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## **Bacteriophage biocontrol: Food and environmental applications**

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### **Abstract**

*Bacteriophages are viruses whose hosts are bacteria. The bactericidal nature of virulent bacteriophage has been exploited by researchers for decades hoping to utilise these viruses in the fight against bacterial infections and antibiotic resistant bacteria in clinical settings. More recently, the potential applications of bacteriophage biocontrol in the food, agriculture and aquaculture industries have been investigated by researchers attempting to develop 'natural' antimicrobial products. Bacteriophages have many advantages over conventional methods of controlling pathogenic bacteria, such as high host specificity, the*

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*ability to self-perpetuate, and the ability to evolve with their hosts to overcome some problems of resistance to the treatment. However, more research is necessary to optimise the parameters for treatment, including temperature, pH, exposure to UV, multiplicity of infection, mode of application and host/phage threshold. It has also been argued that any potential candidate for bacteriophage biocontrol must be genetically sequenced to ensure that it is unable to confer any virulence to the host. Bacteriophages have also been investigated for use as bacterial indicators of the microbiological quality of water, as well as in rapid assays for the detection of pathogens in foods.*

## **1. Introduction**

Bacteriophages (or phages) are viruses that infect bacteria. Although British physician, Ernest Hankin, first observed and reported on the bactericidal effects of filtered water from the river Ganges against *Vibrio cholerae* in 1859, the official discovery of bacteriophages is credited to British bacteriologist Frederick Twort (1915) and French-Canadian Microbiologist, Felix d’Herelle (1917). The two researchers independently described phages as agents capable of bacterial lysis [1], although it was d’Herelle who coined the term ‘bacteriophage’, translating to ‘bacteria eater’ [1]. Prior to the discovery of antibiotics, it was hoped that bacteriophages, being the natural predator of bacteria, would hold the key to curing infectious disease [2].

Bacteriophages are abundant in the environment with an estimated population of up to  $10^{30}$  phage particles [3]. Phages can be found in high concentrations in the ocean, where each marine bacterial species may be infected by up to 10 phage species [4]. Indeed, phages can be found in all environments inhabited by their hosts. There have even been phages isolated from extreme environments inhabited by the Archaea [5,6,7].

## **2. Phage morphology**

Phages are structurally diverse. Unlike bacterial or eukaryotic cells, phage genomes may consist of either single or double stranded DNA or RNA. DNA genomes may be circular or linear, while RNA genomes are linear, but may consist of a single molecule or several ‘segments’ [8]. The genome, which may contain from as few as four to more than 200 genes, is enclosed by a capsid, or protein coat, which may be helical or icosahedral in shape. Many phages, such as the T4 phage of *Escherichia coli*, are complex viruses, comprising an icosahedral nucleocapsid attached to a helical protein ‘tail’ [5]. Some phages, such as the Pseudomonas phage  $\phi 6$ , are enveloped by phospholipid and/or protein [9].

### 3. Replication cycles of phages

Bacteriophages may be classified according to the replication cycle undertaken. Virulent bacteriophages undergo the 'lytic cycle' so termed because the host is killed by cell lysis following infection, while temperate bacteriophage are able to replicate their genome along with that of the host without taking control of and lysing the host cell. The replication cycle undertaken by temperate bacteriophage is called the 'lysogenic cycle', and bacteria that contain temperate bacteriophage are called 'lysogens' [10].

#### 3.1 The lytic cycle

The general stages of virulent bacteriophage replication are as follows (Figure 1):

1. **Attachment.** The bacteriophage attaches or adsorbs to a specific receptor on the host cell. Common receptors include surface proteins, parts of the lipopolysaccharide, pili and flagella.
2. **Penetration.** Phage nucleic acid is "injected" into the host cell. The capsid and other protein structures remain outside. In some tailed phages, the tail sheath contracts to allow the nucleic acid to make its way into the cell.
3. **Synthesis of nucleic acid and protein.** The phage takes over host metabolic machinery to produce its own nucleic acids and proteins. The first (early) proteins to be translated are those responsible for inhibiting host systems and replicating the phage genome. The 'late' proteins are involved in the formation of new phage particles and lysis of the host cell.
4. **Assembly and maturation.** The nucleic acid and protein products are assembled to form mature phage particles.
5. **Release.** The host cell wall is lysed by phage encoded enzymes called lysins, releasing the newly formed phage particles into the environment [11].

#### 3.2 The lysogenic cycle

In the lysogenic cycle, the temperate phage attaches to and penetrates the host cell in the same method as in the lytic cycle. However, once inside, the phage genome integrates with that of the host cell and becomes a dormant prophage [8]. This results in the host bacterium producing a copy of the phage genome, along with its own DNA each time it undergoes cell division, usually for many generations. A bacterial host containing a prophage is called a lysogen, and is protected by the prophage from superinfection by another temperate phage. When the host is exposed to an environmental stressor such

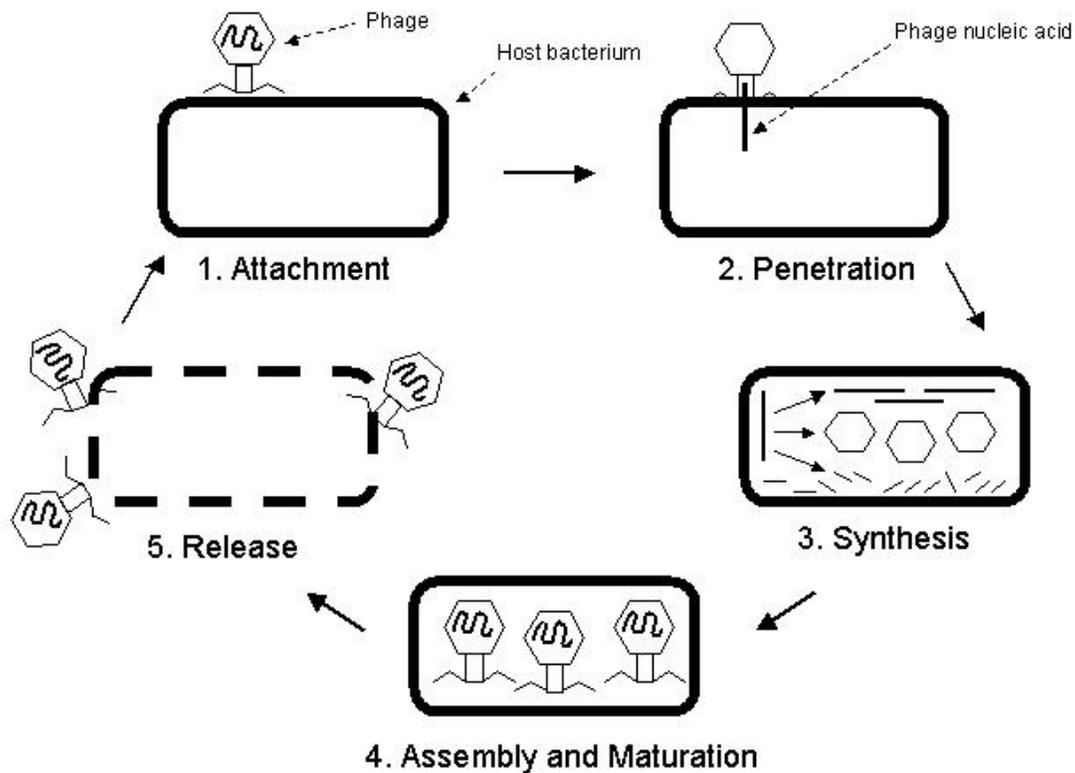


Figure 1

as ultraviolet light, the dormant prophage is induced to carry out the lytic cycle [8]. Although not suitable candidates for phage biocontrol, temperate bacteriophages are very useful tools to the molecular biologist due to the role they play in transduction, recombination and lysogenic conversion [10].

#### 4. The use of phages for typing and detection of bacteria and as indicators of viral contamination

The ability of virulent phages to lyse specific bacterial strains and to produce plaques on artificial media has been exploited by epidemiologists in the characterisation and identification of bacteria involved in disease outbreaks. The technique, known as ‘phage typing’ is used to differentiate between homologous bacterial strains, that may be distinguishable phenotypically only by their susceptibility to certain phages.

Phages have also been used to detect bacterial pathogens in food and food processing plants. Barbalho *et al.* [12] employed listeriophages to confirm suspect colonies of *Listeria* spp. that had been isolated from the gloves and hands of workers involved in handling chicken carcasses at a food processing plant. Following the same principle as phage typing, suspect bacterial colonies were grown as a bacterial lawn and then ‘spot-tested’ against different

virulent listeriophages. Plaque formation in the bacterial lawn following incubation demonstrated susceptibility of the bacteria to the phage, and was considered to be a positive or confirmatory result. This rapid method reduced the time for confirmation of *Listeria* species from four days to eight hours, although phage typing was still necessary to determine the strain [12].

Another avenue of research has been the development of fluorescently labelled bacteriophages for the detection of pathogenic *E. coli*. Goodridge et al. [13] developed a fluorescent bacteriophage assay to detect *E. coli* O157:H7 in inoculated ground beef and raw milk. The bacteriophages were prepared by staining with the fluorescent nucleic acid dye YOYO-1 and then added to samples of ground beef and raw milk that had been inoculated with *E. coli* O157:H7. The samples were analysed by epifluorescence microscopy and flow cytometry. The detection limit of fluorescence microscopy in milk samples was  $10^3$  colony forming units (cfu) ml<sup>-1</sup>, and between  $10^1$  cfu ml<sup>-1</sup> and  $10^2$  cfu ml<sup>-1</sup> for flow cytometry. For beef samples, the detection limit by epifluorescence microscopy was  $10^3$  cfu 25g<sup>-1</sup> and 2.2 cfu 25g<sup>-1</sup> for flow cytometry. Although the sensitivity of the assay was poor, the authors suggested that it could be improved with a longer enrichment period [13]. Oda et al. [14] also employed the use of fluorescence for the rapid detection of viable but non-culturable (VBNC) *E. coli* O157:H7. The study involved labelling the capsid of PP01 bacteriophage with green fluorescent protein (GFP). Following phage infection of susceptible *E. coli* cells, the fluorescence intensity of the culture increased, as observed by fluorescence microscopy. The study has important public health implications, as VBNC bacteria have the potential to cause illness but cannot be detected by traditional screening methods [14].

Bacteriophages have also been investigated as potential indicators of viruses and faecal contamination of water sources [15,16,17,18,19]. F-RNA bacteriophages, a group of phages which adsorb to the host cell via adsorption to the F pili, are homologous in morphology and in terms of survival characteristics of some important human viruses [15], and are easier to culture. Havelaar et al. [16] suggested that coliphages could make useful indicators of faecal contamination of water sources because they often originate from faecal sources and may be more persistent in water sources than traditional bacterial indicators. Somatic coliphages have been suggested for us as predictors of treated wastewater and sludge quality intended for agricultural reuse, due to their ability to survive chlorination during wastewater treatment [17].

Mandilara et al. [17] suggested that the ability of somatic coliphages to survive chlorination during wastewater treatment makes them a good candidate for use as indicators of waste water and sludge quality. This is particularly important where the treated wastewater is intended for agricultural reuse, in order to avoid contamination of crops and groundwater with pathogens that were also able to survive the treatment process.

## 5. Phage therapy

The bactericidal nature of virulent phage sparked interest in their potential for use as antimicrobial agents following their discovery in the early 20<sup>th</sup> century. Phage therapy refers to the application of virulent bacteriophages to treat bacterial infections in humans or animals. The initial trials of phage therapy produced varied results which have been attributed in part to the limited scientific rigour applied to the experiments, including the failure to conduct double-blind trials which were not standard practice at the time, as well as poor understanding of the biological nature of phage and lysogeny [20]. Lysogenic phage are now known to be unsuitable for phage therapy, due to their ability to confer virulence and antibiotic resistance to bacterial hosts via phage-mediated gene transfer and their ability to remain in a dormant prophage state. Examples of virulence factors encoded by bacteriophage genes include the toxins produced by *Corynebacterium diphtheriae*, *Vibrio cholerae* and *Salmonella enterica* [10].

Following the discovery of penicillin in the 1940s and due in part to inconclusive results obtained from early phage therapy trials, this type of research was largely abandoned in western countries in favour of broad spectrum antibiotic production, although it continued in the former Soviet Union unabated [21]. In the past 20 years, Western countries have refocused their phage therapy research efforts to combat the rise in antibiotic resistant bacteria. Human phage therapy is outside the scope of this chapter; however it has been the subject of many recent reviews [20,21,22,23,24,25,26,27].

## 6. Phage biocontrol

A growing focus of phage-related research is in the area of ‘phage biocontrol’, which is the term used to describe the intentional application of host-specific virulent phages to environmental settings for the purpose of controlling pathogenic or spoilage bacteria [28]. Phage biocontrol applications have been investigated for areas as diverse as food safety, agriculture, aquaculture and wastewater treatment. The following sections will review the research in these areas, with a primary focus on phage biocontrol in the food industry.

## 7. Phage biocontrol in the food industry

Foodborne illness is a major cause of morbidity and mortality worldwide [10]. Food production methods are constantly evolving to accommodate growing populations and consumer demands. Globalisation has ensured that consumers are able to access traditionally ‘seasonal’ produce all year round, as well as increasing the availability of imported exotic foods. In addition to these benefits, globalisation and mass production of food have contributed to the increased risk of food contamination and the potential for large-scale foodborne illness outbreaks [29].

Furthermore, changes to the demographic profile of the community, as well as the emergence of new foodborne pathogens, make it necessary to continuously develop new methods of controlling foodborne pathogens at all stages of food production in an effort to reduce the incidence of food-related illness. Phage biocontrol is considered one such method, having many potential applications at all stages of food production (Table 1).

**Table 1.** Applications of phage biocontrol to reduce human pathogens in the food industry\*.

Product	Host Bacteria	Phage	Reference
<i>Live Food Animals</i>			
Chickens	<i>Salmonella</i>	CNPSA1, CNPSA3, CNPSA4	35
Sheep	<i>C. jejuni</i>	CP8, CP34	36
	<i>E. coli</i> O157:H7	DC22	47
		CEV1	48
<i>Poultry</i>			
Chicken	<i>Salmonella</i>	P22	37
	<i>Campylobacter</i>	29C	37
	<i>Campylobacter</i>	φ2	34
	<i>Salmonella</i>	PHL4	39
<i>Meat</i>			
Beef	<i>Pseudomonas</i>	C5, C25, C35, C40, C46 C48, C61	55
Beef (Steak)	<i>E. coli</i> O157:H7	e11/2, e4/1c, pp01	49
	<i>L. monocytogenes</i>	LH7	52
<i>Fresh Produce</i>			
Honeydew melon, apples	<i>L. monocytogenes</i>	LM-3, LMP-102 (phage mixtures)	60
	<i>Salmonella</i>	SCPLX-1 (phage mixture)	61
<i>Cheese and Processed Food</i>			
Chicken frankfurters	<i>Salmonella</i>	Felix O1	62
Cheddar cheese	<i>Salmonella</i>	SJ2	63
Red smear soft cheese	<i>L. monocytogenes</i>	P100	64
<i>Removal of Biofilms and Cells from Surfaces</i>			
Stainless steel	<i>L. monocytogenes</i>	Bred 23074-B1	66
Stainless steel, polypropylene	<i>L. monocytogenes</i>	H387, H387-A, 2671	67
Stainless steel	<i>E. coli</i> O157:H7	KH1	65
Glass coverslips	<i>E. agglomerans</i>	φ1.15	68
	<i>E. clocae</i>	Philipstown	68

\* Selected phage biocontrol applications are described in the text.

Proper implementation of food safety procedures and vigilance by food regulation authorities, food producers and consumers, will continue to be the most important methods of reducing the incidence of foodborne illness. However, many manufacturers and researchers are looking for innovative methods to control problem pathogenic bacteria specific to certain foods during processing.

Phages are ubiquitous in the environment and have been isolated from various food products, suggesting that they are normal inhabitants of food ecosystems [30]. The potential advantages and disadvantages of bacteriophage biocontrol of foodborne bacteria are listed in Table 2 [31].

**Table 2.** Potential advantages and disadvantages of bacteriophage biocontrol of foodborne bacteria (Adapted from Greer et al, [31]).

Advantages	Disadvantages
1. Self-perpetuating	1. Limited host range
2. Stable in foods and able to survive processing	2. Phage-resistant bacterial mutants
3. Ubiquitous, natural and readily isolated	3. Requires large numbers of target bacteria
4. Cost-effective	4. Transduction of undesirable characteristics
5. Easily prepared	5. Lysogenic conversion (temperate phages)
6. Non-toxic to eukaryotic cells	6. Consumer perception of adding viruses to foods
7. No effect on food quality	

## 7.1 Phage biocontrol to reduce *Campylobacter* and *Salmonella* in chicken and chicken products

Although not typically pathogenic to chickens, *Campylobacter* and *Salmonella* are among the most commonly identified causes of bacterial gastroenteritis in humans, with contaminated poultry a common vehicle for infection [32]. Phage biocontrol has been considered a potential method for reducing colonisation of broiler chickens by *Campylobacter* and *Salmonella*. *Campylobacter* is a commensal inhabitant of chickens, whereas *Salmonella* is a common environmental organism which causes asymptomatic infection in chickens but which is pathogenic to humans. Both *Salmonella* and *Campylobacter* frequently colonise the gastrointestinal tract of chickens. *Campylobacter*-specific phages have been isolated from the caeca of slaughtered chickens and from refrigerated retail poultry, indicating that they may already play a natural role in the control of *Campylobacter* in chickens [33,34]. Phage therapy researchers aim to exploit this natural process to effect a reduction or elimination of colonisation of broiler chickens by *Campylobacter* and *Salmonella* prior to harvest through the deliberate

application of specific phages. Studies involving phage therapy of broiler chickens intentionally infected with *Campylobacter jejuni* and *Salmonella enteritidis* have found that success of reduction of host bacteria is dependent on the phage dosage and specificity, and the length of time between infection and treatment [35,36]. For example Loc Carrillo et al. [35] found that the longer the time period between infection with *C. jejuni* and oral administration of phage, the lower the reduction of host bacteria. Phage cocktails were more effective than using a single phage strain, and resulted in a lower percentage of resistant mutants [35,36].

Post-harvest biocontrol studies have also investigated the potential application of virulent phage to the carcasses and skin of chickens to reduce contamination by *Salmonella* and *Campylobacter*. This type of application has potential advantages over pre-harvest applications, since the phages used in the treatment would be unlikely to find their way back to the farms and live animals, therefore reducing the potential for bacteria to mutate to resist infection by the phages [37]. Goode et al. [37] investigated the ability of lytic bacteriophages to reduce the number of artificially inoculated *Salmonella* and *Campylobacter* on the skin of chicken at different multiplicities of infection (MOI) ranging from 1 to 1,000. At low levels of contamination and a low MOI bacterial numbers were reduced by less than 1 log<sub>10</sub> unit. Phages applied at a high MOI (100 to 1000) rapidly reduced the recoverable bacterial numbers by up to 2 log<sub>10</sub> units over 48 hours. At an MOI of 10<sup>5</sup>, no *Salmonella* was recovered following treatment.

In a similar study by Atterbury et al. [38] chicken skin was artificially inoculated with *Campylobacter jejuni* (PT14) and bacteriophage  $\phi$ 2 and incubated at either 4°C or -20°C for a period of 10 days. Over the test period, the number of recoverable *Campylobacter* cells decreased by 1 log unit for samples incubated at 4°C and 2 log units in the samples incubated at -20°C. Bacteriophage  $\phi$ 2 recovery from samples incubated at 4°C remained relatively constant for the duration of the experiment, and while the recovery of phage stored at -20°C was initially similar to recovery from skin at 4°C, the rate of recovery was reduced following freeze-thaw. The application of *Campylobacter*-specific bacteriophage in high enough titres to the surface of chicken skin inoculated with *Campylobacter* clearly reduced the number of recoverable cells, by 1 log unit for inoculated skin stored at 4°C. The authors concluded that a higher reduction in *Campylobacter* would be desirable if this practice was to yield commercial benefit in the future as a sole control measure. However, in its present state, it might be useful alongside other contamination control practices. It was also suggested that the phages used in the experiment were able to adsorb to *Campylobacter* cells prior to refrigeration and replicate only when the host increases its metabolic activity [38].

Higgins *et al.* [39] conducted experiments in which commercially processed chicken carcasses were sprayed with a wash solution containing bacteriophage PHL4 to reduce the number of recoverable *Salmonella enteritidis* cells that had been artificially inoculated onto the carcasses. It was observed that treatment of broiler carcasses with  $5.5 \times 10^{10}$  plaque forming units (pfu)  $\text{ml}^{-1}$  of PHL4 caused an 85% reduction in the frequency of *Salmonella* recovery as compared with untreated controls. The rate of *Salmonella* recovery was further reduced when higher concentrations of phage were used. The authors concluded that while large numbers of bacteriophage must be applied to markedly reduce *Salmonella enteritidis* recovery from inoculated carcasses, there is no evidence to suggest that highest possible concentrations should not be used [39].

## 7.2 Biocontrol to reduce *E. coli* O157:H7 in sheep and contaminated meat

Phage biocontrol has also been investigated as a potential measure to reduce carriage of *E. coli* O157:H7 in sheep, which are a major reservoir of the organism [40]. *E. coli* O157:H7 is an important emerging foodborne pathogen, capable of causing gastroenteritis, which often has more serious complications including haemolytic uraemic syndrome (HUS) and thrombocytopenic purpura (TTP), particularly in the elderly, immunocompromised and children [41]. The main cause of infection in humans is consumption of undercooked beef, although cases have been documented where infection has been caused by consumption of lettuce, potatoes and radish sprouts contaminated with bovine manure [42,43,44]. *E. coli* O157:H7 is persistent in the environment once introduced, and is able to survive in feed, water, soil and manure [45]. It has the potential to be transferred to many environmental sources by direct transfer in manure for fertilisation, or through vectors such as flies [46]. Phage biocontrol studies have focused on eradicating *E. coli* O157:H7 from the gastrointestinal tract of ruminants in an attempt to reduce shedding in faeces, consequently reducing the potential for environmental and meat contamination.

Bach *et al.* [47] conducted a study into the effect of a bacteriophage (DC22) on the survival of *E. coli* O157:H7 in an artificial rumen system (Rusitec) and experimentally infected sheep. DC22 was found to have a bactericidal effect on *E. coli* O157:H7 *in vitro*. However, an MOI of  $10^5$  was required to significantly reduce *E. coli* O157:H7 in the artificial rumen system. The authors suggested that due to the decline of DC22 in ruminal fluid, it is possible that the high MOI resulted in the phenomenon known as lysis from without, which occurs when a bacterial cell is attacked by a large number of phage particles resulting in disruption of the cell wall rather than direct infection and lysis of the *E. coli*. Regrowth of resistant *E. coli* O157:H7 in

ruminal fluid was also observed. The bacteriophage DC22 had no effect on the faecal shedding of *E. coli* O157:H7 by artificially infected lambs. This study indicates the limitations of using a single bacteriophage in biocontrol applications.

Recently, Raya et al. [48] isolated and characterised a new T-even bacteriophage, CEV1, and investigated its potential to reduce *E. coli* O157:H7 in sheep. It was observed that CEV1 was able to lyse 17 out of 19 *E. coli* O157:H7 strains tested and a limited number of commensal *E. coli* strains, therefore creating minimal disturbance of the gut biota. *In vitro* tests showed that CEV1 was able to infect the test strain of *E. coli* O157:H7 (strain 933) both in aerobic and anaerobic conditions. *In vivo* tests involved infecting sheep with *E. coli* O157:H7 (933) and then treating them with a single oral dose of CEV1. Following treatment, the ruminal, caecal and rectal contents of the animals were examined and it was observed that *E. coli* O157:H7 had been reduced by 2 to 3 log units in the caeca and rectum of sheep that were treated with CEV1 compared with the untreated controls. The authors emphasised the importance of extensive characterisation of candidate phages before any widespread application is implemented.

Biocontrol of *E. coli* in culture and on artificially contaminated meat has also been investigated using phage cocktails [49,50]. In these studies, it was also found that a high MOI was required to significantly reduce numbers of *E. coli* O157:H7 *in vitro*. According to Kudva et al. [50] the factors that are critical for rapid cell lysis in culture include aeration, incubation at 37°C, a high MOI and simultaneous infection with the three phages used (KH1, KH4 and KH5). In a study by O'Flynn et al. [49] it was observed that out of nine meat samples artificially inoculated with *E. coli* O157:H7, seven were found to be completely free of *E. coli* O157:H7 after treatment with a phage cocktail containing e11/2, e4/1c, and pp01, determined by viable plate count after enrichment. It was found that the ability of the phage cocktail to reduce *E. coli* was significantly reduced at temperatures below 12°C. The authors suggested that phage biocontrol could be introduced as a control measure to eliminate *E. coli* O157:H7 from the carcass surface during slaughter, or via the addition of a phage cocktail to cattle hides.

### **7.3 Phage biocontrol to reduce *Listeria* on chilled meat**

*Listeria* is an important environmental pathogen due to its serious and often fatal consequences for susceptible individuals as well as its ability to survive and grow in foods stored under refrigeration [51]. Dykes & Moorhead [52] investigated the effect of the bacteriocin nisin and listeriophage LH7 on the growth of two strains of *L. monocytogenes*, L62 and L99, in broth, chilled

buffer and on raw beef. Nisin is a broad-spectrum bacteriocin active against many gram-positive bacteria at low pH values. In phosphate buffered saline inoculated with L62 and L99, addition of LH7 alone did not decrease the number of cells of either strain of *L. monocytogenes*. Addition of nisin alone, however, resulted in an immediate and significant reduction of cells to a level that was maintained over the 28 days of the experiment at 4°C. Addition of LH7 and nisin in combination resulted in a further significant decrease in *L. monocytogenes* cells after 1 day that was maintained over 28 days compared with the nisin treated control. Similar effects were observed when the experiments were replicated using vacuum-packed meat. Regrowth of *L. monocytogenes* to levels equivalent to those of untreated controls were observed, however, this was more obvious at 30°C than at 7°C. The authors concluded that future biocontrol studies should differentiate between growing and non-growing cells and different strains of a species of bacteria to ensure effective control. Bacteria at different growth phases may react differently to antimicrobial agents and so sufficient understanding of growth kinetics and phage host interactions is necessary if phage biocontrol is to be practically applied [52].

#### **7.4 Phage Biocontrol to reduce spoilage bacteria and increase product shelf life**

The psychrotrophic *Pseudomonas* bacteria have been targeted for phage biocontrol due to their ability to grow in and cause spoilage of refrigerated foods such as milk and meat [11]. It has been previously identified that phages already constitute a measurable component of the natural flora of a number of refrigerated foods including meat, fish and poultry [53]. Patel and Jackman [54] first suggested that deliberate application of *Pseudomonas* phages to milk and milk products could be used to increase shelf life. Greer and Dilts [55] investigated the ability of a bacteriophage pool containing seven lytic phages to control pseudomonads linked to beef spoilage. Beef steaks were inoculated with a phage pool containing seven different *Pseudomonas* phages and were then incubated under simulated retail conditions under refrigeration at 8°C. Although it was observed that the phage pool was able to produce a limited but statistically significant reduction in bacterial growth, this was not sufficient to result in any demonstrable increase in shelf life. The authors suggested that the results of the study supported the contention that phage control of spoilage of perishable foods would be unlikely since critical concentrations of sensitive bacteria may not be encountered under natural conditions [55]. Furthermore, given the diversity of *Pseudomonas* associated with beef spoilage and the limited host range of the *Pseudomonas* phages used in the phage pool, it is necessary to isolate phages with broad host ranges to have better success in controlling a greater number of *Pseudomonas* species.

## 7.5 Phage biocontrol to reduce bacterial contamination of fresh produce

Raw fruits and vegetables are particularly vulnerable to contamination by pathogenic bacteria because they are often grown outdoors in soil and as such are exposed to many environmental pathogens either through direct contact or through contact with vectors harbouring bacteria [56]. Irrigation with untreated sewage or fertilisation with poorly composted manure may also contribute to the risk of contamination [56]. Numerous outbreaks have been attributed to the consumption of contaminated fruits and vegetables [56,57,58]. Although fruits and vegetables possess skin or rinds which confer protection against contamination by pathogens, damage to this outer skin can allow penetration of bacteria which may be difficult to remove by chemical sanitisation. Fresh-cut produce is also susceptible to contamination if the skin is not thoroughly washed prior to slicing [58]. The two most common approaches to decontaminating fruits and vegetables involve washing with water and washing with sanitising agents such as trisodium phosphate or sodium hypochlorite. The routine use of the latter in food processing environments has led to various bacteria developing resistance to these agents, causing a decline in their efficacy [59].

Bacteriophage biocontrol of raw fruits and vegetables has been investigated as a potential method for reducing the risk of contamination and foodborne illness. Leverentz et al. [60] examined bacteriophage as a biocontrol method for *Salmonella* on fresh cut melon and apple slices. Melon and apple slices were artificially inoculated with *Salmonella* at a concentration of  $10^6$  cfu ml<sup>-1</sup> and then treated with a phage mixture (SCPLX-1) containing four distinct lytic phages specific for *Salmonella enteritidis* at a concentration of  $2 \times 10^8$  pfu ml<sup>-1</sup>. The fruit slices were incubated at 5, 10 and 25°C and *Salmonella* counts determined at 0, 3, 24, 48, 120, and 168 hours after phage application. *Salmonella* was able to survive on fruit at each of the incubation temperatures although growth only occurred at 10 and 25°C and the most vigorous growth was observed on the fruit incubated at 25°C. *Salmonella* populations on experimentally contaminated melon slices were significantly reduced at each temperature by applying SCPLX-1 compared to the negative control. Conversely, the concentration of phage was reduced to undetectable limits within 48 hours of application on the apple slices due to the lower pH and as such no significant reduction in *Salmonella* was observed after phage treatment at any of the incubation temperatures.

In a similar study, Leverentz et al. [61] investigated the use of bacteriophages in combination with nisin as a biocontrol treatment for *Listeria monocytogenes* on fresh-cut produce. Melon and apple slices artificially contaminated with *Listeria monocytogenes* were treated with phage mixtures designated LMP-103 and LMP-102 which contained 14 and 6 distinct lytic

phages, respectively, specific for *L. monocytogenes*. In similar experiments, melon and apple slices artificially contaminated with *L. monocytogenes* were treated with nisin alone and in combination with LMP-102 or LMP-103. The phage mixture reduced *L. monocytogenes* populations by 2.0-4.6 log units over the control on honeydew melons. On apples, the reduction was below 0.4 log units. In combination with nisin, the phage mixture reduced *L. monocytogenes* populations by up to 5.7 log units on honeydew melon slices and by up to 2.3 log units on apples compared to the control. Nisin alone reduced *L. monocytogenes* populations by up to 3.2 log units on the honeydew melon slices and by up to 2.0 log units on apple slices. The phage titre was stable on honeydew slices but declined rapidly on apple slices. It was concluded by the authors that treatment of fresh-cut fruit with a lower pH with a phage cocktail in combination with a higher concentration of nisin was effective in controlling *L. monocytogenes*. In contrast, fruit with a neutral pH could be effectively controlled with a phage cocktail alone or in combination with nisin. LMP-102<sup>TM</sup>, developed by the biotechnology company Intralytix, has recently become the first bacteriophage-based food safety product approved by the United States Food and Drug Administration.

## 7.6 Phage biocontrol during food processing

Modi *et al.* [62] investigated the effect of a *Salmonella*-specific phage, SJ2, on the survival of *Salmonella enteritidis* on cheddar cheese made from raw and pasteurised milk, during manufacture and storage. Raw and pasteurized milk were inoculated with  $10^4$  cfu ml<sup>-1</sup> of a luminescent strain of *Salmonella enteritidis* (lux) and  $10^8$  pfu ml<sup>-1</sup> SJ2 phage. The milks were processed into cheddar cheese and samples were examined for *Salmonella enteritidis* (lux) as well as other bacteria, moulds and yeasts over a period of 99 days. Counts of *Salmonella enteritidis* (lux) decreased by 1 to 2 log units in raw and pasteurized milk cheeses containing phage. In the non-phage treated controls, *Salmonella* counts increased by about 1 log unit. *Salmonella* did not survive in pasteurized milk cheese after 89 days in the presence of phage. However, *Salmonella* counts of approximately 50 cfu g<sup>-1</sup> were observed in raw milk cheese containing phage even after 99 days of storage. The authors concluded that while addition of SJ2 to raw and pasteurised milk may be a useful adjunct to reduce the ability of *Salmonella* to survive in cheddar cheese, it does not prevent survival within the 60-day storage specification required by legislation in Canada where the study was conducted.

Whichard *et al.* [63] compared the ability of wild-type bacteriophage Felix O1 and a large-plaque variant of the same phage to suppress the growth of *Salmonella typhimurium* on chicken frankfurters, which were chosen as a model food system due to their homogenous composition. Chicken frankfurters were inoculated with 300 cfu g<sup>-1</sup> of log phase *Salmonella*

*typhimurium* and then subsequently treated either wild-type or large plaque variant Felix O1. Both phage treatments effected a 2.0 log unit reduction of *Salmonella* at room temperature. The authors recommended that further tests be conducted to determine the effect of the phage under more realistic conditions, including lower temperatures and a lower level of contamination.

Carlton et al. [64] used the broad-host range phage P100 to successfully control *L. monocytogenes* on the surface of artificially contaminated soft, red-smear cheese. Unripened cheeses were contaminated with low concentrations of *L. monocytogenes* to simulate natural contamination of  $2 \times 10^1$  cfu/cm<sup>2</sup>. During 13 days of the ripening period, P100 was applied at different concentrations in a brine wash solution. It was found that the effect of P100 on the growth of *L. monocytogenes* was dose-dependent, with low concentrations ( $1.5 \times 10^8$  pfu ml<sup>-1</sup>) able to significantly decrease *Listeria* viable counts, while higher concentrations ( $3 \times 10^9$  pfu ml<sup>-1</sup>) were able to completely eradicate *Listeria* from the surface of the cheese. It was also observed that none of the *Listeria* isolated from the cheeses receiving low concentrations of P100 revealed resistance against the phage. The authors concluded that knowledge of such properties is crucial for preparing phages and developing phage application protocols for the control of unwanted bacteria in any environment.

## 7.7 Bacteriophages to remove biofilms and bacteria from food processing environments

Bacteriophages have also been employed to remove biofilms in food processing environments. Biofilms can form on inadequately cleaned surfaces that have been contaminated with bacteria and confer protection against disinfectants and sanitizers, making the bacteria difficult to eliminate [65]. Hibma et al. [66] employed phage breeding techniques to produce a bacteriophage specific for L-forms of *Listeria monocytogenes*. The bred bacteriophage was able to prevent L-form biofilm formation on stainless steel and was as effective at inactivating L-form biofilm on stainless steel as 130 ppm lactic acid. A study by Roy et al. [67] investigated the use of listeriaphages (2671, H387, and H387-A) as a means of disinfecting contaminated stainless-steel and polypropylene surfaces. The study compared the effectiveness of using a chemical disinfectant (a quaternary ammonium compound, QUATAL), a phage solution and a combination of both phage and QUATAL in inhibiting the growth of *L. monocytogenes* which had been added to stainless-steel and polypropylene surfaces. It was found that a mixture of the three listeriaphages at concentrations up to  $3.5 \times 10^8$  pfu ml<sup>-1</sup> was about as efficient as a 20 ppm solution of QUATAL in sanitising the artificially contaminated surfaces. It was also found that the listeriaphages could maintain their infectivity when exposed to various concentrations of QUATAL (1 to 50 ppm) for up to 4 hours.

Sharma et al. [65] also investigated the use of bacteriophages to inactivate biofilms formed by two strains of *E. coli* O157:H7 ATCC 43895 and FRIK 816-3, and attached cells (not in biofilms) on stainless steel at 4°C. When used in conjunction with an alkaline cleaner containing sodium hypochlorite, bacteriophage KH1 was able to significantly reduce free *E. coli* O157:H7 cells and cells attached to stainless steel but not in biofilms. It was concluded that KH1 did not possess significant lytic or enzymatic activity at 4°C to cause lethality.

The previous studies investigated the use of bacteriophages to inactivate biofilms formed by a single species of bacteria. However, many biofilms contain a number of species of bacteria and therefore may be more difficult to eliminate using this method. Tait et al. [68] investigated the efficacy of bacteriophage as a method of eradicating single and dual-species biofilms. Single and dual species biofilms containing one or both of *Enterobacter cloacae* (NCTC 5920) and *E. agglomerans* (strain *Ent*) were inoculated with bacteriophage  $\phi$ 1.15, lytic against *E. cloacae*, or Philipstown, lytic against *E. agglomerans*, to allow 1:10, 1:100 and 1:1000 ratios of phage and biofilm bacteria. Biofilms containing both species of bacteria were not significantly affected by treatment with any concentration of phages. The use of phage cocktails containing three phages specific for *E. cloacae* was able to reduce the number of *E. cloacae* cells in a dual-species biofilm but was unable to eliminate them completely and had no effect on *E. agglomerans* cells. It was concluded by the authors that the use of bacteriophage in real life settings would be difficult due to the complex nature of mixed species biofilms and the fact that a number of bacteriophages were required to reduce even one species in a multi-species biofilm. The presence of a non-susceptible bacterial population within a biofilm could protect phage-susceptible strains from phage attack.

## **8. Environmental applications of phage biocontrol**

### **8.1 Phage biocontrol of plant pathogens**

Phage biocontrol has been investigated for agricultural practices to reduce bacterial infection of food crops by plant pathogens. Although not of direct public health significance, bacterial plant pathogens can cause widespread crop losses and have serious economic consequences [69]. Of particular concern are bacteria which cause 'bacterial spot' and blight diseases. *Xanthomonas campestris* pv. *vesicatoria* causes bacterial spot of tomatoes and peppers and has the potential to cause up to 50% losses in crop production [70]. Conventional chemical control strategies have limited efficacy due to the emergence of resistant bacteria [71], and so alternative methods of control are being investigated. Flaherty et al. [72] compared the effectiveness of a bacteriophage preparation at controlling bacterial spot on tomato caused by

*X. campestris* pv. *vesicatoria* with the commonly used chemical control strategy, a solution containing copper and mancozeb. Tomato seedlings were treated with either  $10^8$  pfu ml<sup>-1</sup> bacteriophage cocktail or copper/mancozeb which was applied during irrigation over three weeks. One week into the experiment, seedlings were inoculated with *Xanthomonas campestris* pv. *vesicatoria*. Seedlings treated with either bacteriophage or copper/mancozeb displayed significantly lower disease incidence than the untreated control. The bacteriophage treated plants produced significantly better yields than the untreated controls and copper/mancozeb treated plants. The authors concluded that due to the sensitivity of bacteriophages to environmental factors such as rain and UV light, further studies were needed to examine the effectiveness of compounds that may extend the life of bacteriophages in the field [72].

Balogh et al. [73] attempted to develop formulations to increase the efficacy and longevity of four previously isolated bacteriophages, lytic against *X. campestris* pv. *vesicatoria*. The formulations included pregelatinised corn flour (PCF) and sucrose; casecrete, sucrose and PCF; and skim milk and sucrose. When the phage cocktail was added to the above formulations, the concentration of phage populations two days after the application increased 4,700-, 38,500-, and 100,00-fold, respectively. It was observed that evening applications resulted in better control than morning applications. None of the formulations was able to improve the yield size.

McKenna et al. [69] tested the efficacy of a highly virulent and polyvalent *Streptomyces* phage ØAS1, to disinfest seed potato tubers that had been artificially inoculated with a common scab-causing streptomycete. *Streptomyces scabies* causes significant income losses to potato growers due to reduced crop yields and diminished aesthetic quality of potatoes. Symptoms of infestation by *S. scabies* include deep or superficial lesions on the surface of potatoes. *In vitro*, 'spot testing' by addition of 200µl ØAS1 containing  $1 \times 10^{12}$  pfu ml<sup>-1</sup> to *S. scabies* caused a clear plaque of lysis of 33mm in diameter. Four other strains of *S. scabies* were also susceptible to lysis by the phage. For the *in vivo* experiments, a solution containing  $10^9$  pfu ml<sup>-1</sup> of ØAS1 was used to bathe diseased potatoes over 24 hours. When planted into steam-pasteurised field soil, the potatoes produced tuber progeny with significantly reduced levels of surface lesions of scab compared with the tubers harvested from untreated controls.

## 8.2 Phage biocontrol in aquaculture

Phage biocontrol has also been investigated to control infectious diseases affecting fish and shellfish. Conventional chemotherapeutic treatment of bacterial infections in fish and shellfish has led to the development of antibiotic resistant strains [74]. Nakai & Park [75] reviewed their attempts to control two fish pathogens by phage biocontrol. The potential for phage

biocontrol of the opportunistic pathogen *Lactococcus garvieae* which infects the saltwater fish, yellowtail, was investigated. Outbreaks caused by this pathogen have resulted in serious economic damage to the aquaculture industry in Japan [75]. Phage specific for *L. garvieae*, designated PLgY and PLgW, were isolated from diseased fish and seawater obtained from fish culture cages and were administered to yellowtails experimentally infected with *L. garvieae* via intra-peritoneal injection or oral administration. It was observed that fish receiving the i.p. injection had a much higher survival rate than control fish over 24 hours. Fish that were orally administered the phage preparation via phage-impregnated feed also obtained improved protection, with phage detectable in the intestines and spleens of the treated fish 3 to 48 hours later. Importantly, no phage resistant mutants were recovered from the treated fish [75].

The same authors also investigated the potential for phage biocontrol of the freshwater fish pathogen *Pseudomonas plecoglossicida* [75]. This pathogen causes bacterial haemorrhagic ascites in the Japanese fish, ayu, and has been one of the most devastating diseases in the ayu culture industry in Japan. When ayu were experimentally infected with *P. plecoglossicida* and then treated with host-specific phage (designated PPpW-3 and PPpW-4) via phage-impregnated feed, the rate of survival increased significantly compared to the untreated controls. In addition, *P. plecoglossicida* was isolated from the kidneys of all dead fish (both phage-treated and control) but not in any of the surviving phage-treated fish. The phage preparation containing both PPpW-3 and PPpW-4 was more effective in providing protection against *P. plecoglossicida* than using either phage alone. No phage-resistant hosts were recovered from the treated fish.

Based on the studies above, the authors concluded that oral administration of phages via phage-impregnated feed was a practical method of delivering phage to a large number of fish, particularly in cases where the oral route is the major route for pathogen transmission.

Another major area of biocontrol research in aquaculture is investigating the potential for phage control of *Vibrio harveyi*, the causative agent of luminous vibriosis in shrimp. Luminous vibriosis has a high mortality rate and can be economically devastating for hatcheries. Vinod *et al.* [76] isolated *V. harveyi*-specific virulent phage from shrimp farm and hatchery waters to be used for phage biocontrol in laboratory and field trials. Laboratory trials involved creating microcosms in plastic tubs by addition of 18 day old shrimp postlarvae, filter-sterilized sea water and then experimental infection by addition of *V. harveyi*. Tubs were treated with phage either initially at day zero or at day zero and again after 24 hours. In the tubs treated with phage only once, a 2 log unit reduction in *V. harveyi* counts was observed in addition to 70% larvae survival. In the tubs that received two doses of phage, *V. harveyi* counts were reduced by 3 log units with an 80% survival rate of

larvae. In the hatchery trial, phage biocontrol was compared with antibiotic treatment. When treated daily with phage, the larvae survival rate was 86% compared with 40% survival in antibiotic-treated tanks and 17% for untreated controls.

### **8.3 Phage biocontrol in aqueous environments**

Phage biocontrol of bloom-forming cyanobacteria and red tide-causing dinoflagellates has been investigated. Cyanobacteria can occur in mass as ‘algal-blooms’ as a result of eutrophication of water bodies, causing intoxication of fish and reducing water quality [10]. Cyanophage biocontrol has been attempted with some success in controlled environments [77,78] but not in natural environments. A suggested explanation for this is that continuous lysis of sensitive cyanobacterial hosts by proliferating phage establishes population equilibrium between phage and host [79]. Gons et al. [80] observed filamentous cyanobacterial population collapse in water taken from a eutrophic lake and contained in laboratory scale enclosures (LSEs), but not in the lake from which the water was taken. It was suggested that the complex environmental parameters such as UV light, wind and sedimentation in the natural lake decreased the chance of phage attachment to a host, compared to the LSEs where UV exposure was limited and sediments were washed out.

Recently, Yang et al. [81] reviewed the potential for application of phages to control red tide in seawater. The dinoflagellates which cause red tide can result in mass mortality of fish and toxigenic illness in humans which is transmitted via consumption of contaminated shellfish. As such, research into phage biocontrol of red-tide could have important implications for public health.

Finally, Withey et al. [82] reviewed the potential for bacteriophage biocontrol in wastewater treatment processes for the purpose of reducing pathogenic bacteria and improving effluent and sludge emissions. Sludge produced by biological wastewater treatment processes contains high numbers of microorganisms including bacteria and is often reused by application to agricultural land. It has been highlighted that such use of sludge has the potential to transmit human diseases and so proper treatment and regulation is necessary, specifically regarding the required reduction in concentration of pathogens. The use of virulent bacteriophages to assist in the treatment process could reduce the costs associated with such treatment.

## **9. Conclusion**

Clearly the potential applications of phage biocontrol are enormous. While research to date has provided cause for optimism, there are a number of important practical issues that must be addressed before widespread

commercial implementation can occur. First, researchers must be confident that any phage that is being considered for biocontrol is lytic and does not confer virulence to its host. This is something that can only be achieved by complete genetic characterization. Secondly, the optimum host/phage threshold, mode of delivery, length of treatment and environmental factors such as temperature, pH and exposure to UV must be known to ensure success under 'real' conditions.

Mutation of host bacteria to resist infection by lytic bacteriophage is another threat to the success of phage therapies. Although the rate of host mutation is typically low, it is an important consideration in order to avoid proliferation of resistant strains of bacteria that would render phage biocontrol ineffective. It may therefore be necessary to use 'phage cocktails' to decrease the possibility of host mutation and also to rotate the phages used in biocontrol order to maintain efficacy. This would, however, increase the associated costs of such treatments.

Finally, perhaps the most important considerations regarding the feasibility of phage biocontrol in the food industry and environment are the perceptions and attitudes of consumers toward such methods. In light of debate surrounding genetically modified foods, it would be advantageous to conduct research into the acceptability by consumers of using viruses obtained from environments such as sewage as biocontrol agents.

## References

1. Adams, M. 1959, Bacteriophages, InterScience Publishers, New York.
2. Sharp, R., 2001, J. Chem. Technol. Biotechnol., 76, 667
3. Chibani-Chennoufi, S., Bruttin, A., Dillmann, M. and Brussow, H. 2004, J. Bacteriol., 186, 3677.
4. Wommack, K. & Colwell, R., 2000, Microbiol. Mol. Biol. Rev., 64, 69.
5. Voyles, B., 2002, The Biology of Viruses, McGraw Hill, New York.
6. Zillig, W., Reiter, WD., Palm, P., Gropp, F., Neumann, H. and Rettenberger, M. 1988, The Bacteriophages, vol 1, R. Calendar (Ed.), Plenum Press, New York.
7. Schleper, C., Kubo, K. and Zillig, W. 1992, Proceedings of the National Academy of Sciences of the United States of America, 89, 7645.
8. Hogg, S., 2005, Essential Microbiology, John Wiley and Sons, Ltd., Chichester
9. Murphy, F., Fauquet, C., Bishop, D., Ghabrial, S., Jarvis, A., Martelli, G., Mayo, M. and Summers, M., (Ed.) 1995, Virus Taxonomy, Springer-Verlag Wien, New York.
10. Prescott, L., Harley, J. and Klein, D. 2005, Microbiology, McGraw Hill, New York.
11. Madigan, M. and Martinko, J., 2006, Brock Biology of Microorganisms, Pearson Prentice Hall.
12. Barbalho, T. C. F., Almeida, P. F., Almeida, R. C. C. and Hofer, E. 2005, Food Cont., 16, 211.

13. Goodridge, L., Chen, J. and Griffiths, M. 1999, *Appl. Environ. Microbiol.* 65, 1397.
14. Oda, M., Morita, M., Unno, H. and Tanji, Y. 2004, *Appl. Environ. Microbiol.*, 70, 527.
15. Turner, S. and Lewis, G. 1995, *Water Sci. Technol.*, 31, 85.
16. McLaughlin, M. & Rose, J., 2006, *Estuar Coast*, 29, 246.
17. Mocé-Llivina, L., Lucena, F. and Jofre, J., 2005, *Appl. Environ. Microbiol.*, 71, 6838.
18. Havelaar, A. H., Van Olphen, M. and Drost, Y. C. 1993, *Appl. Environ. Microbiol.*, 59, 2956.
19. Mandilara, G., Smeti, E., Mavridou, A., Lambiri, M., Vatopoulos, A. and Rigas, F. 2006, *FEMS Microbiol. Lett.*, 263, 119.
20. Summers, W. 2001, *Ann. Rev. Microbiol.*, 55, 437
21. Sulakvelidze, A., Alavidze, Z. and Morris, G., Jr 2001, *Antimicrob. Agents Chemother.*, 45, 649.
22. Alisky, J., Iczkowski, K., Rapoport, A. and Troitsky, N. 1998, *J. Infect.*, 36, 5.
23. Carlton, R. 1999, *Arch. Immunol. Ther. Exp.*, 47, 267.
24. Duckworth, D and Gulig, P, 2002, *BioDrugs*, 16, 57.
25. Inal, J. 2003, *Arch Immunol. Ther. Exp.*, 51, 237.
26. Kropinski, A. 2006, *Can. J. Infect. Dis. Med. Microbiol.*, 17, 297.
27. Skurnik, M. and Strauch, E. 2006, *Int. J. Med. Microbiol.*, 296, 5.
28. Goodridge, L. 2004, *Trends biotechnol.*, 22, 384
29. Todd, E. 2001, *Guide to Foodborne Pathogens*, R. Labbé, and S. García (Ed.), John Wiley and Sons Inc., New York
30. Hudson, J., Billington, C., Carey-Smith, G. and Greening, G., 2005, *J. Food Protect.*, 68, 426.
31. Greer, G., 2005, *J. Food Protect.*, 68, 1102.
32. Hall, G. and Kirk, M. 2005, *Foodborne Illness in Australia: Annual Incidence Circa 2000*, Australian Government Department of Health and Ageing Canberra.
33. Atterbury, R., Connerton, P., Dodd, C., Rees, C. and Connerton, I. 2003, *Appl. Environ. Microbiol.*, 69, 4511.
34. Atterbury, R., Dillon, E., Swift, C., Connerton, P., Frost, J., Dodd, C., Rees, C. and Connerton, I. 2005, *Appl. Environ. Microbiol.*, 71, 4885.
35. Fiorentin, L., Vieira, N. and Barioni Jr, W. 2005, *Avian Pathol.*, 34, 258.
36. Loc Carrillo, C., Atterbury, R., El-Shibiny, A., Connerton, P., Dillon, E., Scott, A. and Connerton, I. 2005, *Appl. Environ. Microbiol.*, 71, 6554.
37. Goode, D., Allen, V. and Barrow, P. 2003, *Appl. Environ. Microbiol.*, 69, 5032.
38. Atterbury, R., Connerton, P., Dodd, C., Rees, C. and Connerton, I. 2003, *Appl. Environ. Microbiol.*, 69, 6302.
39. Higgins, J., Higgins, S., Guenther, K., Huff, W., Donoghue, A., Donoghue, D. and Hargis, B. 2005, *Poult. Sci.*, 84, 1141.
40. Kudva, I., Hatfield, P. and Hovde., C. 1996, *J. Clin. Microbiol.*, 34, 431.
41. Griffin, P. and Tauxe, R. 1991, *Epidemiol. Rev.*, 13, 60.
42. Ackers, M., Mahon, B., Leahy, E., Goode, B., Damrow, T., Hayes, P., Bibb, W., Rice, D., Barrett, T., Hutwagner, L., Griffin, P. and Slutsker, L. 1998, *J. Infect. Dis.*, 177, 1588.
43. Chapman, P., Siddons, C., Cerdan Malo, A. and Harkin, M. 1997, *Epidemiol. Infect.*, 119, 245.

44. Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Akio, O. and Yanagawa, H. 1999, *Am. J. Epidemiol.*, 150, 787.
45. Johnson, R., Wilson, J., Michel, P., Rahn, K., Renwick, S., Gyles, C. and Spika, J. 1999, *Escherichia coli* O157 in Farm Animals, Stewart, C. and Flint, H. CABI Publications, New York.
46. Janisiewicz, W., Conway, W., Brown, M., Sapers, G., Fratamico, P. and Buchanan, R. 1999, *Appl. Environ. Microbiol.*, 65, 1.
47. Bach, S., McAllister, T., Veira, D., Gannon, V. and Holley, R. 2003, *Anim. Res.*, 52, 89.
48. Raya, R., Varey, P., Oot, R., Dyen, M., Callaway, T., Edrington, T., Kutter, E. and Brabban, A. 2006, *Appl. Environ. Microbiol.*, 72, 6405.
49. O'Flynn, G., Ross, R., Fitzgerald, G. and Coffey, A. 2004, *Appl. Environ. Microbiol.*, 70, 3417.
50. Kudva, I., Jelacic, S., Tarr, P., Youderian, P. and Hovde, C. 1999, *Appl. Environ. Microbiol.*, 65, 3767.
51. Hudson, J., Mott, S. and Penney, N. 1994, *J. Food Protect.*, 57, 204.
52. Dykes, G. and Moorhead, S. 2002, *Int. J. Food Microbiol.*, 73, 71.
53. Whitman, P. and Marshall, R. 1971, *Appl. Microbiol.*, 22, 220.
54. Patel, T. and Jackman, D. 1986, *Appl. Environ. Microbiol.*, 51, 446.
55. Greer, G. and Dilts, B. 1990, *Int. J. Food Microbiol.*, 10, 331.
56. Beuchat, L. 2002, *Microb. Infect.*, 4, 413.
57. Burnett, S. and Beuchat, L. 2000, *J. Ind. Microbiol. Biotechnol.*, 25, 281.
58. Bowen, A., Fry, A., Richards, G. and Beuchat, L. 2006, *Epidemiol. Infect.*, 134, 675.
59. Mokgatla, R., Brozel, V. and Gouws, P. 1998, *Lett. Appl. Microbiol.*, 27, 379.
60. Leverentz, B., Conway, W., Alavidze, Z., Janisiewicz, W., Fuchs, Y., Camp, M., Chighladze, E. and Sulakvelidze, A. 2001, *J. Food Prot.*, 64, 1116.
61. Leverentz, B., Conway, W., Camp, M., Janisiewicz, W., Abuladze, T., Yang, M., Saftner, R. and Sulakvelidze 2003, *Appl. Environ. Microbiol.*, 69, 4519.
62. Modi, R., Hirvi, Y., Hill, A. and Griffiths, M. 2001, *J. Food Prot.*, 64, 927.
63. Whichard, J., Sriranganathan, N. and Pierson, F. 2003, *J. Food Prot.*, 66, 220.
64. Carlton, R., Noordman, W., Biswas, B., De Meester, E. and Loessner, M. 2005, *Regul. Toxicol. Pharm.*, 43, 301.
65. Sharma, M., Ryu, J.-H. and Beuchat, L. 2005, *J. Appl. Microbiol.*, 99, 449.
66. Hibma, A., Jassim, S. and Griffiths, M. 1997, *Int. J. Food Microbiol.*, 34, 197.
67. Roy, B., Ackermann, H., Pandian, S., Picard, G. and Goulet, J. 1993, *Appl. Environ. Microbiol.*, 59, 2914.
68. Tait, K., Skillman, L. and Sutherland, I. 2002, *Biofouling*, 18, 305.
69. McKenna, F., El-Tarabily, Hardy, G. and Dell, B. 2001, *Plant Pathol.*, 50, 666.
70. Pohronezny, K. and Volin, R. 1983, *HortSci.*, 18, 69.
71. Marco, G. and Stall, R. 1983, *Plant Dis.* 67, 779.
72. Flaherty, J. E., Jones, J. B., Harbaugh, B. K., Somodi, G. C. and Jackson, L. E. 2000, *HortSci.* 35, 882.
73. Balogh, B., Jones, J., Momol, M., Olson, S., Obradovic, A., King, P. and Jackson, L. 2003, *Plant Dis.*, 87, 949.
74. Kusuda, R. and Kawai, K. 1998, *Fish Pathol.* 33, 22.
75. Nakai, T. and Park, S. 2002, *Res. Microbiol.*, 153, 13.

- 
76. Vinod, M., Shivu, M., Umesha, K., Rajeeva, B., Krohne, G., Karunasagar, I. and Karunasagar, I. 2006, *Aquaculture*, 255, 117.
  77. Safferman, R. and Morris, M. 1964, *J. Am. Water Works Association*, 56, 1217.
  78. Jackson, D. and Sladeczek, V. 1970, *Yale Sci. Mag*, 44, 16.
  79. Padan, E. and Shilo, M. 1973, *Bacteriol. Rev.*, 37, 343.
  80. Gons, H., Ebert, J., Hoogveld, H., van den Hove, L., Pel, R., Takkenberg, W. and Woldringh, C. 2002, *Ant. van Leewenhoek Int. J. Gen. Mol. Microbiol.* 81, 319.
  81. Yang, X., Zheng, T., Su, J., Yu, Z. and Song, X. 2005, *Chin. J. Appl. Environ. Biol.*, 11, 651.
  82. Withey, S., Cartmell, E., Avery, L. and Stephenson, T. 2005, *Sci. Total Environ.*, 339, 1