The use and mode of action of bacteriophages in food production

Scientific Opinion of the Panel on Biological Hazards

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Public consultation

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SUMMARY

Following a request from the Health and Consumer Protection, Directorate General, European Commission, the Panel on Biological Hazards was asked to deliver a scientific opinion on "*The use and mode of action of bacteriophages in food production*". In accordance with the terms of reference, this report does not consider the safety assessment of the use of bacteriophages on foods.

Modern microbial food safety assurance is based on a farm-to-fork principle that involves a wide range of coordinated control measures applied at all relevant steps in the food chain. A large number of different food decontamination treatments have been described in the literature. Some of them involve the application of live microorganisms to inhibit or eradicate pathogenic and/or spoilage bacteria in/on foods. To this regard, the use of bacteriophages has recently attracted a growing interest. This Opinion deals only with bacteriophage-based treatments of food products, and its main focus is on their mode of action when used for the most important types of foods of animal origin (i.e. meat and meat products, milk and dairy products).

The Panel on Biological Hazards made following main conclusions: Bacteriophages may be temperate or virulent; they can induce lysis of the bacterial host-cell by 2 mechanisms: "*lysis from within*" and/or "*lysis from without*". The bacteriophages have narrow host-ranges and replicate best on growing bacterial cells. Naturally occurring bacteriophages can be isolated in considerable numbers from foods of animal origin. Virulent bacteriophages are the ones of choice for phage-based food decontamination, and some of these, under specific conditions, have been demonstrated to be very effective in the targeted elimination of specific pathogens from foods. In general terms, the higher the ratio of bacteriophages to host cells, the greater the

reduction in the target bacterial population. The persistence in/on food varies with each bacteriophage, and with the conditions of application, including dose, and physical and chemical factors associated with the food matrix. Based on data currently available in peer-reviewed literature, it cannot be concluded whether bacteriophages are able or unable to protect against recontamination of food with bacterial pathogens. This is likely to vary with each bacteriophage, each food matrix, and with conditions of application including environmental factors. Research for specific bacteriophage-pathogen-food combinations should be encouraged to ascertain these issues.

The Panel on Biological Hazards recommends that, if bacteriophage treatments are to be used for removal of surface contamination of foods of animal origin, then a Guidance Document on the submission of data for their evaluation is to be provided.

Key words: Bacteriophages, food of animal origin, food-borne zoonoses.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission has become aware of a developing issue on the use of bacteriophages to counter *Listeria* contamination in food. Bacteriophages are viruses which infect bacteria and kill them, they are abundantly present in nature and, as a consequence, in food. Different bacteriophages work against specific bacteria. When a bacteriophage encounters its specific bacterium, it attaches itself to the cell wall of the bacterium using its tail fibres. Once a bacteriophage attaches to the bacterium, it penetrates the cell wall and its DNA is drawn into the bacterium, effectively taking over the cell and destroying the bacterium's ability to function or replicate. When the replication of bacteriophage weakens the cell wall structure and exceeds the available space within the bacterium cell, the cell wall bursts (lyses) and new bacteriophages are released into the environment to further infect their specific bacteria if they are present.

The products which are reportedly under development are utilising bacteriophages which reproduce via the lytic cycle whereby the virus invades the bacterium and toxins are released thus killing the bacterium. Some other bacteriophages operate by lysogeny (lysogenic cycle) where the nucleic acid of the bacteriophage fuses with the DNA of the host bacterium. Such a transfer of DNA could lead to a modification of the host bacteria such as an increase in the pathogenicity and/or virulence of the host bacteria.

Regulatory framework

Council Directive 89/107/EEC provides a definition of food additive as 'any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has a nutritive value the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods'.

Processing aids are specifically excluded from Council Directive 89/107/EEC. For that purpose, the definition of processing aid is 'any substance not consumed as a food ingredient by itself, intentionally used in the processing of raw materials foods or their ingredients, to fulfil a certain technological purpose during treatment or processing and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final, product provided that these residues do not present any health risk and do not have any technological effect in the finished product'.

Whilst processing aids are generally excluded from the food additive legislation described above, they are with some exceptions subject to national legislation. The exceptions being the use extraction solvents which is harmonised by Council Directive 88/344/EEC and other areas of food legislation where the use of processing aids are regulated, such as legislation on wine or the hygiene legislation (Regulation (EC) No 853/2004). The latter states that 'Food business operators shall not use any substance other than potable water… to remove surface contamination from products of animal origin, unless use of the substance has been approved in accordance with the procedure referred to in… [The Comitology procedure].'

The possibility to use substances other than potable water for surface decontamination is a new development brought about by the recently adopted hygiene package. Previously only potable water was permitted.

In response to a request from the Member State the Commission has further examined the matter and considers that bacteriophages when used on food of animal origin (including cheese) could be considered either as food additives or as substances used for reducing surface contamination (and thereby requiring approval under Regulation 853/2004).

The crux of the issue is the manner in which the bacteriophages exert their effect i.e. whether they preserve against recontamination or whether the effect is short lived and no continual functioning of the bacteriophages can be expected. In order to clarify their status the Commission is seeking technical assistance from EFSA on the way in which the bacteriophages work. Following this assistance from EFSA the Commission will consider which of the two regulatory frameworks apply so that the manufacturer can make the necessary request for authorisation.

The Commission is not at this stage seeking advice with regard to the safety in use of such bacteriophage solutions because either as food additives or as antimicrobial treatments an EFSA evaluation on the safety will be necessary before they can be considered for authorisation.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 31 of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide technical assistance in relation to the use and mode of action of bacteriophages on food of animal origin.

The European Food Safety Authority is asked to:

(i) From the literature provided and/or a literature search, if deemed necessary, to describe the mode of action expected from the use of bacteriophage solutions on food of animal origin (including but not exclusively use on animal carcases, meat products and dairy products).

(ii) Advise whether the use of bacteriophages may lead to a continual functioning in the food, thereby protecting against recontamination or whether the effect can be expected to be short lived with no continuing action effect in the final food.

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ASSESSMENT

1. Introduction

Modern microbial food safety assurance is based on a farm-to-fork principle that involves a wide range of coordinated control measures applied at all relevant steps in the food chain. For didactic reasons, such control measures can be grouped into two global approaches, "proactive" or "reactive". The former approach is of preventative nature and comprises hygiene-based measures aimed at the total avoidance or minimisation of the microbial contamination of food. The latter approach employs various treatments aimed at the elimination of microorganisms that already contaminated the food. The "proactive" approach is universally and mandatorily used, but can be complemented with the "reactive" approach in some situations within the regulatory frame.

Based on the knowledge accumulated to date, it is assumed that currently available decontamination treatments, generally, can only reduce the microbial contamination level in/on, but cannot completely eliminate microbial pathogens from, foods. It is recognised that the ultimate effectiveness of antimicrobial treatments, when assessed through the levels of surviving microflora remaining on treated foods, depends on the initial microbial load to a great extent. Better ultimate results of the antimicrobial treatment are achieved when applied to cleaner foods. Furthermore, many factors affect the efficacy of antimicrobials, including the concentration of the antimicrobial substance, duration of exposure, temperature, pH and hardness of the water, firmness of bacterial attachment to the carcasses, biofilm formation and the presence of fat or organic material in water (http://ec.europa.eu/food/fs/sc/scv/out63_en.pdf). A large number of different antimicrobial treatments ("decontamination") of foods, developed and applied mostly under experimental conditions only and, comparably, rarely to a commercial application level, have been described in the literature (Acuff, G.R., 2005; Bacon, R.T. *et al.*, 2000; Feirtag, J.M. and Pullen, M.M., 2003; Guan, D. and Hoover, D.G., 2005; Huffman, R.D., 2002; Smulders, F.J. and Greer, G.G., 1998; Sofos, J.N. and Smith, G.C., 1998).

Physical treatments include water treatments (cold or hot water washing/rinsing), electrolysed water treatments, steam treatments (pasteurisation; sub-atmospheric; steam vacuum), high pressure treatments, irradiation treatments (electron beam; gamma rays), electromagnetic treatments (pulsed visible light; ultraviolet; microwave; infrared; dielectric or radiofrequency), electric treatments (pulsed electric field) and gas plasma treatments.

Chemical treatments are based on the use of chlorine, organic acids (e.g. lactic, acetic, or citric acid), peroxyacetic acids, acidified sodium chlorite, acidic calcium sulphate, activated lactoferrin, trisodium phosphate, cetylpyridinium chloride, ozone and carbon dioxide.

In addition to their antimicrobial effectiveness, relevant aspects of physical and chemical treatments also include issues concerning their undesirable effects. These include potential changes of sensory qualities of foods (e.g. after heat or irradiation treatments) and a possibility of residues remaining in the food (e.g. after chemical treatments). To minimize these risks, the intensity of the treatments has to be limited, which limits their effectiveness. To overcome this problem, different treatments can be used in a sequence, which may yield synergistic or additive decontaminating effects termed as a "multiple hurdles" decontamination approach (Bacon, R.T. *et al.*, 2000; Sofos, J.N. and Smith, G.C., 1998).

On the other hand, some treatments are based on "natural" antimicrobials, such as plant extracts or microbial products (e.g. bacteriocins) that allow the manipulation of the microbial ecology of

foods. Furthermore, some antimicrobial treatment technologies involve the application of live microorganisms e.g. "protective" bacterial cultures or bacteriophages to inhibit or eradicate pathogenic and/or spoilage bacteria in/on foods. To this regard, the use of bacteriophages has recently attracted a growing interest (Hudson, J.A. *et al.*, 2005) from researchers and industry as well.

This Opinion deals only with bacteriophages-based treatments of food products, and its main focus is on their mode of action when used for the most important types of foods of animal origin (i.e. meat and meat products, milk and dairy products). The safety assessment of bacteriophages will not be considered here.

2. Biology of bacteriophages

2.1. Description, types of bacteriophages, and life cycle

In the last years several multi-authored books on bacteriophages have been published, see for example (Calendar, R., 2006; McGrath, S. and van Sinderen, D., 2007; Waldor, M.K. *et al.*, 2005); the reader is referred to them for comprehensive information on bacteriophage biology, applications and problems associated to them. The information summarized bellow has been taken from the previously mentioned books.

Bacteriophages (comes from the Greek for bacteria eaters) are the viruses of bacteria. Like all other viruses they are intracellular obligate parasites. Their extracellular form (the virion) behaves as an inert particle composed of a nucleic acid (usually double stranded DNA) surrounded by a protein coat (the capsid). Most dsDNA bacteriophages present an injection apparatus (the tail) to allow passage of the nucleic acid through the bacterial cell wall and plasma membrane. Unlike animal viruses, enveloped bacteriophages are rare.

Bacteriophages are abundant in saltwater, freshwater, soil, plants and animals and they have been shown to be unintentional contaminants of milk and even some commercially-available vaccines and sera. They are also found in the human digestive and genitourinary tracts and even in the skin. Furthermore, they were used as therapeutic agents almost since their discovery up to the advent of antibiotics in the western countries and still are in Poland and many of the nations that have arisen from dismemberment of the Soviet Union, for the treatment of internal and superficial infections without any consistent record of adverse effects imputable to their use.

In general, virions are able to remain in the environment for long periods of time due to their lack of metabolism. Consequently they are frequently an important cause of failures in the food and drug fermentation industries due to the contamination of the raw materials and the factory setting, which allows the infection of the starter cells. However, most bacteriophages tend to be very susceptible to the exhaustion of divalent cations which are essential for the stability of capsids, and to the attack of proteases, frequently produced by environmental microorganisms. Ultimately, inactivated bacteriophage particles will be broken down into common biological particles (amino acids and nucleosides) that are naturally absorbed back in the environment.

The encounter of the bacteriophage with its host is a random event and is followed by the specific recognition between surface cell-receptors and bacteriophage anti-receptors located at the tip of the tail. This implies that bacteriophages have narrow host ranges, rarely expanding further than the species or genus level for Gram positive and Gram negative bacteria respectively. Consequently, they are unable to infect eukaryotic cells.

Bacteriophages may follow a **lytic cycle**; and those that can only follow the lytic cycle are known as **virulent** bacteriophages. Lysis of the host bacterial cell can occur as a result of two possible mechanisms indicated below:

(i) "Lysis from within". In this case, lysis of the host cell occurs as a result of phage replication. The genetic material is the only component of the virion that enters into the host cell, which may occur through injection (bacteriophages with contractile tails) or following the enzymatic breakage of the cell wall. In both cases, the pore generated in the membrane will affect its electric potential, although this harm is easily repaired. Once inside the cell, the genetic material of the bacteriophage is replicated hundreds of times, the coat proteins are synthesized and new particles are assembled that will constitute the viral progeny (usually between several tens and a few hundreds per infected cell). Release of the progeny is the consequence of the collaborative action of the holin, a hydrophobic polypeptide that forms pores in the cell membrane, through which the lysin (a muramidase) reaches the cell wall, thus provoking the lysis of the host-cell.

(ii) "*Lysis from without*". In this case, lysis of the host cell occurs in the absence of phage replication. This happens when a sufficiently high number of phages particles adhere to the cell, and lyse it through alteration of the membrane electric potential, and/or the activity of cell-wall degrading enzymes.

Some dsDNA bacteriophages, however, have the capacity to synthesize a repressor protein that silences most bacteriophage genes and results in abortion of the lytic cycle. Under these circumstances the bacteriophage DNA (the prophage) synchronizes its replication to that of the host to be inherited by its offspring. In most cases this is brought about through integration of the bacteriophage DNA into the host genome via site-specific recombination. This alternative method of bacteriophage propagation is called the **lysogenic cycle** and the bacteriophages able to pursue it are known as **temperate.**

The expression of the repressor gene throughout the lysogenic cycle leads to superinfection immunity (i.e. the inability of newcomer related bacteriophage to develop in the host cell). Frequently, temperate bacteriophages harbour other genes that are also expressed during lysogeny. These may confer new properties on their hosts (lysogenic conversion) this being especially relevant for those that encode virulence factors, such as the diphtheria toxin encoded by the β bacteriophage of *Corynebacterium diphteriae, bacteriophages of verocytotoxin–producing E. coli* and many others.

Also, bacterial DNA can be transferred from cell to cell, inside viral capsids (transduction). The extremes of the concatemers formed during the rolling-circle replication followed by most dsDNA bacteriophages, are specifically identified to initiate packaging. In cohesive-end bearing bacteriophages the terminase recognizes the same sequence at the end of the incoming genome and introduces a staggered cut, so that the resulting outer extreme can be identified, thus keeping a tight control of the DNA that enters the capsid. Other bacteriophages package as much DNA as can be admitted into the capsid, which is usually more than the unit genome. This results in circularly permuted molecules and in a more relaxed control of the DNA to be packaged, reason why they tend to be better transductants than cohesive end bacteriophages.

Bacterial host cells are not defenceless against phage attack. The heavy burden put on the susceptible bacteria may select cell variants that are refractory to bacteriophage infection (bacteriophage insensitive mutants, BIMs). This is usually accomplished by loss, modification, or masking of the bacteriophage receptors located at the cell wall. However, genes specifically devoted to neutralize bacteriophage infection have been described in bacteria that are frequently challenged by bacteriophages, such as fermentation starters. These genes comprise the ones involved in restriction-modification (R-M systems) and in abortive infection (abi systems) which inhibit specific steps of the cell metabolism upon infection, resulting in the inability of the bacteriophage to generate a progeny and, usually, in death of the infected cell, thus blocking spread of the infection. Resistance mechanisms identified so far are mainly plasmid encoded.

For more detailed information on resistance mechanisms, readers are referred to publications by (Emond, E. *et al.*, 1997 ; Garcia, L.R. and Molineux, I.J., 1995 ; Hudson, J.A. *et al.*, 2005).

Bacteriophage treatments could provide the conditions for selection of bacteriophage-resistant clones of the target bacteria, that could occupy niches in processing equipment/environment, and continue to be a source of cross-contamination during food processing. A number of strategies that may be used to overcome or limit resistance development have been indicated in the literature, including the prevention of the recycling of the bacteriophages in the reservoir of the pathogen by alternating use of different bacteriophages (either in a cocktail of several bacteriophages, or in consecutive treatments).

While bacteria have developed specialized bacteriophage-defence mechanisms, phages also continuously adapt to these altered host systems. Spontaneous mutations conferring bacteriophage resistance may actually have deleterious effects on these bacteria, and not necessarily confer an evolutionary advantage in the absence of phages. In one study, it was found that bacteriophage-insensitive mutants revert to phage sensitivity in the absence of selective pressure (O'Flynn, G. *et al.*, 2004).

2.2. General remarks on the mechanism (mode) of action of bacteriophages in foods

Bacteriophages generally exhibit a narrow host range, which is usually restricted to one genus of bacteria (Ammann, A. *et al.*, 2008; O'Flaherty, S. *et al.*, 2005a), but more frequently restricted to either a limited number of species within a genus or to a limited number of bacterial strains within a species (Jarvis, A.W. *et al.*, 1991). The best virulent bacteriophages for biocontrol applications are those with the broadest possible host range. These are termed polyvalent bacteriophages (O'Flaherty, S. *et al.*, 2005a) or WHR (wide host range) bacteriophages (Bielke, L.R. *et al.*, 2007) as they are usually active against many species within a bacterial genus. Thus they can be applied to specifically target and eliminate that genus in foods or other environments.

As bacteriophages rely on host bacteria to replicate, it is essential that they come in contact with their bacterial host, and that they survive well in the environment until they do so. This stage in the infection cycle can be considered an extracellular “search stage”, which is constrained by bacteriophage and host-cell migration rates and is also dependent on host-cell and bacteriophage numbers. This stage is followed by bacteriophage adsorption, which combines reversible bacteriophage binding, irreversible bacteriophage binding and bacteriophage genome transfer into the host, which typically occurs rapidly following collision between a bacteriophage particle and a bacteriophage-susceptible bacterium. Bacteriophage replication within the bacterial cell and release of progeny bacteriophage, are dependent on the metabolic status of the bacterial cell.

A variety of extrinsic factors can influence the ability of bacteriophages to adsorb onto and infect their bacterial host. Among the most important are bacterial cell and bacteriophage numbers. Much information on the use of bacteriophages to eliminate bacteria comes from experiments where researchers have typically mixed a high titre of a single bacteriophage strain with a single bacterial strain at about 10^7 or 10^8 cells per ml. Nevertheless, laboratory experiments with coliphage T4, *Bacillus* and *Staphylococcus* bacteriophages have shown bacteriophage propagation on bacterial cells occurred with as low as 10^4 host cells per ml (Wiggins, B.A. and Alexander, M., 1985). Furthermore, studies with *Pseudomonas* bacteriophages (Greer, G.G., 2006; Kokjohn, T.A. *et al.*, 1991) indicated bacteriophage replication with as little as 10^2 target cells per ml. (O'Flynn, G. *et al.*, 2004) used a cocktail of three different bacteriophages to treat beef contaminated with 10^3 CFU per g of *E. coli* O157:H7; in the majority of samples, no viable *E. coli* cells could be retrieved after storage. In

the case of *Salmonella,* (Bigwood, T. *et al.*, 2008) also showed effective elimination of *Salmonella* cells where 10^4 cells per g were employed. The above studies indicate that the application of bacteriophages in food to eliminate undesirable bacteria, which may be present at low numbers, could well be successful. However, this is likely to be dependent on the amount of fluid present in the food, which will contribute to bacteriophage mobility.

Bacteriophage infection and replication is influenced by the physiological and nutritional status of the host bacterium. Many bacteria undergo a variety of metabolic and structural changes in stationary-phase conditions that facilitate long-term survival in hostile conditions (McCann, M.P. *et al.*, 1991) and it is widely accepted that most bacteriophages cannot productively infect stationary-phase bacteria (Brussow, H. and Kutter, E., 2004). Nevertheless, the existence of a high abundance of bacteriophage in natural ecosystems (Bergh, O. *et al.*, 1989; Torrella, F. and Morita, R.Y., 1979) would appear to disagree with this, as many bacteria are understood to be in a physiological state similar to the stationary phase of growth. Indeed, one study clearly showed bacteriophage replication, albeit at a reduced rate, on stationary-phase *E. coli* and *Pseudomonas aeruginosa* cells (Schrader, H.S. *et al.*, 1997).

It is important to understand that the precise properties exhibited by one bacteriophage cannot be assumed to be identical for other bacteriophages. Each bacteriophage will have its own characteristic properties including host range, burst size, and ability to maintain its physical integrity in different environments.

3. Bacteriophages in foods of animal origin

3.1. Ecology of bacteriophages in food (natural abundance)

Bacteriophages may be present on the surface of foods, including carcasses and meat, wherever the bacterial host is or has been present. Bacterial hosts include intestinal and skin bacteria, both pathogens and non-pathogens, colonising food animals. It is not surprising therefore that bacteriophages have been found frequently on the surface of red and white meat, fish and other foods. In addition, many fermented foods are likely to be contaminated with bacteriophages, either from the environment or from the host bacteria themselves if these are lysogenic.

There is not an absolute correlation between the presence of bacteriophages and the target host since the latter may be inactivated by processing.

Bacteriophages can be isolated from foods by their ability to lyse indicator bacteria. Where these are not available samples may be tested for their ability to lyse the predominant bacteria isolated from the samples, the so-called bacteriophage-host systems. This latter method is very convenient although it will not necessarily detect bacteriophages which have been released from lysogenised bacteria since the bacteria will normally be resistant to the bacteriophages which have been released. In this case co-culture with an indicator organism is required, again necessitating availability of an indicator.

Over a number of years bacteriophages have been studied in foods for a number of reasons, including (i) their influence on spoilage bacteria and as a means to prevent this, (ii) as indicators of contamination with intestinal/faecal bacteria, (iii) their detrimental effects on the production of certain foods by fermentation, or (iv) the recent resurgence in interest in bacteriophages for control of bacterial food-borne pathogens..

Early studies had the aim of using the presence of enteric bacteria or their bacteriophages (in addition to enteric viruses) as an indication of faecal contamination with the advantage that bacteriophage detection was a quicker process than bacterial culture. Poultry and pig meat has the capacity to be contaminated extensively given the conditions prior to and immediately after

slaughter and the fact that skin is retained on the carcass. Very little published information is available for pork meat. In contrast there is evidence that bacteriophages active on *E. coli* and *Campylobacter* can be isolated frequently from poultry. Enteric bacteriophages have been isolated from poultry for these reasons (Hsu, F.C. *et al.*, 2002; Kennedy, J.E., Jr. and Bitton, G., 1987; Kennedy, J.E. *et al.*, 1986). Bacteriophage counts of between $<10^1$ and 6 x 10^2 PFU (plaque forming units) per g tissue were found in chicken, turkey or ground beef (Kennedy, J.E. *et al.*, 1986). In some cases pilus-specific bacteriophages have been sought (which may limit the range of bacteriophages and host organisms that can be detected) and these have been found in between 63% and 100% of samples of ground beef and chicken meat (Hsu, F.C. *et al.*, 2002). Coliphages were isolated from between 69 and 88% samples and *Salmonella* bacteriophages were found in 65% samples (Hsu, F.C. *et al.*, 2002). The study by (Atterbury, R.J. *et al.*, 2003b) included a validation of the method of isolation indicating that recovery of *Campylobacter jejuni* bacteriophages inoculated experimentally on to fresh or frozen chicken skin remained constant at 42-44% over a 6 day period thereafter falling to 17% by day 10. The method, using a standard indicator strain, was also sensitive enough to detect ca. 10^3 PFU/cm^2 of skin. Recovery decreased markedly from 100% immediately after inoculation to 22% following refreezing and thawing. Given the poor growth of *C. jejuni* at refrigeration temperatures it is not surprising that bacteriophage recovery was not affected by the presence of *C. jejuni* on the skin surface. *Campylobacter* bacteriophages were recovered from 11% of 300 skin samples. The recovery rates were 79% for free-range chickens and 15% and 6% for standard and economy products. The mean bacteriophage numbers isolated were 4.6 x 10^5 PFU/cm^2 (range, 1 x 10^2 to 4 x 10^6). Bacteriophage recovery from skin from frozen chicken was not successful. A more recent study (Tsuei, A.C. *et al.*, 2007) demonstrated isolation of coliphages from 90.2% of 51 samples of chicken skin in a study from New Zealand. Most bacteriophage counts were in the range of 1-10 PFU/g with the highest count 2.6 x 10^2 PFU/g. The figure for *C. jejuni* bacteriophages was 0% for skin samples and 28.2% for whole bird rinses.

No studies have been carried out on the relationship between numbers of specific bacteriophages present in the intestine and which are active on bacteria such as lactobacillus and the obligate anaerobes, and their number on skin after slaughter or during retail.

A number of other early studies have shown bacteriophages active on *Pseudomonas* spp., psychrotrophic bacteria, *Staphylococcus aureus*, enterobacteria, including *E. coli* and *Salmonella*, to be isolated from poultry, red meat, fish and shellfish and raw milk (see (Kennedy, J.E., Jr. and Bitton, G., 1987) for review) in addition to fermentation products derived from milk, including cheese (Gautier, M. *et al.*, 1995; Suarez, V.B. and Reinheimer, J.A., 2002).

As a result of storage of meats and other foods at low temperature isolation of bacteriophages from such products has been largely confined to psychrotrophic bacteria that can be isolated from and are associated with spoilage of chilled meats. Thus (Greer, G.G., 1983) isolated a total of 21 virulent bacteriophages active on a wide range of strains of *Brocothrix thermosphacta* from steak rib washings. (Whitman, P.A. and Marshall, R.T., 1971b) used the bacteriophage-host system to study a variety of refrigerated products. Bacteriophages which were active on the host bacteria isolated from the same sample were isolated from ground beef (11/17 samples), pork sausage (4/7), chicken (4/8), raw skim milk (2/5), oysters (1/1), but they were not isolated from 2 samples of egg white and 5 samples of luncheon meat. In most cases more than one bacteriophage type was isolated from each sample. The range of bacteriophage counts was wide between $<10^2$ PFU/g to 6.3 x 10^7 PFU/g. Bacterial counts were greater than 2.2 x 10^5 CFU/g in all except one sample. The bacteriophages were fairly specific, generally lysing only the hosts on which they were isolated which were *Pseudomonas*, enterobacteria or *Leuconostoc* spp..

Similar studies were carried out by (Delisle, A.L. and Levin, R.E., 1969) with bacteriophage-hosts systems involving *Pseudomonas* isolated from fish meat.

It is unclear whether the primary source of bacteriophages on seafood is the resident microflora of the organisms at catch or from the processing environment. Bacteriophages have been isolated from mussels and oysters (Croci, L. *et al.*, 2000; Kennedy, J.E. *et al.*, 1986). Oysters contained $<10^1$ PFU/g coliphages and similar numbers of *E. coli*.

Bacteriophages have also been isolated from processed meats including sausage although it is again unclear whether this is a result of contamination during processing (Whitman, P.A. and Marshall, R.T., 1971a, b). (Kennedy, J.E. *et al.*, 1986) found low numbers of bacteriophage ($<10^2$ PFU/100g and $<10^3$ PFU/100g respectively) from luncheon meat and chicken pot pie.

3.2. Use of bacteriophages in the biocontrol of microorganisms in food

Bacteriophages can be used following two different approaches, in a passive or in an active treatment.

(i) used in a passive treatment

In this approach bacteriophages are added in sufficient quantities to overwhelm all target organisms by primary infection, or by lysis from without. Although much higher numbers of the bacteriophages are required, they should be able to eliminate even sparse populations of susceptible bacteria. One other advantage of this approach is that, since much of the effect is a result of lysis from without, natural resistance due to restriction enzymes present in host bacteria will not be an issue. Since the attachment antigen may be shared between several bacterial taxa which may not normally be susceptible to bacteriophage multiplication, the use of this method can widen the range of susceptible bacteria

(ii) used in an active treatment

A relatively small dose of bacteriophages may be required for efficacious elimination of the undesirable bacteria, since most are killed by secondary infections due to replication and transmission from neighbouring organisms. This is dependent on the bacteriophages being able to spread between susceptible bacterial hosts, which may be hindered by the surrounding material being viscous or by the presence of outnumbering inert bacteria.

The timing of bacteriophage application appears to be important in active treatment, and the host cells must be in excess of a predicted critical replication threshold to propagate enough bacteriophages to kill all target cells. If this threshold is not reached the bacteriophages are unable to multiply and may disappear.

Three scenarios have been proposed for the use of bacteriophages in biocontrol:

(a) control of pathogenic bacteria in foods

(b) prevention of bacterial food spoilage

(c) reduction of antibiotic resistance by suppressing resistance gene expression by using bacteriophages to deliver antisense DNA. This is purely in the experimental phase.

Bacteriophages used for the first application (a) usually originate from non-food sources where the pathogens may also be found, such as waste water, faeces, sewage, soil etc.; those used for the second application (b) generally derive from foods and food-processing environments. Most data available to date come from experimentally inoculated foods in laboratories, and in many of the experiments, optimum control of pathogens were achieved at high multiplicity of infection values (ratio of bacteriophage to target bacteria).

3.2.1. Examples of use in dairy products

Bacteriophages are naturally present in raw milk as reported by (Bruttin, A. *et al.*, 1997; Quiberoni, A. *et al.*, 2006). These bacteriophages were identified as a result of their potential role in lysing starter cultures used in dairy fermentations. The presence of bacteriophages that target the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* is a problem in dairy fermentations (Sturino, J.M. and Klaenhammer, T.R., 2004). In addition to the wide body of research on this industrially important bacteriophage issue, a number of studies have been carried out where bacteriophages, which are inhibitory to pathogenic or spoilage bacteria have been deliberately added with the intention of demonstrating their efficacy in eliminating undesirable bacteria from dairy products. These are described below. Interestingly, two papers report observations that bacteriophage were unable to lyse their target bacteria in raw milk (Gill, J.J. *et al.*, 2006; O'Flaherty, S. *et al.*, 2005b) due to heat-labile factors present in raw milk, but which were inactivated in pasteurized milk. (O'Flaherty, S. *et al.*, 2005b) proposed that the inhibition was due to immune factors present in milk which brought about agglutination of the bacterial cells rendering them inaccessible to the bacteriophages.

On the topic of longevity of phages in milk, one recent study showed that phage preparations constituted in milk-based formulations were protected from physical damage brought about by UV irradiation and other factors associated with phage survival on leaf surfaces such as dessication and temperature (Iriarte, F.B. *et al.*, 2007). Phages generally survived longer when composed in the formulation, which contained 7.5g/L skim-milk powder. For example, in the absence of formulation, fluorescent light eliminated phage within two weeks. In the presence of the formulation the reduction in phage numbers was eliminated (Iriarte, F.B. *et al.*, 2007). It is noteworthy that sugar and protein have long been known to have a protective effect on phage (Ehrlich, R. *et al.*, 1964; Prouty, C.C., 1953).

Studies where bacteriophage have successfully been used to inhibit undesirable bacteria in milk and dairy products include those by (Ellis, D.E. *et al.*, 1973) and (Patel, T.R. and Jackman, D.M., 1986) who showed that bacteriophage could reduce the numbers of the psychrotrophic *Pseudomonas* in milk. In a different study focusing on staphylococci, the anti-staphylococcal bacteriophages employed were found to be very stable and active in decreasing numbers of this bacterium. They were more effective during enzymatic (rennet) manufacturing of curd than during acid curd manufacturing (Garcia, P. *et al.*, 2007) suggesting that pH had a negative effect on bacteriophage activity in this case. In another study, addition of anti-*Salmonella* bacteriophages to cheese milk was shown to reduce the numbers of *Salmonella* Enteritidis in cheese made from both raw and pasteurised milk (Modi, R. *et al.*, 2001). In the case of *Enterobacter sakazakii*, bacteriophages were able to effectively suppress the growth of this pathogen in reconstituted infant formula milk both at 24 and 37^{o}C (Kim, K.P., 2007). Another example is the pathogen *Listeria monocytogenes* which is a significant problem in many dairy products, especially raw-milk cheeses. In this research, treatment with anti-*Listeria* bacteriophage lead to complete eradication of this pathogen in soft cheese (Carlton, R.M. *et al.*, 2005) and in mozzarella cheese (Guenther, S. and Loessner, M.J., 2006). *Listeria* disappeared to titers below the detection limit up to 21 d after cheese packaging when applying bacteriophage frequently and at a high dose (Schellekens, M.M. *et al.*, 2007). These studies all indicate a strong potential for success when applying bacteriophages to eliminate undesirable bacteria in milk and dairy products.

Another interesting study looked at the possibility of deliberately applying bacteriophages, which targeted lactic acid bacteria, to mediate lysis of specific components of a cheese starter culture. The aim here was to bring about release of intracellular bacterial enzymes into the cheese curd: namely peptidases and lipases, which are known to generally have a positive

impact on cheese flavour during cheese ripening. This approach was demonstrated by (Crow, V.L. *et al.*, 1995). In the same context, a study by (O'Sullivan, D. *et al.*, 2000) demonstrated that a wide range of dairy starter cultures associated with autolysis (and thus good flavour characteristics) in cheese curd harboured prophage determinants. It was proposed that the "cooking" stage of cheese manufacture brought about prophage induction and release of bacteriophages (and thus cell lysis) into the cheese curd. Note, the "cooking" stage typically involves heating the curd to 40^{o}C in the fermentation tank. "Cooking"-induced lysis of a starter culture with concommitant detection of bacteriophage particles by electron microscopy was demonstrated by (Feirtag, J.M. and McKay, L.L., 1987).

3.2.2. Examples of use in carcasses, meats and meat products

Bacteriophages have been applied to meat and meat products with the main aim of selectively reducing target populations of pathogenic or spoilage bacteria. Although the application of bacteriophages as a biocontrol has been investigated in a variety of food matrices, most studies have focussed on chicken, beef and pork. Some mathematical models of phage-host interactions suggest that a minimum density of host cells is required in order to support phage replication and significantly reduce the target population of bacteria (Payne, R.J. and Jansen, V.A., 2001; Payne, R.J. *et al.*, 2000). One study concluded that bacteriophages do not affect the number or activity of bacteria in liquid environments where the population density of the host species is below approximately 10^4 CFU per ml (Wiggins, B.A. and Alexander, M., 1985). However, these conclusions are not universally accepted (Kasman, L.M. *et al.*, 2002) and studies on the control of spoilage bacteria on meat surfaces suggest that bacteriophages can be effective biocontrol agents when the population of host cells is as low as 46 CFU per cm^2 (Greer, G.G., 1988). These conflicting findings may be a result of factors such as different phage/host combinations, the matrix used, the presence of non-host decoys (i.e. particles to which the phage will attach, other than the bacterial host) or the assumptions made when modelling. As such, the efficacy of phage-based biocontrol should be determined empirically on a case-by-case basis as the predictive power of current mathematical models is limited.

3.2.2.1. Examples of use in chicken products

Poultry products have arguably been the most widely-used meats to study the efficacy of bacteriophage-mediated biocontrol in foods. Members of the *Campylobacter* and *Salmonella* genera have been the most frequently targeted pathogens on chicken meat. Significant reductions in *C. jejuni* and *S.* Enteritidis numbers following phage treatment have been recorded on artificially contaminated chicken skin (Atterbury, R.J. *et al.*, 2003a; Goode, D. *et al.*, 2003). Freezing of the chicken skin after the application of phage was more effective in reducing *C. jejuni* numbers than either treatment used independently (Atterbury, R.J. *et al.*, 2003a). In an effort to represent a more accurate distribution of pathogens on the surface of chicken carcasses, (Atterbury, R. *et al.*, 2006) took skin sections from slaughtered chickens which had been experimentally infected with *S.* Enteritidis or Typhimurium during rearing. The application of a high titre phage suspension reduced *S.* Enteritidis numbers to below detectable levels in the majority of contaminated skin sections. A significant reduction in the proportion of broiler chicken and/or turkey carcasses contaminated with *Salmonella* following phage treatment was reported by (Higgins, J.P. *et al.*, 2005) and (Chighladze, E. *et al.*, 2001). The higher bacteriophage titres used in these experiments were generally much more effective in reducing *Salmonella* numbers than the lowest titres. A small number of studies have examined the efficacy of bacteriophages against *Salmonella* in chicken portions and processed products. Bacteriophages have been used to reduce numbers of *S.* Typhimurium DT104 inoculated onto chicken legs (Kostrzynska, M. *et al.*, 2002) and chicken sausages (Whichard, J.M. *et al.*, 2003).

570 3.2.2.2. Examples of use in beef products

571 Studies using bacteriophages to treat beef products have targeted both spoilage and pathogenic
572 bacteria. Spoilage organisms such as *Pseudomonas* spp. have been controlled on artificially-
573 contaminated beef surfaces using bacteriophages, with a concomitant increase in the shelf life
574 of the product (Greer, G.G., 1982, 1986). However, experiments using bacteriophages to treat
575 meat surfaces naturally-contaminated with *Pseudomonas* have thus far proved unsuccessful
576 (Greer, G.G. and Dilts, B.D., 1990). (O'Flynn, G. *et al.*, 2004) and (Abuladze, T. *et al.*, 2008)
577 were able to significantly reduce the numbers of *E. coli* O157 on artificially-contaminated beef
578 surfaces and ground beef respectively following phage treatment. The control of *Listeria*
579 *monocytogenes* in meats raises additional difficulties due to the ability of this pathogen to grow
580 at low temperatures. In a study by (Dykes, G.A. and Moorhead, S.M., 2002), bacteriophages
581 alone had no effect on the growth of *L. monocytogenes* in beef broth at 4^{o}C. However, an
582 enhanced effect was seen when bacteriophages and nisin were combined, although this could
583 not be replicated on a vacuum-packed beef model. (Bigwood, T. *et al.*, 2008) investigated the
584 use of bacteriophages against *Salmonella* Typhimurium and *Campylobacter jejuni* in cooked
585 and raw meat at different temperatures. The greatest reduction in *Salmonella* numbers was
586 obtained when both the population density of target bacteria and multiplicity of infection were
587 high. The incubation temperature also appeared to be important, with greater reductions in
588 pathogen numbers occurring at higher temperatures (~24^{o}C). The reduction in pathogen
589 numbers following phage treatment could be maintained for up to eight days when the meat
590 samples were incubated at 5^{o}C. This was despite no recorded increase in phage numbers after
591 24 h.

592 3.2.2.3. Examples of use in pork products

593 Relatively few studies have used pork as a model for phage treatments. Bacteriophages have
594 been used to significantly reduce the growth of *Brochothrix thermosphacta* on pork adipose
595 tissue over two days (Greer, G.G. and Dilts, B.D., 2002). However, prolonging incubation of
596 the phage-treated tissue samples to ten days resulted in the growth of BIMs. A recent study
597 demonstrated that phage could significantly reduce the numbers of *Listeria* on hot dogs
598 (Guenther, S. *et al.*, 2009). The largest reductions in *Listeria* were recorded when the highest
599 titres of phage were applied. The bacteriophages remained viable on the food surface for six
600 days when stored at 6^{o}C, with only a negligible reduction in titre during this period.

601 3.2.2.4. Examples of use in seafood

602 There are few examples of bacteriophage treatments in seafood. One study reported significant
603 reductions in *Listeria monocytogenes* in mixed seafood following phage treatment and
604 incubation at 6^{o}C for six days (Guenther, S. *et al.*, 2009). A small reduction in *L.*
605 *monocytogenes* was also achieved on the surface of smoked salmon following phage treatment.
606 However, this reduction was not sustained over the six days of incubation. Generally speaking,
607 higher phage numbers applied to the food surface resulted in greater reductions in pathogen
608 numbers. Similar findings were reported by (Hagens, S. and Loessner, M.J., 2007) who
609 demonstrated that the application of a high titre phage suspension could result in appreciable
610 reductions in *Listeria* numbers in artificially-contaminated salmon. The application of lower
611 phage titres did not lead to reductions in *Listeria* numbers.

612 3.2.2.5. Examples of use in food processing environments

613 A limited number of studies have investigated the use of bacteriophages to control pathogen
614 numbers in processing plants or metallic surfaces. This could be particularly important in high-

throughput meat processing plants which receive animals from a wide geographical area (e.g. large broiler chicken processors) and are difficult to thoroughly clean and disinfect. Due to its propensity for growth at low temperatures and incorporation into biofilms, *Listeria monocytogenes* has been the focus of bacteriophage treatment of biofilms in food processing plant surfaces. (Hibma, A.M. *et al.*, 1997) showed that the formation of *Listeria* biofilms on metal discs was reduced in the presence of phage. Moreover, phage treatment was as effective as 130 ppm lactic acid at removing *Listeria* from mature biofilms. Similar findings were reported by (Roy, B. *et al.*, 1993) who found that the numbers of *Listeria* in biofilms on stainless steel discs could be reduced significantly following phage treatment. The combined use of bacteriophages and a disinfectant further reduced *Listeria* numbers in the biofilm by approximately 100-fold.

4. Factors affecting the survival of bacteriophages in foods and food-processing facilities

While some bacteriophages may degrade during storage, it is impossible to generalize on their ability to survive intact independently of their host bacterium. This needs to be defined for individual bacteriophages, as do all their properties (Carlton, R.M. *et al.*, 2005). In one study by (Guenther, S. *et al.*, 2009), survival of *Listeria* bacteriophages was described. On most foods, these bacteriophages appeared very stable (maximum decrease of infectivity 0.6 logs). The added bacteriophages retained most of their activity during storage of foods of animal origin, whereas plant material caused inactivation by more than one $\log_{10}$. It is important to mention that although bacteriophage were sometimes not inactivated, they were apparently immobilized relatively soon after addition to non-liquid foods and therefore became inactive by limited diffusion (Guenther, S. *et al.*, 2009).

Bacteriophages have no metabolism and inactivation is likely to follow first order kinetics, although rates of inactivation will differ depending on various factors. The conditions of relevance are those to which food is subjected post-slaughter and during processing. Survival and persistence may be affected by a combination of physical factors such as pH, temperature, water content etc. in association with food composition including fat, sugar, protein and salt content. Thus is the same way that *Streptococcus cremoris* (*Lactococcus lactis* subsp *cremoris*) bacteriophages are more heat-resistant in milk than in broth (Koka, M. and Mikolacjik, E.M., 1967), survival on carcasses or in meat is also likely to be enhanced by close association with host proteins.

The aims of studies determining bacteriophage survival, are to look at persistence of naturally contaminated and applied bacteriophages, so that they would remain protective during processing and prevent re-contamination.

4.1. pH

A number of studies have indicated that bacteriophages are generally stable between pH 5 and 8, this being broadened to a pH range between 4 and 10 at lower temperatures (Adams, M.H., 1959). In a study to determine stability following oral administration to calves, survival at between 3.5 and 6.8 in milk whey was found, followed by increasingly rapid inactivation below pH 3 (Smith, H.W. *et al.*, 1987). pH is also likely to be relevant to survival in fermented foods..

4.2. Temperature

Thermotolerance of bacteriophages is in correlation with the environment/host system from which they are derived. Thus bacteriophages found in cheese and yoghurt tend to be highly

thermotolerant, whereas those from psychrotrophic bacteria are less so (Hudson, J.A. *et al.*, 2005). Inactivation of coliphages takes place between 60^{o} and 75^{o}C depending on the surrounding medium (Adams, M.H., 1959). Bacteriophages are generally more thermotolerant than the host bacteria indicating that they may survive after the host bacteria has been killed. T4 bacteriophages were fed to crabs which were then boiled for 5 min; the internal temperature reached 70^{o}C with 80% inactivation of the bacteriophages. However, 2.5% of bacteriophage survived 20 min at an internal temperature at 84^{o}C (DiGirolamo, R. and Daley, M., 1973). Bacteriophages can survive the pasteurisation process this being bacteriophage strain dependent (Suarez, V.B. and Reinheimer, J.A., 2002).

Bacteriophage activity is generally only evident when the environmental and nutritional conditions are conducive to growth of the host. At refrigeration temperatures growth rates of enteric pathogens may be much lower and the length of the bacteriophage infection cycle, including the latent period, will be longer. However, psychrotrophic bacteriophages may multiply on their hosts at 1^{o}C (Greer, G.G., 1982, 1988, 2005). Furthermore, bacteriophage multiplication on the host whilst on the carcass is not necessary for lysis from without. In addition, early studies showed that at 0^{o}C abortive infections occur in 80% of bacteriophage T2 absorption events (Adams, M.H., 1955). Although there is now evidence of bacteriophage activity by lysis from without, a more detailed determination of the exact nature of the relationship between host and bacteriophage would assist in defining the optimal conditions for their activity at low temperatures.

There is experimental evidence for survival of *Salmonella* bacteriophages on chicken skin for 48 h at 4^{o}C (Goode, D. *et al.*, 2003), and of *C. jejuni* bacteriophages on chicken skin for up to 10 d at 4^{o}C (Atterbury, R.J. *et al.*, 2003b). Survival at low temperature may also be of value, since the bacteriophages can enter the lytic cycle once products are warmed or ingested (Greer, G.G. and Dilts, B.D., 1990). Survival on cheeses at 14^{o}C for several days has also been reported (Schellekens, M.M. *et al.*, 2007).

4.3. Light

Bacteriophages are inactivated exponentially by ultra violet light at variable rates (Adams, M.H., 1959) which is probably the reason for inactivation by sunlight in water. This is generally due to DNA damage which may also be repaired after infection by bacterial DNA repair mechanisms. In another study by (Iriarte, F.B. *et al.*, 2007), fluorescent light eliminated *Xanthomonas* bacteriophages within 2 weeks.

4.4. Osmotic shock and pressure

Osmotic shock generally produces bacteriophage ghost particles, in which the DNA has been lost (Adams, M.H., 1959). This would affect the ability to multiply in the host bacterial cell, but not to attach and cause lysis from without..

4.5. Disinfectants and other chemicals

A number of antiseptic chemicals inactivate bacteriophage particles rapidly, including periacetic acid, ethanol and sodium hypochlorite (Binetti, A.G. and Reinheimer, J.A., 2000; Suarez, V.B. and Reinheimer, J.A., 2002). Although bacteriophages are generally more resistant than bacteria to inactivation by chemical and physical stresses, there is a wide range of resistance to chlorine amongst coliphages (Kennedy, J.E., Jr. and Bitton, G., 1987). Bacteriophages are more resistant than *E. coli* to waste water treatment. It seems likely

therefore, that bacteriophages could become persistent in processing plants and that disinfection regimens may need to be developed to monitor efficacy of their application in the food industry.

4.6. Other factors

Information on the effects of fermentation, freeze-drying or irradiation on bacteriophage stability is scarce. A proportion of bacteriophages survive in fermented sausage. Freeze-drying reduces titres initially but the lower titres persist for many weeks. Bacteriophages are more resistant to gamma irradiation than are the host bacteria (see (Kennedy, J.E., Jr. and Bitton, G., 1987) for review). The food matrix can have an important protective effect on bacteriophages. For instance, a milk-based formulation protected a bacteriophage against dessication and UV (Iriarte, F.B. *et al.*, 2007).

4.7. Interpretation of industry data

Two types of experiments were presented in the documents provided by Industry to test the stability and persistence of activity of bacteriophage applied on foods.

(i) Stability measured after recovery of the bacteriophage from the inoculated foods.

The bacteriophage P100, isolated from sewage effluents from a dairy plant, was tested in soft cheese to control *Listeria monocytogenes* (Carlton, R.M. *et al.*, 2005). One day after cheese making, the rind was inoculated with *L. monocytogenes* and the bacteriophage was spread on the surface of the cheese rind to achieve $6x10^7$ pfu/cm^2. The cheeses were kept at 14°C for ripening, then at 6°C during storage. The bacteriophage numbers recovered from the cheese surface by homogenisation of the rind was then measured every day until day 6. The authors reported no decrease or increase in the bacteriophage number over this period. Industry technical reports (for details see section *Documents provided to EFSA*), not published in the scientific literature, concerned a commercial preparation of the bacteriophage P100 (Listex^{TM}) and gave more details on the stability of P100 on soft cheese surfaces. The bacteriophage numbers, initially around $6x10^7$ pfu/cm^2, remained stable until day 9, and then decreased to approximately $5x10^6$ pfu/cm^2 until the end of the experiment at day 21. In one study by (Guenther, S. *et al.*, 2009), survival of two *Listeria* bacteriophages (including P100 as in the works cited above) was described. On all foods of animal origin tested (meat, dairy and seafoods), these bacteriophages appeared very stable over the 6 days at 6°C tested (maximum decrease of infectivity 0.6 logs). In contrast, on lettuce and cabbage bacteriophages were inactivated by more than one $\log_{10}$.

(ii) Persistence of the activity of the bacteriophage on the food surface.

The technical reports described the activity of the bacteriophage P100 against *L. monocytogenes* on the surface of soft cheese and meat products (ham and turkey breast). (Guenther, S. *et al.*, 2009) studied P100 and another bacteriophages on meats, dairy products, seafoods and fresh-cut vegetables. In all these works, *L. monocytogenes* was initially inoculated on the foods at levels around 10^3 cfu/g. The bacteriophages added at the start of the experiment at levels around 10^8 pfu/cm^2 or g, reduced *L. monocytogenes* by 10-fold to 1000-fold within the first day of incubation. The surviving fraction of *L. monocytogenes* started growing after 1 to 3 days in the case of solid foods, depending on the food and the incubation temperature, at the same rate as the control, not treated with the bacteriophage. These growing bacteria were not resistant to the bacteriophages. These results indicate that the bacteriophages rapidly lost their activity against the residual population of *L. monocytogenes*. On cheese, growth started only after 6 days. However, until 6 days the cheese pH was presumably too low for *L. monocytogenes* growth.

Association of both experiments i) and ii) (Guenther, S. *et al.*, 2009) shows that although bacteriophages rapidly lost their effect on the target bacteria on food surfaces, they were still active when recovered and tested outside the food. Therefore, bacteriophages were not inactivated, they were apparently immobilized relatively soon after addition to non-liquid foods and therefore could not came into contact with the surviving bacteria by limited diffusion (Guenther, S. *et al.*, 2009). However, whether these immobilized, but still active, bacteriophages could lyse target bacteria re-inoculated on the foods was not tested.

In conclusion, the documents provided by industry show that the methods used to measure the persistence of the bacteriophage (either persistence of the activity of the bacteriophage on the food or stability of the bacteriophage on the food) may give different results. With regards to the terms of reference of the mandate addressed in this opinion, it should be stressed that ability of the bacteriophages to protect the food against re-contamination with the target bacteria was not tested.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Conclusions relating to the mode of action expected from the use of bacteriophages solutions on food of animal origin (including but not exclusively use on animal carcases, meat products and dairy products). Terms of Reference number 1.

- Bacteriophages may be virulent or temperate. Upon infection, the first group kills their host bacteria, so they are the ones of choice for bacteriophage-based food decontamination. Temperate bacteriophages do not always kill their hosts, and may confer unforeseen properties to their host bacteria.
- Bacteriophages can induce lysis of the bacterial host-cell by "*lysis from within*" and/or "*lysis from without*".
- Bacteriophages have narrow host-ranges, generally restricted to either a limited number of species within a genus, or to a limited number of bacterial strains within a species.
- While bacteriophage replicate best on growing bacterial cells, they have also been shown to reproduce on stationary phase cells.
- The ratio of bacteriophages to host cells is critical to the success of bacteriophage treatment. The higher this ratio, the greater the reduction in the target bacterial population.
- Naturally occurring bacteriophages have a broad range of habitats and may be isolated in considerable numbers from meat, milk and products thereof.
- Some bacteriophages, under specific conditions, have been demonstrated to be very effective in the targeted elimination of specific pathogens from meat, milk and products thereof.

Conclusions relating to whether the use of bacteriophages may lead to a continual functioning in the food, thereby protecting against recontamination or whether the effect can be expected to be short lived with no continuing action effect in the final food. Term of reference 2.

- Bacteriophages in the environment behave as inert particles and tend to persist longer than their hosts. However, their long-term antibacterial activity is compromised on dry surfaces.
- The persistence in/on food varies with each bacteriophage, and with the conditions of application, including dose, and physical and chemical factors associated with the food matrix.
- Refrigeration temperatures enhance persistence of bacteriophages on the surface of meat and on/in dairy products.
- Based on data currently available in peer-reviewed literature, it cannot be concluded whether bacteriophages are able or unable to protect against recontamination of food with bacterial pathogens. This is likely to vary with each bacteriophage, each food matrix, and with conditions of application including environmental factors.

RECOMMENDATIONS

- In order to assess the issue of bacteriophage persistence in foods, and their ability to prevent recontamination with bacterial pathogens, research for specific bacteriophage-pathogen-food combinations should be encouraged.
- If bacteriophages treatments are to be used for removal of surface contamination of foods of animal origin, then it is recommended that a Guidance Document on the submission of data for their evaluation is provided.

DOCUMENTATION PROVIDED TO EFSA

1. Which path to go? Carl von Jagow and Tobias Teufer EFFL 3/2007 p136
2. The great puzzle, Bacteriophages in the production of foodstuffs: a legal introduction (In DE with EN translation)
3. Carlton et al. Regulatory Toxicology and Pharmacology 43 (2005) 301-312
4. 'The Bacteriophages preparation Listex P100 has no effect on the final product' Dr Steven Hagens September 2007
5. Bacteriophages: brief background information (classification, omnipresence, lytic cycle)
6. Listex P100: Legal status (input from Mr Schipper, Chairman Dutch Expert Committee on Food Labelling)
7. Legal opinion on the application of Listex P100 as a processing aid for foodstuffs, Dr Carl von Jagow, Krohn Rechtsanwalte, Sept 2005
8. Persistence and inactivation of bacteriophages, Prof Dr Martin Loesner, ETH Sept 2006
9. Technical background information on rapid inactivation through adsorption of LISTEX P100 bacteriophages

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