dose can be lower and secondly eradication of the target organism at this point avoids dissemination of the target bacterium throughout the facility, which in turn can reduce contamination of machinery and food contact surfaces and thus recontamination of un-contaminated foods. While phage resistance through mutation does not have to be a problem if food alone is treated, tsome care has to be taken that phage pressure is kept low on the niches and reservoirs where the target organism may reside. Harsh chemical cleaners may not be appropriate for use on foods, but they can be very effective in environmental application, are cheap, and can penetrate anywhere a phage can.

Given that phages can be efficacious in their biocontrol of foodborne bacteria, but that mutation to phage resistant can occur plus phage penetration to bacteria may be inefficient as well as other concerns, there exist various strategies by which phage biocontrol of foodborne bacteria

may be optimized. The actual application strategies can include dipping, spraying, or adding phages as a liquid to a larger volume of food material. Application can also occur at different points or even multiple points in a food processing facility. One can also consider the timing of phage application relative to the point of slaughter and/or packaging.

If foods are dipped (or otherwise washed) then phage application at this point can be convenient. However, if volumes of wash are high, then this strategy could be wasteful of phage materials. In addition, if the washing fluids themselves are places where bacteria can propagate, then there exists a potential for bacterial evolution of phage resistance, which could necessitate modification of the formulation of phage preparations over time. An additional consideration is that phage survival within heterogeneous environments, such as dips, could be variable, uncontrollable, or not easily determined, and decay could be accelerated as a consequence of inclusion of other materials within the wash fluid, i.e., such as bleach. For all of these reasons, dipping or washing may not serve as the ideal first choice as the means of phage application.

Addition of phages directly to a batch of food may also be problematic for reasons of dilution and evolution of bacterial resistance. The latter may be addressed by regular disinfection of equipment, especially if that disinfection is highly efficacious. Concerns about dilution (and thereby a requirement for addition of very large numbers of phages) may be overcome by applying phages prior to the mixing or disruption of food materials, such as by spraying carcasses prior to processing. Indeed, generally it is advisable to identify those points in the food processing path where bacterial contamination is freshest; where application is most convenient, most economical, and least invasive into the process itself; and where feedbacks back to the contaminating source are unlikely, thereby reducing the potential for bacterial evolution to phage resistance.

CONCLUSIONS AND FUTURE PERSPECTIVES

The increasing number of phage application studies, both by research groups and commercial enterprises, have altogether provided convincing evidence that phages can play an important role in biocontrol of pathogens in food and feed. The direct application of phages to target specific pathogens in or on foodstuffs is a very straightforward approach. Spontaneously occurring phage-resistant mutants are not likely to significantly influence treatment efficacy. Complex phage-resistance mechanisms common in bacteria can be pre-empted when screening for susceptibility of large strain collections, and supplemented by continued screening. The use of strictly virulent phages, unable to perform transduction, and devoid of any virulence or pathogenicity associated genes will ensure that problems concerning bacteriophage safety will not be an issue. As a more general rule, a sufficient concentration of infectious phage particles will have to be applied on foods. Last but not least, an important practical challenge is the incorporation of phage application step at the best possible time point into existing processing and production schemes.

While phages can obviously help to reduce pathogen loads in foodstuffs, not only scientific considerations will determine the success of different possible applications. Cost-effective production vs. efficacy in real-life application will have to be assured. For certain applications, long-term plans for avoiding resistance-associated problems will have to be made in advance. Considering the likely situation that phage products will require inclusion of new phages featuring different host ranges at more or less frequent intervals in order to react to changes in the bacterial flora, transparent and quick approval procedures by regulatory authorities would be beneficial. As stated above, the optimal time point of phage application is likely at (or very close to) the moment bacterial contaminants enter the food matrix. Thus, in the manufacturing chain, it is the food processors who will be the ones using the phages. It should also be noted that the term bacteriophages, when mentioned in the context of food, might be associated with the danger that

phages can pose for starter cultures by food manufacturers. Ironically, it is this ability of phages to ruin batches of yoghurt and cheese which explains that the concept of using other phages, targeted at dangerous pathogens, might work equally well and is therefore an acceptable option.

Lastly, market acceptance by the food industry and the consumer is the most critical hurdle which will need to be overcome in order to use phages on a broad basis for biocontrol of bacterial pathogens within food. Crucial to that acceptance are that phages are both safe and efficacious while providing little (if any) negative impact on food quality. As such they could and should be considered ideal antibacterial agents for use in foods.

ACKNOWLEDGMENTS

ABBREVIATIONS

CFU = Colony-forming unit

EHEC = Enterohemorrhagic *E. coli*

EPA = Environmental Protection Agency

GRAS = Generally recognized as safe

HUS = Hemolytic uremic syndrome

PFU = Plaque-forming unit

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69

743**Table 1**: Synopsis of phage used for biocontrol of bacteria in various foods

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Target species	**Treated food**	**Reference**
Campylobacter jejuni	Chicken skin	[41]
Salmonella spp.	Chicken skin	[41]
	Fruit	[46]
	Frankfurters	[42]
	Cheddar cheese	[43]
E. coli	Beef	[44]
	Vegetables/ground beef	[45]
Listeria monocytogenes	Beef	[52]
	Fruit	[49, 50]
	Cheese	[13]
	RTE foods	[51]
Enterobacter sakazakii	Infant formula	[48]
Pseudomonas spp.	Beef	[47]

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