Isolation and characterisation of bacteriophages for the biocontrol of *Enterococcus faecalis* and *Escherichia coli* on surfaces and in milk

Sarah McLean

A thesis submitted for the degree of
Doctor of Philosophy

2011

Environment and Biotechnology Centre
Faculty of Life and Social Sciences
Swinburne University of Technology
Melbourne, Australia
Abstract

Bacteriophages have been used to treat bacterial infections since their discovery almost 100 years ago. Furthermore, phages have been increasingly investigated for the biocontrol of bacterial pathogens in a diverse range of environmental applications. The major aims of this research were to isolate and characterise bacteriophages for the biocontrol of two organisms, Enterococcus faecalis and Escherichia coli, on surfaces and in milk. While normally harmless, both organisms are capable of causing disease. *E. faecalis* and *E. coli* are commonly isolated from foods of animal origin including dairy products. Furthermore, *E. faecalis* and *E. coli* contaminated surfaces have been indirectly linked to cases of human illness. While many studies have investigated using phages to control *E. coli* O157 on surfaces and in foods, fewer studies have considered other serotypes. In contrast, there are no studies reporting the use of phages in the biocontrol of *E. faecalis*.

In the current study, phages infecting *E. faecalis* and *E. coli* were isolated from environmental sources in Victoria, Australia, including sewage and landfill leachate. Selected isolates were characterised by biological and genetic means. Four enterococcal phages, designated φSUT1, φSUT3, φSUT4 and φSUT6 were found by electron microscopy to belong to the Siphoviridae. Three coliphages, φEC6, φEC9 and φEC11, were morphologically distinct. The coliphage isolates possessed a wider host range compared to the enterococcal phages, including pathogenic strains of *E. coli*.

The ability of the φSUT phages to control their enterococcal hosts on a range of surfaces commonly found in hospitals was assessed. φSUT1 achieved significant reductions in *E. faecalis* on all surfaces following 120 minutes of incubation at ambient temperature. The remaining phages were less effective in reducing their respective hosts on all surfaces. Phage cocktails comprising the φEC phages were used to similarly disinfect glass slides and stainless steel coupons contaminated with one of four strains of *E. coli*. When applied at a concentration of $10^8$ PFU/mL, the phage cocktails eliminated each strain within 10 minutes of application. At lower concentrations, the effect was largely dose and time-dependent.
Abstract

Given the association of \textit{E. faecalis} and \textit{E. coli} with dairy products it was determined that milk, and in particular raw milk, could be a potential novel application for phage biocontrol. While the microbiota of raw milk has been studied extensively worldwide, there is little published information regarding the quality of raw milk produced in Australia. This study found Australian raw milk to contain pathogens including \textit{Salmonella} spp., as well as indicators of faecal contamination including coliforms, \textit{Enterococcus} spp. and \textit{E. coli}. There was considerable variation in the microbiological quality of raw milk samples obtained from three sources indicating that a wider study of Australian milk over time could be beneficial. Given the incidence of \textit{Enterococcus} spp. and \textit{E. coli} in raw milk in the current study, the ability of phages to control these organisms in raw milk was investigated.

\(\phi\)SUT1 and \(\phi\)SUT4 were selected to control two strains of \textit{E. faecalis} in UHT and pasteurized milk. The two phages varied considerably in their ability to reduce their respective hosts. \(\phi\)SUT4 initially inhibited the growth of a vancomycin-resistant \textit{Enterococcus} (VRE) in UHT milk but growth resumed over time. While \(\phi\)SUT1 significantly reduced \textit{E. faecalis} in UHT milk at 10 and 25°C, a complete reduction was not achieved. Both phages were less effective in reducing their hosts in pasteurized milk, although phage titres remained stable. The \(\phi\)EC phages were combined in a cocktail to control the growth of four strains of \textit{E. coli} in raw milk. The cocktail eliminated \textit{E. coli} K12, G106 and O127:H6 in raw milk at both 5-9°C and 25°C. In milk contaminated with \textit{E. coli} O5:NM, the phage cocktail achieved a complete reduction initially but \textit{E. coli} increased at both 5-9°C and 25°C over time. The results of the raw milk studies were promising but further investigations should be conducted to determine the effect of differences in milk composition and microbiota on the efficacy of the phages.

The findings from this research suggested that phage biocontrol of \textit{E. faecalis}, which has not been previously reported, can be successful if the appropriate phages are used. Furthermore, the coliphages described were effective in reducing \textit{E. coli}, including pathogenic strains, on surfaces and in milk. Therefore, further study of these phages in a wider range of applications is warranted.
Acknowledgements

There are many people who I would like to thank for their contributions to my project. Foremost, I am sincerely indebted to my supervisors, Enzo Palombo and Louise Dunn, who encouraged me to pursue a PhD in the first place. They have been unwavering in their encouragement and support and I am grateful for the many opportunities they provided to travel and present my work to the wider scientific community. I thank Enzo for sharing his passion for microbiology – it was contagious (haha). I sincerely appreciate his enthusiasm for my project and the many hours he spent listening to ideas and complaints and providing advice, support and direction. I thank Louise for encouraging my interest in research during my undergraduate and honours studies and for always making the time to listen and provide advice and encouragement over coffee.

I wish to thank Chris Key, Soula Mougos and, in particular, Ngan Nguyen, for sharing their technical expertise with me. I also acknowledge Jason Mackenzie (La Trobe University) and Joan Clark (University of Melbourne) for performing the transmission electron microscopy reported in Chapter Four.

I am lucky to have made so many great friends during my time at Swinburne. In order of appearance, I wish to thank Kelly Walton, Carly Gamble, Jacqui Bermingham, Pete Gollan, Elizabeth Nelson, Mark Ziemann and Elisa Hayhoe for the camaraderie, support, advice and entertainment they provided. It is these enduring friendships that helped make my PhD experience all the more enjoyable.

I am sincerely grateful to my family for always encouraging me to pursue my goals. Mum and Dad, especially, have gone above and beyond over my (many) years of study without ever expecting anything in return. Finally, I dedicate this thesis to my partner, Danniel Smith, without whom I would never have made it. Thank you for your love, patience and support during this rollercoaster journey and for always believing in me.
Declaration

I hereby declare, that to the best of my knowledge, this thesis contains neither material which has been accepted for the award of any other degree or diploma, or any material previously published or written by another person, except where due reference is made in the text of the thesis. Where the work is based on joint research or publications, I have disclosed the relative contributions of the respective workers or authors.

Sarah McLean
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<td>--------------</td>
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<td></td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
<td></td>
</tr>
<tr>
<td>ARE</td>
<td>Antibiotic resistant bacteria</td>
<td></td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
<td></td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered peptone water</td>
<td></td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
<td></td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohaemorrhagic <em>E. coli</em></td>
<td></td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>E. coli</em></td>
<td></td>
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<tr>
<td>ERIC</td>
<td>Enterobacterial repetitive intergenic consensus</td>
<td></td>
</tr>
<tr>
<td>FSANZ</td>
<td>Food Standards Australia New Zealand</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>Gravitational acceleration</td>
<td></td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobase pairs</td>
<td></td>
</tr>
<tr>
<td>mAmps</td>
<td>Milliampere</td>
<td></td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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</tr>
<tr>
<td>PCA</td>
<td>Plate count agar</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
<td></td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse field gel electrophoresis</td>
<td></td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
<td></td>
</tr>
<tr>
<td>PPC</td>
<td>Post-pasteurization contamination</td>
<td></td>
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<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Suspension medium</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SPC</td>
<td>Standard plate count</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptone soya agar</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra high temperature</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>USFDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>Zinc chloride</td>
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</table>
1.1. Introduction

Bacteriophages (phages) have been used to treat bacterial infections since their discovery almost 100 years ago. Although phage therapy diminished following the advent of antimicrobial drugs, interest has been renewed with the rising prevalence of antibiotic resistant bacteria (Summers, 2001). Furthermore, phages have been increasingly investigated for the biocontrol of bacterial pathogens in a diverse range of environmental applications related to food production, agriculture and aquaculture. Reasons for this revival include the continued burden of bacterial illness, the emergence of new pathogens and the increasing resistance of bacterial pathogens to conventional disinfectants (Joseph et al. 2001; Mokgatla et al., 1998).

There are a number of studies that have isolated and characterised bacteriophages for biocontrol of pathogens in foods (Greer, 2005; Hudson et al., 2005). Fewer studies have focused on environmental biocontrol of nosocomial pathogens. This thesis investigated using phages to control two potential pathogens, Enterococcus faecalis and Escherichia coli, on surfaces and in milk. While taxonomically distinct, both organisms are associated with the gastrointestinal tract of mammals, and, while normally harmless, are capable of causing disease (Franz et al., 1999; Croxen and Finlay, 2010). Both organisms are also associated with foods of animal origin including dairy products made from raw milk. Furthermore, E. faecalis and E. coli associated illnesses have been indirectly linked to contaminated surfaces (Huycke et al., 1998; Viazis et al., 2011a).

While many studies have investigated phages for controlling E. coli O157 on surfaces and in foods, fewer studies have focused on other serotypes. Australia has a low incidence of reported E. coli O157 infections compared to other countries including Japan, Argentina and the US (Sakuma et al., 2006; Leotta et al., 2008; Centres for Disease Control and Prevention, 2009). Conversely, pathogenic E. coli belonging to a range of non-O157 serotypes have been implicated in cases of disease in Australia (Combs et al., 2005). Furthermore, while there are several recent papers that describe the use of phages in the treatment of enterococcal infection (Biswas et al., 2002; Letkiewicz et al., 2009) there are no reports of phages being used in the
environmental biocontrol of *E. faecalis* despite a continuing increase in enterococcal-associated morbidity worldwide (Franz *et al*., 2003).

### 1.2. Aim

The major aims of this project were to isolate and characterise phages infecting *E. faecalis* and *E. coli* and to test the ability of these phages to control their hosts on surfaces and in milk under different conditions. To achieve this, a series of tasks were undertaken, which were designed to:

i. Investigate different sources (environmental and clinical) and methods for the isolation of bacteriophages and to characterise selected isolates;

ii. Determine the ability of characterised isolates to reduce *Enterococcus* sp. and *E. coli* on a range of surfaces;

iii. Investigate the prevalence of *Enterococcus* spp. and *E. coli* contamination in Australian raw milk; and

iv. Assess the capacity of the characterised phages to control *Enterococcus* sp. and *E. coli* in a range of bovine milk.

### 1.3. Thesis outline

Section 1.2 identified the four areas of investigation designed to address the aims of the project. Chapter Two provides a review of the literature in order to introduce the major concepts relevant to the project. After a brief introduction to the discovery of bacteriophage, their classification and modes of replication, the review describes the different areas of phage biocontrol research with a specific focus on food-based applications. The review concludes with an overview of the major features of the organisms to be targeted in the current study, *E. faecalis* and *E. coli*. This is followed, in Chapter Three, by a description of the methods undertaken in the current study.

Chapter Four outlines the isolation and characterisation of bacteriophages required for use in subsequent studies of phage biocontrol. The characterised phages were
tested for their ability to control *E. coli* and *E. faecalis* on a range of surfaces under different conditions, the findings of which are described in Chapter Five.

Chapter Six describes a survey of the microbiological quality of selected Australian raw milk samples with specific focus on the prevalence of *Enterococcus* spp. and *E. coli*. Pasteurized milk samples were similarly tested for the presence of these organisms. In addition, genetic fingerprinting using ERIC PCR was employed to analyse *E. coli* contaminants recovered from a pasteurized milk product involved in a recall.

Chapter Seven presents the findings of a number of studies in which the phages characterised in Chapter Four were used to control *E. coli* and *E. faecalis* in different types of milk under a range of conditions.

Finally, Chapter Eight presents a summary of this project’s major findings as well as a general discussion of the broader topic of phage biocontrol and the scope for further research.
CHAPTER 2

Literature Review
2.1 Bacteriophage history

Bacteriophages (phages) are viruses that infect bacteria. The effects of phage were first reported in 1859 by British physician Ernest Hankin, who observed the bactericidal effects of filtered water from the river Ganges against *Vibrio cholerae* (Sharp, 2001). However, the discovery of phages is usually credited to two other researchers, Frederick Twort (1915) and Felix D’Herelle (1917), who independently described phages as filterable, transmissible agents capable of bacterial lysis (Sharp, 2001). While Twort did not pursue his discovery, D’Herelle continued his work with bacteriophages (a term he coined which translates to ‘bacteria eater’) for the rest of his career. He immediately saw the potential for phages to be used as therapeutic agents and became the first proponent of ‘phage therapy’ which he hoped would hold the cure for bacterial diseases.

D’Herelle’s initial field experiments focused on treating avian typhosis caused by *Salmonella gallinarum*. Before conducting human trials, D’Herelle tested the safety of his phage preparations on himself by ingesting and injecting them. His first human trials involved treating bacillary dysentery and cholera. He also had success in treating a few cases of bubonic plague using *Yersinia* phage. These case studies were published in a widely read periodical and interest in phage therapy research subsequently flourished (Summers, 2001). The applications of phage therapy were diverse, ranging from treatment of wound infections to gastrointestinal and respiratory infections. However, many trials yielded variable results. There were many factors that contributed to this inconsistency, including the limited scientific rigour applied to the experimental design. Although consistent with the standards of the time, there were few reported cases where rigorous studies, such as double-blind trials, were carried out. Another issue was the limited understanding of phage biology which was still being debated. As such, temperate phage, which do not consistently produce cell lysis, were possibly used and methods used to propagate and “stabilise” phages may have resulted in reduced viability of preparations (Summers, 2001). For example, many large pharmaceutical companies began preparing commercial quantities of phages for treatment of a range of infections. Some preparations were advertised as containing up to 100 different phages.
However, many of these polyvalent preparations were often found to be inactive (Summers, 2001).

Figure 2.1 Advertisement for a polyvalent bacteriophage preparation for treating gastrointestinal infections (Image reproduced from Hausler, 2006. Original source: Wiener Klinische Wochenschrift, Springer, Vienna, Austria).

These issues along with the commencement of World War II and the advent of penicillin, which was far easier to produce and had a much broader spectrum compared to phage therapy, led to the decline in phage therapy research in Western countries (Summers, 2001). However, it continued unabated in the former Soviet Union, where phage therapy continues to be used routinely. In particular, the Eliava Institute in Tbilisi, Georgia, became, and remains, the principal institute for phage therapy globally (Kutateladze and Adamia, 2008).

Although phage therapy was discontinued in the West, research into phage virology has been integral to the development of genetics and molecular biology due to their simple genomes and ability to be rapidly propagated using simple media and bacterial cultures (Pennazio, 2006). Research involving primarily the T-series phages of *Escherichia coli* helped to identify features of DNA replication, recombination and transduction and many molecular techniques were derived from this research (Cairns *et al.*, 2007). Furthermore, the past 20 years have seen a resurgence of interest in Western countries in phage therapy research efforts to combat the rise in antibiotic resistant bacteria (Chanishvili *et al.*, 2001).
2.2 Bacteriophage taxonomy and classification

Phages are the most ubiquitous life form on the planet, with estimates of their total population reaching $10^{31}$ (Chibani-Chennoufi et al., 2004a). The International Committee on Taxonomy of Viruses (ICTV) conventions group viruses sharing characteristics together in “orders” which can be broken down into subgroups or “families”. These families can be further broken down into “genera” based on genome configuration and size (Nelson, 2004). Figure 2.2 identifies each of the families comprising the phages.

![Bacteriophage morphologies](image)

**Figure 2.2** Bacteriophage morphologies. Adapted from Voyles (2002).

Phage are genetically diverse, with approximately half of newly sequenced phage genes having no known homologues and phage genomes varying in size by at least two orders of magnitude (Petty et al., 2007). It has been suggested that the current taxonomic system fails to classify half of all the phages for which the entire genomic sequence is known. More than 90% of phages are tailed and belong to the order
Caudovirales. These phages possess double-stranded DNA (dsDNA) genomes and are further divided into families based on their tail length. The Myoviridae comprise phages with long, contractile tails; the Siphoviridae comprise those with long, noncontractile tails and the Podoviridae comprise phages with very short tails (Voyles, 2002) (Figure 2.2). These phages possess icosahedral heads made of protein which encapsulates the DNA. Most dsDNA phages have genomes larger than 15 kbp due to the virion structure and assembly genes comprising at least 15 kbp of genome space. The Myoviridae typically have larger genomes (>125 kbp) while Siphoviridae have genomes longer than 20 kbp (Hatfull, 2008).

2.3 Bacteriophage replication cycles

Bacteriophages can be broadly divided into two groups, virulent (lytic cycle of replication) or temperate (lytic and lysogenic cycles of replication). A third and less common group comprises the filamentous phages, which cause persistent infection of bacterial hosts without lysis or integration of genetic material into the host chromosome (Harper and Kutter, 2001). The method of replication is integral to determining suitability of phage for certain applications.

2.3.1 Bacteriophage lytic cycle

The general stages of virulent bacteriophage replication are as follows (Figure 2.3):

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 (Madigan et al., 2003).

![Figure 2.3 Representation of the phage lytic cycle](image)

2.3.2 Bacteriophage lysogenic cycle

In the lysogenic cycle, the attachment and penetration processes are the same as for the lytic cycle. However, once inside the host cell, the phage genome integrates with that of the host and becomes a dormant prophage (Hogg, 2005). This results in the host bacterium producing a copy of the phage genome, along with its own DNA each time it undergoes cell division. 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 as ultraviolet light, the dormant prophage is induced to carry out the lytic cycle (Hogg, 2005).


2.4 Applications of lytic bacteriophages

2.4.1 Phage typing

The ability of virulent phages to lyse specific bacterial strains and to produce plaques in bacterial lawns grown on laboratory media has been employed for the characterisation and identification of bacteria involved in disease outbreaks (Cookson, 1996). 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. Phage typing schemes have been developed for identification and differentiation of Staphylococcus aureus, Salmonella spp., E. coli O157, Listeria and Campylobacter. The Central Public Health Laboratory in London maintains and distributes the Basic Set of Phage and their propagation strains (Sharp, 2001).

2.4.2 Detection of bacteria

2.4.2.1 Clinical applications

Many rapid tests have been devised using phages to identify clinically important bacteria. One example, FASTPlaqueTB assay (FPTB), is a cost-effective, rapid diagnostic tool for identifying M. tuberculosis in sputum samples and is used primarily in developing countries (Monk et al., 2010). The assay uses a bacteriophage preparation with a broad host range among the Mycobacterium spp. The phage preparation is added to a biological sample and the suspension is incubated for a short period to allow infection. Following incubation, a virucidal agent is added to inactivate extracellular phages. The suspension is subsequently mixed with a M. smegmatis indicator culture which is then added to soft agar and set on an agar base plate. Following incubation, plaques within the bacterial lawn are indicative that the target bacteria from the original sample have lysed resulting in release of phage progeny. The released phages are then able to infect M. smegmatis to produce a plaque. PCR has enabled this assay to be used to detect other species of Mycobacteria in a wider range of samples (Stanley et al., 2007; Monk et al., 2010).

Another rapid assay employs bacteriophages to rapidly identify methicillin resistance
in *Staphylococcus aureus* isolates when the use of molecular diagnostics is not practicable. The assay is based on the premise that *Staphylococcus* cells must be viable in order for phages to replicate, and therefore detection of phages following infection of the target cell is also indicative of bacterial growth. The assay is simple in that cells obtained from a swab can be challenged with different antibiotics and bacteriophages will only replicate and increase in number if the cells are resistant (Monk *et al.*, 2010).

### 2.4.2.2 Detection of foodborne bacteria

Bacteriophages have also been used in diverse ways to detect foodborne bacterial pathogens. Following a similar principle to phage typing, virulent phages were used by Barbalho *et al.* (2005) to confirm presumptive *Listeria* spp. isolated from the gloves and hands of workers involved in handling chicken carcasses at a food processing plant. Suspect bacterial colonies were grown as a bacterial lawn which was then ‘spot-tested’ against different virulent *Listeria*-specific phages. Plaque formation in the bacterial lawn following incubation demonstrated susceptibility of the bacteria to the phage, and was considered to be a positive 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.

Lytic phages have been used as biosensors to detect foodborne pathogens including *E. coli* O157 and enterotoxins produced by *Staphylococcus aureus* in experimentally inoculated foods. Goodridge *et al.* (1999) 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 were 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 limits for fluorescence microscopy in milk samples was $10^3$ CFU/mL, and between $10^1$ CFU/mL and $10^2$ CFU/mL for flow cytometry. For beef samples, the detection limit by epifluorescence microscopy was $10^3$ CFU/25g and 2.2 CFU/25g for flow cytometry. Although the sensitivity of the
assay was poor, the authors suggested that it could be improved with a longer enrichment period.

Viable but non-culturable (VBNC) *E. coli* O157:H7 has also been the target for phage biosensors, due to their ability to avoid detection by conventional culture. A recombinant form of phage PP01 containing green fluorescent protein (GFP) was constructed by Oda *et al.* (2004) for one such assay. The recombinant phage was used to infect VNBC *E. coli* O157:H7 which increased the fluorescence intensity of the culture when examined by fluorescent microscopy. The authors also noted that inclusion of non-susceptible *E. coli* O157:H7 cells in the culture did not reduce the sensitivity or selectivity of the assay. Bioluminescence phage biosensors may also be used to detect bacterial pathogens in food samples. Recombinant reporter phages containing luciferase genes such as *luxAB* have been used to transfer these bioluminescent genes to target bacterial cells during infection. The target cells that contain these genes can then be detected using a luminometer. This technique has been used successfully to detect low levels of *Salmonella* (Modi *et al.*, 2001) and *Listeria monocytogenes* (Loessner, 2005) in food samples.

### 2.4.3 Indicators of viral and faecal contamination

Phages have long been considered potential indicators of viral and faecal contamination of water sources. A range of different phages have been investigated for this purpose. F-RNA bacteriophages, a group of phages which attach 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 with the advantage that they are easier to culture (Turner and Lewis, 1995). Havelaar *et al.* (1993) 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. However, a more recent study by Santiago-Rodriguez *et al.* (2010) suggested that enterococcal phages may be better indicators, as other current and proposed indicators, including coliphages, may be limited by their survival characteristics, geographical specificity and prevalence among animal hosts. Santiago-Rodriguez *et al.* (2010) isolated enterococcal phages,
designated ‘enterophages’, from a variety of sources including domestic sewage, human faeces and water. The enterophages were compared with coliphages across a range of characteristics to determine whether they would be a more suitable marker of human faecal pollution. While coliphages were detected in 12 out of 15 samples of cattle faeces, no enterophages were detected. This indicated that enterophages may be more specific to human faeces and would therefore be a better indicator of human faecal pollution. The enterophages were demonstrated to survive for shorter periods of time in fresh and marine water at all temperatures tested compared with coliphages. It was also suggested that enterophages could be used as surrogates of enteric viruses due to their similar patterns of resistance to primary and tertiary treatments and in their die-off rates in fresh and marine waters.

In addition to their potential for use as indicators of recreational and potable water quality, it has also been suggested that somatic coliphages could be good indicators of wastewater and sludge quality intended for agricultural reuse due to their ability to survive chlorination during wastewater treatment (Mandilara et al., 2006).

### 2.5 Bacteriophage biocontrol

A growing focus of phage-related research is in the area of ‘phage biocontrol’, which follows similar principles to bacteriophage therapy but is focused on environmental, rather than clinical, applications. Suggested reasons for this growing shift towards biocontrol include the less complicated regulatory hurdles for phage-based biocontrol products compared with phage therapy products. There are several phage-based biocontrol products currently in use in the agricultural and food industries with approval of the United States Food and Drug Administration (FDA) or the United States Environmental Protection Agency (EPA) (Monk et al., 2010). Phage biocontrol applications have been investigated for areas as diverse as food safety, agriculture, aquaculture and wastewater treatment. The following section will review the research in these areas, with the subsequent sections focused on phage biocontrol applications in the food industry.
2.5.1 Waste water treatment

Sludge produced by biological wastewater treatment processes contains high numbers of microorganisms including an array of pathogenic bacteria. Approximately 7 million tonnes of sludge are produced every year in the USA alone. The most practical solution to utilizing this waste is application to agricultural land (Hettenbach et al., 1998). However, it has been highlighted that such use of sludge has the potential to transmit human diseases and therefore reduction in the concentration of pathogens is necessary before it can be used for agricultural purposes (Withey et al., 2005). The use of virulent bacteriophages to assist in the treatment process could reduce the costs associated with such treatment. Due to the complex ecology of sludge and wastewater, the success of phages in reducing bacterial pathogens is dependent on the prevalence and diversity of pathogens present. While it would be impossible to use phages alone to reduce all pathogens, it could be used in combination with biological sludge stabilization processes to target and reduce specific species (Withey et al., 2005).

2.5.2 Phage biocontrol of aquacultural pathogens

Pathogens of fish and crustaceans cause significant economic damage to aquacultural industries worldwide and chemotherapeutic treatment of bacterial infections has led to the development of antibiotic resistant strains (Kusuda and Kawai, 1998). Nakai and Park (2002) used phages to control the fish pathogens, Lactococcus garvieae and Pseudomonas plecoglossicida, which infect saltwater and fresh water fish, respectively and which have been implicated in numerous outbreaks of disease among fish in Japan. Phages specific for L. garvieae and P. plecoglossicida were isolated from diseased fish and seawater obtained from culture cages. Yellowtail fish were experimentally infected with L. garvieae and phages were subsequently administered by intraperitoneal (i.p.) injection or orally. It was observed that fish receiving the i.p. injection had a much higher survival rate than control fish over 24 hours and that fish that were given phage orally via phage-impregnated feed also obtained improved protection. Similarly, ayu fish were experimentally infected with P. plecoglossicida and then given phage-impregnated feed which significantly increased the rate of survival 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. Based on these results, it was concluded that oral administration of phages was a practical and effective method of delivering phage to a large number of fish, particularly in cases where the oral route is the major route for pathogen transmission (Nakai and Park, 2002).

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. Several studies have described the isolation and characterisation of V. harveyi-specific virulent phages from hatchery waters for use as biocontrol agents (Vinod et al., 2006; Shivu et al., 2007; Crothers-Stomps, 2010). Vinod et al. (2006) conducted laboratory trials by infecting shrimp postlarvae in tubs containing filter sterilized sea water followed by treatment with phages either once at day zero or once at day zero and again after 1 day. In the tubs treated with phages 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 field trials carried out in a hatchery, phage biocontrol was compared with antibiotic treatment. When treated daily with phages, the larvae survival rate was 86% compared with 40% survival in antibiotic-treated tanks and 17% for untreated controls.

### 2.5.3 Phage biocontrol of agricultural pathogens

Phage biocontrol has been explored for agricultural practices to reduce bacterial infection of food crops by plant pathogens. Bacterial plant pathogens can cause widespread crop losses resulting in serious economic consequences (Pohronezny and Volin, 1983). Conventional chemical control strategies have limited efficacy due to the emergence of resistant bacteria and many studies have investigated novel approaches to controlling these pathogens. Pathogenic bacterial species including Erwinia amylovora, Ralstonia solanacearum, Streptomyces scabies and
*Xanthomonas campestris*, which cause varying diseases among edible plants and fruit, have been targeted for phage biocontrol strategies (Monk *et al*., 2010). Overall, studies of phage efficacy against plant pathogens have given promising results, with many authors reporting phage biocontrol to be more effective than no treatment (controls) and in many cases, comparable to, or better than, conventional chemical treatment (Flaherty *et al*., 2000; McKenna *et al*., 2001; Balogh *et al*., 2003).

A phage-based biocontrol product called AgriPhage gained EPA approval for agricultural use in the USA in 2004. This product is marketed primarily for the treatment of tomato and pepper spot in North and South America, and has recently been given licence for use in Asia. It was also identified by the Organic Materials Review Institute as being suitable for organic food production (Monk *et al*., 2010).

### 2.6 Bacteriophage biocontrol of foodborne pathogens

Foodborne illness is a major cause of morbidity and mortality worldwide (Kirk *et al*., 2008). Food production methods are constantly evolving to accommodate growing populations and consumer demands. Globalisation has ensured that consumers have access to traditionally ‘seasonal’ produce 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 (Todd, 2001). Furthermore, changes to the demographic profile of the community, as well as the emergence of new foodborne pathogens, make it necessary to continually 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 (Figure 2.4). Proper implementation of food safety procedures and vigilance by food regulation authorities, food producers and consumers, will continue to be important in 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.
2.6.1 Veterinary phage therapy

Phage biocontrol may be a more accurate term to describe the pre-harvest control of foodborne pathogens as these organisms often colonize rather than infect their animal hosts. However, for ease of differentiating between pre-harvest and post-harvest strategies, the term phage therapy will be used in this context. The majority of animal phage therapy research has focused on *E. coli* O157 colonisation of ruminants in addition to *Campylobacter* and *Salmonella* colonisation of poultry which are major reservoirs of the respective organisms. This section will review phage therapy studies related to these organisms.

2.6.1.1 Phage therapy of *E. coli* O157:H7 in sheep and cattle

*E. coli* O157:H7 is an important 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 (Griffin and Tauxe, 1991). 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 manure-
contaminated vegetables and dairy products made from unpasteurized milk (Chapman et al., 1997; Ackers et al., 1998; Michino et al., 1999). *E. coli* O157:H7 is persistent in the environment once introduced, and is able to survive in feed, water, soil and manure (Johnson et al., 2008). It has the potential to be transferred to many environmental sources by direct transfer in manure for fertilisation, or through vectors such as flies (Janisiewicz et al., 1999). Phage therapy 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 contamination.

Many phages with lytic ability against *E. coli* O157:H7 have been isolated and characterised for potential use as biocontrol agents (Kudva et al., 1999; Oot et al., 2007; Viscardi et al., 2008; Niu et al., 2009; Dini and de Urazza, 2010), and several studies have investigated the effects of *E. coli* O157:H7-specific phages *in vivo* using sheep or cattle with varied success. Studies have differed in terms of the mode of phage application, length of treatment and number of doses, the multiplicity of infection used, and the use of single phages or cocktails. KH1, an *E. coli* O157:H7-specific phage was orally administered to sheep in a single high concentration ($10^{11}$ PFU) dose, 1, 9, 10 and 11 days post infection with *E. coli* O157:H7. No significant reduction in *E. coli* O157:H7 carriage was observed even though phage was found to be present in the sheep faeces at concentrations of $10^5 - 10^6$ PFU/g during the study period (Kudva et al., 1999). Similarly Bach et al. (2003) found phage DC22 to be effective in significantly reducing *E. coli* O157:H7 *in vitro* and in an artificial rumen system but found that faecal shedding was not reduced in animal trials. Conversely, Raya et al. (2006) showed that phage CEV1 was able to infect the test strain of *E. coli* O157:H7 (strain 933) *in vitro* 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.
Different approaches have been adopted to avoid potential phage degradation in the gastrointestinal tract following oral delivery. As the primary site of *E. coli* O157:H7 colonisation in cattle is via the recto-anal junction, phages KH1 and a newly isolated phage SH1 were administered to cattle by rectal swabbing. Phages were rectally administered on days 0, 1, 2 and 4 post-inoculation with *E. coli* O157:H7 which was enumerated from faecal specimens for 21 days after infection. Although faecal *E. coli* O157:H7 declined significantly compared to the untreated controls over the test period, it was not completely cleared in 4 out of 5 steers. Furthermore, phage concentrations in faeces were reduced by an average of 3.0 log units over the test period. A more recent study comprehensively investigated oral and rectal administration of an *E. coli* O157:H7 phage in an open area feedlot to assess the potential value of the therapy in the presence of animal-animal and animal-environment interactions (Rozema *et al.*, 2009). Phages were administered to steers either orally, rectally or both orally and rectally two days prior to inoculation with *E. coli* O157:H7 and 0, 2, 6 and 9 days post-inoculation. Faecal samples were collected and monitored for *E. coli* O157:H7 shedding over 12 weeks. All animals in treatment groups shed phages in their faeces, with phage levels decreasing throughout the experiment. The control group also shed phages with plaque morphology similar to the treatment phages at levels similar to those of the steers who received phages rectally. Faecal shedding of *E. coli* O157:H7 was lowest for the orally treated group overall, but was not significantly lower than the control group. The authors concluded that while the recto-anal junction may be the primary site for *E. coli* O157:H7 colonisation, phage application at this site would fail to reach the upper regions of the gastrointestinal tract where the bacteria are also able to colonise. They also suggested that oral delivery facilitated retention of phages for a longer period, increasing the opportunity to interact with target cells prior to shedding (Rozema *et al.*, 2009).

After determining oral delivery of phages to be more effective than rectal delivery, Stanford *et al.*, (2010) used the same phages as Rozema *et al.* (2009) to develop an oral delivery system for encapsulated phage (Ephage) whereby phages were incorporated into gelatine capsules containing corn starch. Ephage was administered to feedlot cattle one day prior to inoculation with *E. coli* O157:H7 and on days 1, 3,
6, and eight days post-inoculation. Treatment groups were given phage either orally via a bolus or via an Ephage-barley-silage mixture added to feed. *E. coli* O157:H7 shedding was monitored for 10 weeks. The prevalence of samples positive for *E. coli* O157:H7 did not differ between the two Ephage treatments and the control. Similarly, *E. coli* O157:H7 counts did not differ among treatment groups. The bolus treatment reduced the length of faecal shedding by 14 days compared to the control. Shedding of phages for both treatment groups did not differ and persisted intermittently for 42 days after inoculation. While the bolus delivery system resulted in viable phages being released into the gastrointestinal tract as expected, the effect on faecal shedding of *E. coli* O157:H7 was not significant. The authors highlighted the need for further investigation into the relationships between endemic and experimental phages, effective doses of treatment phages and the relative importance of colonisation of different regions of the gastrointestinal tract on shedding of *E. coli* O157:H7 (Stanford *et al.*, 2010).

### 2.6.1.2 Phage therapy of *Campylobacter* in broiler chickens

*Campylobacter* is a common cause of gastroenteritis in humans worldwide and a common vehicle for infection is poultry (Kirk *et al.*, 2008). *Campylobacter* colonizes the gastrointestinal tract of broiler chickens resulting in high rates of carcass contamination during slaughter (Johnson *et al.*, 2008). *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 (Atterbury *et al.*, 2003a). Phage therapy researchers aim to exploit this natural process to effect a more significant reduction or elimination of colonisation of broiler chickens by *Campylobacter* prior to slaughter through the deliberate application of experimental phages.

The first report of phage therapy for *C. jejuni* in broiler chickens compared the efficacy of phages administered prophylactically and therapeutically in reducing *Campylobacter* colonisation of broiler chickens (Wagenaar *et al.*, 2005). In the preventative trial, 10 day old broiler chickens were administered phage 71 by oral gavage daily for 10 days and were inoculated with *C. jejuni* four days after the first
dose of phage. For the therapeutic study, chicks were challenged with *C. jejuni* on day 10 and phage 71 was administered by oral gavage daily for 6 consecutive days starting on day 15. Phage and *Campylobacter* were enumerated from caecal contents until chickens were 39 days old. Results of the preventative study showed that prophylactic phages were able to delay but not prevent colonization. Levels of *C. jejuni* were initially 2 log_{10} units lower than controls but then stabilised to approximately 1 log_{10} unit lower than controls. In the treatment group, an immediate 3 log_{10} unit reduction in CFU was observed, with bacterial counts stabilising to approximately 1 log_{10} unit lower than controls after phage treatment had ceased. The initial experiments indicated that phage therapy was more effective than prophylaxis and a subsequent therapeutic study was conducted using older birds. Chickens were challenged with *C. jejuni* 10 days before the usual age of slaughter (approximately 42 days). Birds were orally administered a mixture of two phages, 71, used in the primary studies, and 69, for four days commencing 7 days after challenge. Levels of *C. jejuni* reduced by approximately 1.5 log_{10} units during the course of phage treatment, and stabilized to approximately 1 log unit lower than controls. The results suggested that phage therapy shortly before slaughter could be effective in reducing contamination of chicken carcasses and could therefore contribute to reductions in cases of human *Campylobacteriosis*.

Phages CP8 and CP34 were tested *in vitro* for lytic ability against a commensal strain of *C. jejuni*, HPC5 using three different MOI. Although the lowest MOI resulted in the greatest reduction in HPC5, the final counts after 24 hours for all MOI were within 0.5 log_{10} units of each other. For *in vivo* studies, chicks were inoculated with *C. jejuni* at 18-20 days of age and treatment groups were orally administered a single dose of 10^5, 10^7 or 10^9 PFU CP8 or CP34. *Campylobacter* and phage were enumerated from the caecal contents and upper and lower intestinal contents of birds at 24 hour intervals. Treatment of HPC5 colonised chickens with phage CP34 resulted in significant reductions of *C. jejuni* counts at one or more of the intestinal sample sites over the course of the experiment. Overall, CP34 was more effective in reducing *C. jejuni* HPC5 at all intestinal sites compared to CP8, despite *in vitro* evidence that CP8 was virulent for the strain. After the first day of CP8 treatment, the *Campylobacter* counts fell in the upper and lower intestines but the caecal...
Contents were not significantly different from the control birds. However, the efficacy of CP8 against another host, *C. jejuni* GIIC8 was greater, and effected a $5.6 \log_{10}$ reduction in caecal contents within 24 hours (Loc Carrillo et al., 2005). These results emphasise the need for potential therapeutic phages to be thoroughly evaluated both *in vitro* and *in vivo*.

Promising results were reported by Carvalho et al. (2010) who evaluated the efficacy of a phage cocktail against *C. coli* and *C. jejuni* in broiler chickens. The cocktail containing three previously characterised phages was administered to 1 week old chicks colonised by either *C. jejuni* or *C. coli* either by oral gavage or phage impregnated feed. *Campylobacter* and phage titres were enumerated from the chicken faeces every day for 7 days. *Campylobacter* counts for both treatment groups were significantly lower ($p < 0.05$) during the experimental period compared to the controls. Reductions of $\sim 2 \log_{10}$ units in treatment groups were maintained for 7 days. Phage impregnated feed effected greater reductions in *Campylobacter* compared to oral gavage. Furthermore, phage titres remained stable in both treatment groups throughout the study, indicating that the phages were able to replicate in the gastrointestinal tract of the birds. The authors suggested that using cloacal swabs and enumerating *Campylobacter* and phage from faecal samples has the advantage of being able to study the kinetics of colonisation as multiple samples can be taken from single birds.

### 2.6.1.3 Phage therapy of *Salmonella* in broiler chickens

*Salmonella* is a common environmental organism which can cause asymptomatic infection or acute infection of chickens. Poultry products are a major source of human salmonellosis due to high rates of carcass contamination during slaughter (Johnson et al., 2008). Control of *Salmonella* in broiler chickens has historically relied on antibiotics and chemical sanitisers such as chlorine (Atterbury et al., 2003a; Mokgatla et al., 1998). However, many antibiotics and growth promoters are now banned in most Western countries and prolonged use of chlorine has led to resistant strains (Mokgatla et al., 1998). Several studies have investigated pre-harvest application of phages to reduce *Salmonella* carriage in broilers.
Atterbury et al. (2007) isolated and characterised three virulent *Salmonella* phages, \( \phi 10, \phi 25 \) and \( \phi 151 \), with broad lytic ability for use in phage therapy studies. 36 day old broiler chickens were challenged with \( 8.0 \log_{10} \) CFU of one of three *Salmonella* strains, Enteritidis P125109, Hadar 18 or Typhimurium 4/74. Chickens were orally administered with either \( 9.0 \) or \( 11.0 \log_{10} \) PFU of phage \( \phi 151 \) (*S. enterica* serotype Enteritidis P125109), \( \phi 25 \) (*S. enterica* serotype Hadar 18), or \( \phi 10 \) (*S. enterica* Typhimurium 4/74) two days post-challenge. Caecal contents were monitored for *Salmonella* every day for 3 days post-treatment. At the lower titre, no significant reduction in CFU of any serotype was observed. The higher titre produced significant reductions in *S. enterica* serotypes Enteritidis and Typhimurium recovered from caeca after 24 hours compared to the controls. No significant differences were recorded for birds colonised by *S. enterica* serotype Hadar 18 and the controls. A longer term study evaluating a phage cocktail containing \( 10^{11} \) PFU of three phages designated CNPSA1, CNPSA3 and CNPSA4 found that it was also able to reduce *Salmonella* Enteritidis PT4 colonisation of broiler chickens by up to \( 3 \log_{10} \) units. Chickens were experimentally infected with *Salmonella* Enteritidis and were administered the phage cocktail 7 days post-infection. *Salmonella* was enumerated from caecal contents of birds every 5 days until 25 days post-treatment. The authors suggested that administering a single, high titre dose of phage would be more effective than continuous administration which may lead to resistance among host cells.

Two studies have evaluated *Salmonella* phages and competitive exclusion products or probiotics for their ability to reduce *Salmonella* colonisation of chickens. The first study administered a low dose of three broad host range phages to broilers 3 days prior to challenge with *S. Typhimurium* and three days post challenge via oral gavage. While reductions in *S. Typhimurium* were observed, these reductions were not significant. Furthermore, the competitive exclusion product, Protexin, was equally effective, and no synergistic effect was observed when the phages and Protexin were administered together (Toro et al., 2005). The second study used two phage cocktails, CB4Ø and WT45Ø, to reduce *Salmonella* Enteritidis colonisation of chickens, and compared oral gavage and cloacal application as modes of
administering the treatments. The authors also investigated the effect of the phage cocktails when administered cloacally in combination with the probiotic, Floramax-B11 (Andreatti Filho et al., 2007). Orally administered phage produced reductions in *Salmonella* at 24 hours but numbers increased after 48 hours. When WT45Ø and probiotic were administered cloacally, they both caused a significant reduction in *Salmonella*. The authors noted, however, that this mode of administration would not be practical commercially. Furthermore, there was no additive effect observed when WT45Ø and probiotic were administered in combination.

The results of the above phage therapy studies are difficult to evaluate and compare, due to the range of different phages, doses, *Salmonella* strains, methods of administering treatment and durations of treatment used. However, phage therapy to reduce *Salmonella* of chickens appears to show some promise.

### 2.6.2 Biocontrol

#### 2.6.2.1 Raw meat and fish

Phage application post-slaughter has been studied as an alternative to therapy. This type of application has potential advantages over pre-harvest applications, since the phages used in the treatment would be less likely 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 (Goode et al., 2003). The same food animals and target organisms have been investigated for this type of biocontrol as outlined in the previous section.

Biocontrol of *E. coli* in culture and on artificially contaminated meat has been investigated using phage cocktails (Kudva et al., 1999; O’Flynn et al., 2004). In these studies, it was found that a high MOI was required to significantly reduce numbers of *E. coli* O157:H7 *in vitro*. According to Kudva *et al.* (1999) 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). O’Flynn *et al.* (2004) observed that seven out of nine meat samples artificially inoculated with *E. coli* O157:H7 were found to be completely free of *E.*
coli O157:H7 after treatment with a high titre dose of phage cocktail containing e11/2, e4/1c, and pp01. 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. A similar study tested the ability of a bacteriophage cocktail designated ECP-100 lytic for E. coli O157:H7 for its ability to reduce contamination of a range of hard surfaces and foods, including ground beef (Abuladze et al., 2008). Ground beef samples were experimentally inoculated with 3.4 log\textsubscript{10} CFU of E. coli O157:H7 and stored at 10°C for 1 hour before application of ECP-100 with a spray bottle. Incubation was continued at 10°C for 24 hours after which E. coli was recovered. The cocktail was able to significantly reduce E. coli on the meat, results comparable to those reported by O’Flynn et al. (2004).

Post-harvest biocontrol studies have also explored the potential application of virulent phage to the carcasses and skin of chickens to reduce contamination by Salmonella and Campylobacter. Goode et al. (2003) investigated the ability of lytic bacteriophages to reduce the number of artificially inoculated Salmonella and Campylobacter on the skin of chicken at different 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\textsubscript{10} unit. Phages applied at a high MOI (100 to 1000) rapidly reduced the recoverable bacterial numbers by up to 2 log\textsubscript{10} units over 48 hours. At an MOI of 10\textsuperscript{5}, no Salmonella was recovered following treatment.

Higgins et al. (2005) 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 x 10\textsuperscript{10} PFU/mL 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.

In a similar study by Atterbury *et al.* (2003b) chicken skin was artificially inoculated with *Campylobacter jejuni* (PT14) and bacteriophage ϕ2 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 $1 \log_{10}$ unit for samples incubated at 4°C and $2 \log_{10}$ units in the samples incubated at -20°C. Bacteriophage ϕ2 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 sufficiently high titres to the surface of chicken skin inoculated with *Campylobacter* clearly reduced the number of recoverable cells, by $1 \log_{10}$ 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 increased its metabolic activity.

A novel application of phage biocontrol is the use of phages to reduce *L. monocytogenes* contamination of fresh channel catfish. Studies have shown high rates of contamination of fresh channel catfish fillets, ranging from 23.5% to 47% (Chou *et al.*, 2006; Pao *et al.*, 2008). Although there have been no documented cases of listeriosis associated with consumption of catfish, the risk remains due to the high prevalence of *L. monocytogenes* contaminated fish (Soni *et al.*, 2010a). Fish fillet pieces were inoculated with *L. monocytogenes* followed by inoculation with the phage preparation Listex P100. The fish fillet pieces were then incubated at 4°C, 10°C and 22°C for up to 10 days. *L. monocytogenes* was enumerated after 15 minutes, 30 minutes, 1 hour, 2 hours, 1 day, 4 days or 10 days of incubation. Phage was able to reduce *L. monocytogenes* on catfish fillets by $1.5 \log_{10}$ units after 30 minutes at both 4°C and 10°C. While no further inhibition was observed, *L.*
monocytogenes levels in phage treated fish fillets remained approximately 1.0 – 1.5 log_{10} units lower than for the untreated controls over the 10 day experiment. The phages produced significant reductions in *L. monocytogenes* on fish fillets at all temperatures tested, indicating that phage technology could potentially be used at all stages of catfish processing. These results were replicated in another study using Listex P100 to reduce *L. monocytogenes* on raw salmon fillet tissue (Soni and Nannapaneni, 2010b).

### 2.6.2.2 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 (Beuchat, 2002). Irrigation with untreated sewage or fertilisation with poorly composted manure may also contribute to the risk of contamination (Beuchat, 2002). Numerous outbreaks have been attributed to the consumption of contaminated fruits and vegetables (Burnett & Beuchat, 2000; Beuchat, 2002; Bowen *et al.*, 2006). 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 (Bowen *et al.*, 2006). 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 (Mokgatla *et al*., 1998).

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.* (2001) examined bacteriophage as a biocontrol method for *Salmonella* on fresh cut melon and apple slices. Melon and apple slices were experimentally inoculated with *Salmonella* at a concentration of $10^6$ CFU/mL and
then treated with a phage mixture (SCPLX-1) containing four distinct lytic phages specific for *Salmonella* Enteritidis at a concentration of $10^8$ PFU/mL. 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 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.* (2003) 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 $\log_{10}$ units over the control on honeydew melons. On apples, the reduction was below 0.4 $\log_{10}$ units. In combination with nisin, the phage mixture reduced *L. monocytogenes* populations by up to 5.7 $\log_{10}$ units on honeydew melon slices and by up to 2.3 $\log_{10}$ units on apples compared to the control. Nisin alone reduced *L. monocytogenes* populations by up to 3.2 $\log_{10}$ units on the honeydew melon slices and by up to 2.0 $\log_{10}$ 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 which has an intrinsic low pH with a phage cocktail and 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\(^\text{TM}\), developed by the biotechnology company Intralytix, was the first
bacteriophage-based food safety product approved by the United States Food and Drug Administration.

The use of *Listeria* phages, P100 and a new isolate, A5111, to reduce *L. monocytogenes* contamination of the salad vegetables cabbage and iceberg lettuce was reported by Guenther et al. (2009). High concentrations of the phage combination were able to significantly reduce *L. monocytogenes* contamination of lettuce and cabbage during storage at 6°C over 6 days. Furthermore, storage time and temperature were found to have very little effect on the efficacy of the phages used. When the experiment was extended to evaluate the effect of phage during storage at 20°C, the log_{10} reductions in *L. monocytogenes* were similar to those produced at 6°C, although the rate of growth increased for both controls and phage treated samples.

Although usually associated with consumption of ground beef, there have been many outbreaks of *E. coli* O157:H7 attributed to consumption of fresh produce. ECP-100 was able to significantly reduce (*p < 0.05*) *E. coli* O157:H7 on tomatoes, broccoli and spinach after 24 hours, 10 hours and 168 hours of incubation at 10°C. Reductions between 94 and 100% were observed and surviving *E. coli* O157:H7 were found to remain sensitive to lysis by ECP-100. The pH of the tomatoes was not reported, but it is assumed that the skin, which has a more neutral pH than the inner flesh was inoculated with phage, as it was used as an example of a “smooth” food (Abuladze et al., 2008).

### 2.6.2.3 Dairy and other ready-to-eat (RTE) products

Modi et al. (2001) 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 of a luminescent strain of *Salmonella* Enteritidis (lux) and \(10^8\) PFU/mL 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$_{10}$ units in raw and pasteurized milk cheeses containing phage. In the non-phage treated controls, *Salmonella* counts increased by about 1 log$_{10}$ 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 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 survival of *Salmonella* 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.* (2003) compared the ability of a 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 3.0 log$_{10}$ units CFU/g of log phase *Salmonella* Typhimurium and then subsequently treated with either wild-type or large-plaque variant Felix O1. Both phage treatments effected a 2.0 log$_{10}$ 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.

*Staphylococcus aureus* has been targeted by phage investigators due to its association with raw and pasteurized milk. Bacteriophage K was found to inhibit *S. aureus* in heat-treated milk but not raw milk (O’Flaherty *et al.*, 2005a). Garcia *et al.* (2009) used dairy-derived phages, φA5 and φA72, to reduce *S. aureus* in pasteurized full cream milk as well as full cream and semi-skimmed raw milk incubated at 37°C. The phage mixture was able to inhibit the growth of *S. aureus* in heat-treated milk but was less effective in the raw milk. The phages were also used in a study designed to simulate a breakdown in cold storage temperature. While no reduction in *S. aureus* was observed in phage-treated UHT milk during storage at 4°C, a 5 log unit reduction was observed at the end of the study following a shift in storage temperature to 18°C.
In a similar approach to Leverentz et al. (2003) described in the previous section, a study was performed to assess the effect of a phage cocktail in combination with nisin in reducing *S. aureus* in pasteurized milk (Martinez et al., 2008). A synergistic effect was observed when nisin was used in combination with the phages. However, a nisin-adapted isolate was obtained which also exhibited partial resistance to the phages used. When the adapted isolate reverted to the nisin-sensitive phenotype, phage susceptibility was similarly restored. Conversely, phage insensitive mutants did not exhibit nisin-resistance. The authors suggested that changes in the bacterial cell surface linked with nisin resistance could have interfered with phage attachment.

*Listeria monocytogenes* is a foodborne pathogen that commonly contaminates dairy products, beef, pork, poultry, and seafood. However, fresh fruits and vegetables can also become contaminated with *L. monocytogenes*, and some of these products have been implicated in outbreaks of foodborne listeriosis (Farber et al., 1996). The ability of *L. monocytogenes* to grow at low temperatures makes refrigerated ready-to-eat products particularly vulnerable to contamination (Farber et al., 1996). Phage biocontrol has been investigated for controlling *L. monocytogenes* in a range of RTE foods discussed below.

Carlton et al. (2005) 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* (20 CFU/cm²) to simulate natural contamination. During the 13 days 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 (8.0 log₁₀ units PFU/mL) able to significantly decrease *Listeria* viable counts, while higher concentrations (9.0 log₁₀ units PFU/mL) 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 showed 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.
The commercial phage preparation, Listex P100, was used with a protective culture, \textit{Lactobacillus sakei} TH1, to reduce growth of \textit{L. monocytogenes} on sliced, cooked ham. Ham slices were inoculated with \textit{L. monocytogenes}, and were subsequently treated with Listex P100, \textit{L. sakei}, or a combination of the two. Ham slices were incubated at 10°C or 4°C for 25 days. At 10°C, \textit{L. monocytogenes} numbers recovered from control ham slices increased by 2 log\(_{10}\) units after 14 days. In phage treated hams, \textit{L. monocytogenes} was rapidly reduced by 1 log\(_{10}\) unit. However, surviving \textit{L. monocytogenes} grew to the same level as controls after 14 days. Ham slices treated with both Listex P100 and \textit{L. sakei} TH1 produced a similar initial drop in \textit{L. monocytogenes} but in contrast to the phage-only treated slices, the surviving \textit{L. monocytogenes} was further inhibited by the fast growing \textit{L. sakei} TH1. After 28 days, \textit{L. monocytogenes} levels were 2 log\(_{10}\) units lower than the controls. Similar results were observed when incubated at 4°C. The authors concluded that as \textit{L. sakei} grows well at low temperatures, is able to prevent growth of \textit{L. monocytogenes} and has no negative effects on the organoleptic properties of ham, it could be employed as a hurdle together with phages (Holck and Berg, 2009).

The phage combination described in section 2.6.2.2 containing P100 and A511 was also used to reduce \textit{L. monocytogenes} in liquid foods including chocolate milk and mozzarella cheese brine and solid foods including hot dogs, sliced turkey and smoked salmon. In liquid foods, the phage combination was able to completely eliminate \textit{L. monocytogenes} and on solid foods, an average 5 log\(_{10}\) unit reduction was achieved. The authors suggested that the greater efficacy of phage treatment in liquid foods was due to the ability of phages to diffuse further compared to solid foods. Interestingly, the phages retained most of their infectivity during storage on foods of animal origin whereas phage numbers on plant material reduced by more than 1 log\(_{10}\) unit (Guenther \textit{et al.}, 2009).

\subsection*{2.6.3 Biosanitation}

Sanitisation of food processing surfaces helps prevent pathogens from contaminating finished food products (Assanta and Roy, 2001). Improper sanitisation of surfaces and equipment can lead to economic losses due to product recall. The use of
chemical sanitisers has led to resistance among some major pathogens (Mokgatla, 1998) and novel approaches to sanitisation are needed. Phages have been studied for their ability to control bacteria on inert surfaces commonly found in food processing environments. The ability of phage cocktail ECP-100 when applied at three concentrations ($10^8$, $10^9$ and $10^{10}$ PFU/mL) to reduce *E. coli* O157:H7 contamination of hard surfaces was investigated by Abuladze *et al.* (2008). The surfaces comprised hard (glass slides) and porous (gypsum slides) which were first contaminated with 5% (w/v) skim milk to simulate real life settings where surfaces are often covered with dried organic matter (Abuladze *et al.*, 2008). Dirtied surfaces were contaminated with 1.0 X 7.0 CFU of a mixture of three *E. coli* O157:H7 strains. Surfaces were dried in a laminar flow cabinet before addition of ECP-100. *E. coli* O157:H7 was enumerated from surfaces following 5 minutes of incubation at RT to allow adsorption of phages to bacterial cells. The authors suggested that phage-bacterium contact time may be critical for some applications but not for others. For example, when used to decontaminate surfaces, phages may be left on surfaces for prolonged periods to allow for maximum efficacy, whereas in other applications, where rapid decontamination is required, shorter contact times such as those used in this study would be preferable. The phage cocktail was able to significantly reduce ($p < 0.05$) *E. coli* O157:H7 on glass slides at all concentrations tested. However, for gypsum slides, significant reductions were observed only for the two highest concentrations of phage tested ($10^9$ and $10^{10}$ PFU/mL).

A similar study determined the effect of another phage cocktail, BEC8, on *E. coli* O157:H7 contaminated surfaces including stainless steel, ceramic tiles and high density polyethylene chips (HDPE). This study varied the level of *E. coli* O157:H7 contamination ($10^4$, $10^5$ and $10^6$ CFU/chip) and used a constant concentration ($10^6$ PFU/chip) of BEC8 to effect MOI of 100, 10 and 1. A range of incubation temperatures were also used, including 4, 12, 23 and 37°C. The efficacy of phage treatment increased with time, temperature and MOI. At the highest MOI, no *E. coli* was recovered from stainless steel or ceramic tiles following incubation at 37°C for 10 minutes or RT for 1 hour. However, even at low MOI, *E. coli* O157:H7 was reduced by $\geq 1$ log$_{10}$ unit on ceramic tiles and stainless steel following incubation for 24 hours at 4°C. This study demonstrated that statistically significant reductions in
E. coli O157:H7 could be achieved using a lower concentration of phage cocktail than has previously been reported. However, time and incubation temperature considerably influenced phage efficacy (Viazis et al., 2011a).

A novel study by Roy et al. (1993) investigated the use of Listeria phages (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 Listeria phages at concentrations 8.0 log units PFU/mL was as efficient as a 20 ppm solution of QUATAL in sanitising the artificially contaminated surfaces. Furthermore, a synergistic activity was observed when phages were suspended in QATAL. This combined approach enabled the concentration of QATAL to be reduced while achieving the same total log reduction as 50 ppm QATAL alone. It was also reported that the Listeria phages could maintain their infectivity when exposed to various concentrations of QUATAL (1 to 50 ppm) for up to 4 hours.

In addition to contaminating surfaces in a planktonic state, some bacteria can create biofilms on surfaces. Biofilms can form when surfaces that have been inadequately cleaned are contaminated with bacteria. These biofilms confer protection against disinfectants and sanitizers making the bacteria difficult to eliminate (Sharma et al., 2005). Phages have been studied for their potential to kill bacteria attached to surfaces or present in biofilms as an alternative or adjunct to chemical sanitisation. In another study of Listeria specific phages, Hibma et al. (1997) employed phage breeding techniques to produce a bacteriophage specific for L-forms of L. 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.

Sharma et al. (2005) 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. Bacteriophage KH1 was able to reduce *E. coli* O157:H7 cells attached to stainless steel by 1.2 log<sub>10</sub> units but no significant reduction in *E. coli* O157:H7 cells enmeshed in biofilms was observed. It was suggested that while KH1 had lytic activity to kill free cells, it did not possess the EPS-degrading ability exhibited by other lytic bacteriophages to be effective in killing cells in biofilm.

The previous studies used bacteriophages for the inactivation of biofilms formed by a single species of bacteria. However, many biofilms contain a number of species of bacteria and may therefore be more difficult to eliminate using this method. Tait *et al.* (2002) investigated the efficacy of bacteriophage as a method of eradicating single and dual-species biofilms. Biofilms containing one or both of *Enterobacter cloacae* (NCTC 5920) and *E. agglomerans* (strain Ent) were inoculated with bacteriophage φ1.15, 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 bacteriophages 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.

### 2.6.4 Biopreservation

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 (Madigan and Martinko, 2003). 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 (Whitman and Marshall, 1971).
Patel and Jackman (1986) first suggested that deliberate application of *Pseudomonas* phages to milk and milk products could be used to increase shelf life. Greer and Dilts (1990) 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 using phage to reduce spoilage of perishable foods would be unlikely since critical concentrations of sensitive bacteria may not be encountered under natural conditions. 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 would be necessary to isolate phages with broad host range to have better success in controlling a greater number of *Pseudomonas* species.

The bacteria of particular interest to this thesis are *E. coli* and *Enterococcus* sp. Therefore, a brief summary of the major features of these bacteria, including their role as foodborne pathogens, is presented below. In addition, studies investigating phage biocontrol against these bacteria are reviewed.

### 2.7 E. coli

*E. coli* is one of the most studied and well characterised bacterial organisms and has an estimated total population of $10^{20}$ (Whitman et al., 1998). While *E. coli* is a commensal organism of warm-blooded animals, some strains are pathogenic. The pathogenic *E. coli* can be divided into six pathotypes: enteropathogenic, enterotoxigenic, enteroinvasive, enterоaggregative, diffusely adherent and enterohaemorrhagic (Croxen and Finlay, 2010). The most studied pathogenic *E. coli* are the Shiga-toxin producing *E. coli* (STEC) which belong to the enterohaemorrhagic group. *E. coli* O157 belongs to this pathotype. This group of
bacteria are important because of the potential to cause serious illness (Pennington, 2010).

### 2.7.1 Taxonomy and biology

*E. coli* is a member of the family Enterobacteriaceae which comprises Gram-negative, non-sporulating facultative and aerobic rods. *E. coli* that are motile possess peritrichous flagella. A characteristic feature of the Enterobacteriaceae is the ability to ferment lactose with the production of acid and gas. Serotyping of *E. coli* is often used as a tool to classify the huge volume of isolates within the genus. *E. coli* is serotyped based on 173 somatic (O), 56 flagellar (H) and 80 capsular (K) antigens (Feng, 2001). *E. coli* is closely related to *Salmonella* and is thought to share a clonal lineage with *Shigella*. There is enormous genetic diversity among strains of *E. coli*. Analysis of a number of *E. coli* genomes revealed that the average genome size is approximately 5 Mb (representing about 4700 protein-encoding genes). However, within the species, genomes can differ in size by more than 1 Mb, or 1000 genes (Touchon *et al*., 2009). Furthermore, only about 2000 of the 4700 genes encoded by a typical *E. coli* were present in all of the 20 genomes analysed. It is thought that it is this genetic diversity that enables *E. coli* to survive in diverse environments (Gordon, 2010).

### 2.7.2 Reservoirs of *E. coli*

*E. coli* are universally found as commensal organisms in the gastrointestinal tract of warm-blooded animals and serve a beneficial function by synthesising vitamin K and suppressing ingested pathogenic bacteria. It is one of the dominant aerobic bacteria found in human faeces and has therefore been used as an indicator of faecal contamination of water and food for decades (Feng, 2001). It is also shed in the faeces of a number of animals including ruminants and is therefore widespread in the environment. In addition to carriage of non-pathogenic strains of *E. coli*, ruminants and in particular cattle, are reservoirs of enterohaemorrhagic *E. coli* including *E. coli* O157:H7. There are no reported animal reservoirs of the other *E. coli* pathotypes (Feng, 2001).
2.7.3 *E. coli* in food

*E. coli* can be isolated from many foods of animal origin. While outbreaks of foodborne illnesses attributed to pathogenic *E. coli* have been associated primarily with minced beef, many outbreaks have been attributed to consumption of contaminated fresh produce or dairy products. Selected outbreaks of significance are outlined in Table 2.1.

<table>
<thead>
<tr>
<th><em>E. coli</em></th>
<th>Associated food</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>Ground beef</td>
<td>USA</td>
<td>(Tuttle <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>O157</td>
<td>Raw milk</td>
<td>USA</td>
<td>(Guh <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>O157</td>
<td>Unpasteurized apple juice</td>
<td>USA/Canada</td>
<td>(Cody <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>O157</td>
<td>Alfalfa</td>
<td>USA</td>
<td>(Ferguson <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>ETEC</td>
<td>Sushi</td>
<td>USA</td>
<td>(Jain <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>O111:NM</td>
<td>Mettwurst</td>
<td>Australia</td>
<td>(Paton <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>O92:H33</td>
<td>Raw milk pecorino cheese</td>
<td>Italy</td>
<td>(Scavia <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>O145 and</td>
<td>Ice-cream made from</td>
<td>Belgium</td>
<td>(De Schrijver <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>O26</td>
<td>pasteurized milk</td>
<td></td>
<td>(De Schrijver <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>O104:H4</td>
<td>Tomatoes, cucumbers</td>
<td>Germany</td>
<td>(Gault <em>et al.</em>, 2011)</td>
</tr>
</tbody>
</table>

2.7.4 Human health hazards from food derived *E. coli*

While commensal strains of *E. coli* rarely cause disease in healthy individuals, pathogenic *E. coli* are increasingly implicated in outbreaks of foodborne disease. While pathotypes other than enterohaemorrhagic *E. coli* typically cause self-limiting illness, the enterohaemorrhagic *E. coli* cause haemorrhagic colitis, which is characterised by abdominal cramps and bloody diarrhoea (Feng, 2001). Furthermore, in approximately 3-7% of cases, infection can lead to serious complications such as haemolytic uraemic syndrome (HUS) particularly among children (Pennington, 2010). STEC is the most frequently reported foodborne group
of pathogenic *E. coli* and O157 is the most common serotype to be implicated in foodborne outbreaks (Fegan and Desmarchelier, 2010). STEC is transmitted via the faecal-oral route and the dose required for infection is very low (10-100 cells). Therefore, contamination of RTE foods is particularly hazardous (Feng, 2001).

### 2.7.5 The use of phages in the control of *E. coli*

The potential for using phages to control *E. coli* in a variety of environments has already been discussed. However, considering the increasing prevalence of pathogenic *E. coli* in the environment and the increasing burden of *E. coli* associated disease, further research is justified. Moreover, since most research to date has focused on phage biocontrol of *E. coli* O157:H7, other serotypes capable of causing disease have been neglected. In the Australian context, many different EHEC serotypes, including O157 and non-O157 serotypes have been associated with disease (Combs *et al.*, 2005; McPherson *et al.*, 2009). Therefore, research focusing on these local isolates could be beneficial.

### 2.8 Enterococci

In contrast to *E. coli* and in particular *E. coli* O157:H7, there are very few studies reporting phage biocontrol of *Enterococcus* spp., despite the fact that this genus is of clinical importance and is commonly found in many foods. This section will provide a summary of the important features of the genus and a justification for further study into alternate methods of controlling this opportunistic pathogen in foods and on surfaces.

#### 2.8.1 Taxonomy

The genus *Enterococcus*, formerly known as the faecal streptococci or Lancefield’s group D *Streptococcus*, comprises Gram-positive, catalase negative, facultatively anaerobic lactic acid bacteria (LAB). Enterococci are chemoorganotrophic and produce L-lactic acid from hexoses by homofermentative lactic acid fermentation. Enterococci can grow in media containing 6.5 % sodium chloride, 40 % bile or 0.1%
methylene blue. They will usually survive at 60°C for 30 min and can grow at extremes of pH (up to 9.6) and temperature (range of 10-45 °C) (Chenoweth and Schaberg, 1990). On the basis of comparative 16S rRNA sequence analysis, the genus *Enterococcus* belongs to the Gram-positive bacteria with low (< 50 mol %) G+C content in the DNA. Typical members of this phylum are clostridia and bacilli (Franz *et al.*, 2003). Approximately 40 species have been assigned to the genus *Enterococcus* based on chemotaxonomic and phylogenetic studies (Ogier and Serror, 2008). However, *E. faecium* and *E. faecalis* are the most prevalent species and play an important role in enterococcal-associated human disease. They are also the most prominent species associated with foods (Franz *et al.*, 2003).

### 2.8.2 Reservoirs of enterococci

Enterococci are normal inhabitants of the mammalian gastrointestinal tract. *E. faecalis* is usually the predominant species found in the human bowel followed by *E. faecium*, although, this trend is reversed in some individuals and in some countries (Ruoff *et al.*, 1990). Studies have found that the numbers of *E. faecalis* and *E. faecium* in human faeces range from $10^5$ to $10^7$ CFU/g and $10^4$ to $10^5$ CFU/g respectively (Chenoweth and Schaberg, 1990). Other human reservoirs of the enterococci include the oral cavity, hepatobiliary tract and vagina of asymptomatic women. *Enterococcus* spp. can also be found in the gastrointestinal tracts of cattle, swine and poultry as well as in soil, surface waters and on plants and vegetables (Giraffa, 2003). The ability of enterococci to survive under adverse conditions enables them to colonise areas that are hostile to other organisms (Huycke *et al.*, 1998).

### 2.8.3 Enterococci as nosocomial pathogens

Enterococci are recognised as major nosocomial pathogens that cause bacteraemia, endocarditis, urinary tract and other infections. They are among the most prevalent organisms encountered in hospital infections, accounting for approximately 12% of nosocomial infections in the USA. Enterococci are opportunistic and usually cause infections in patients that have severe underlying disease or who are
E. faecalis predominates among enterococci isolated from human infections (more than 80%) while E. faecium is associated with the majority of the remaining infections (Huycke et al., 1998).

A major factor contributing to the pathogenicity of enterococci is its intrinsic resistance to low concentrations of aminoglycosides, beta-lactams and quinolones (Ogier and Serror, 2008). Furthermore, the past two decades have seen a substantial increase in enterococci with acquired resistance to glycopeptides including vancomycin and teicoplanin, which are often used as a last resort for treatment of multi-resistant enterococci (Huycke et al., 1998). The increasing proportion of enterococcal isolates that are vancomycin-resistant may have a major impact on the mortality of hospitalized patients (Chavers et al., 2003). The spread of vancomycin-resistant enterococci (VRE) has also had a significant impact on the health care system, with VRE patients spending more time in hospital and with higher associated costs compared to patients infected with vancomycin–sensitive enterococci (VSE) (Stosor et al., 1998).

Contaminated instruments have been implicated in several outbreaks of VRE (Livornese et al., 1992; Porwancher et al., 1997) and admission of a VRE-free patient to a hospital room recently occupied by a VRE-colonised patient was found to be an independent risk factor for nosocomial acquisition of VRE by the previously uncolonised patient (Jernigan et al., 1997). The ability of an organism to survive on environmental surfaces is a critical factor for transmission. Several studies have demonstrated that enterococci are able to survive on hard and porous surfaces for extended periods (Wendt et al., 1998; Neely and Maley, 2000; Noskin et al., 2000).

### 2.8.4 Enterococci in food

#### 2.8.4.1 Enterococci in dairy products

Enterococci have been isolated from bovine faeces as well as the raw milk of dairy cattle with mastitis infection. Enterococci can be found naturally in many traditional European cheeses and can form part of the starter culture or contribute to the ripening process through proteolytic and lipolytic traits (Franz et al., 2003).
Numbers of enterococci in Mediterranean-type cheese curds range from $10^4$ to $10^6$ CFU/g and in fully ripened cheeses from $10^5$ to $10^7$ CFU/g (Franz et al., 1999). Enterococci can grow in this restrictive environment of high salt content and low pH. Some enterococci have additional beneficial effects such as producing bacteriocins which inhibit the growth of other pathogens including *Listeria monocytogenes* (Giraffa et al., 1997). However, in contrast to other LAB, enterococci are not ‘generally regarded as safe’ (GRAS) as their presence in foods is an indication of faecal contamination (Giraffa, 2003). The source of enterococci in dairy products is likely due to faecal contamination of raw milk or contamination of milking or storage equipment (Mannu et al., 2003). *E. faecalis* and *E. faecium* are the species most frequently isolated from dairy products. A high prevalence of antibiotic resistance including vancomycin-resistance among enterococci isolated from raw milk was reported by Citak et al. (2005).

### 2.8.4.2 Enterococci as probiotics

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (WHO, 2002). While most probiotics belong to the genera *Bifidobacterium* and *Lactobacillus*, some strains of *E. faecium* have also been used for this purpose. For instance, *E. faecium* SF68 has been used as an alternative to antibiotics in the treatment of diarrhoea, with studies showing it is able to decrease the duration of diarrhoeal symptoms in adults and children (Bellomo et al., 1980; Bruno and Frigerio, 1981; D’Apuzzo and Salzberg, 1982). The probiotic culture Causido® contains a mixture of *S. thermophilus* and *E. faecium* and is used to ferment the yogurt product Gaio®. Short term studies have shown that the product is able to reduce total and low-density lipoprotein (LDL) cholesterol levels in plasma (Agerholm-Larsen et al., 2000). However, the long term effects have not been investigated. Strains of *E. faecium* and *E. faecalis* have also been used as growth promoters or to treat enteric disease in livestock (Underdahl, 1983). The use of *Enterococcus* spp. as probiotics is controversial due to concern that antimicrobial resistance genes or genes encoding virulence factors may be transferred to these strains in the gastrointestinal tract (Franz et al., 2003).
2.8.4.3 Enterococci in meats

Enterococci are present in high numbers in the gastrointestinal tracts of animals which can lead to contamination of meat during slaughter. *Enterococcus* spp. are consistently recovered from raw meat originating from pigs and poultry (Franz et al., 2003). A study by Knudtson and Hartman (1993) found that enterococci recovered from pig carcasses had mean log$_{10}$ unit counts of $10^4$-$10^8$ CFU/100 cm$^2$ of surface area, with *E. faecium* and *E. faecalis* the predominant species. Enterococci also frequently contaminate processed meat products. As enterococci are among the most thermotolerant of the non-sporeforming bacteria, they may survive the heating processes which kill competitive microbiota. *E. faecalis* and *E. faecium* have been implicated in the spoilage of cured meat products (Bell and Gill, 1982), and have been isolated from fermented sausage products such as salami and Landjäger in numbers ranging from $1.0 \times 10^2$ to $2.6 \times 10^5$ CFU/g (Teuber et al., 1996).

2.8.5 Human health hazards from food derived enterococci

The principal concern for enterococci in the food supply is their pathogenic potential based on horizontal transfer of genes for factors associated with virulence and antibiotic resistance (Franz et al., 1999). The use of antimicrobial agents such as avoparcin as growth promoters in food animals has contributed to cross-resistance to antibiotics used in human therapy. As such, this practice was banned in the European Union. Antibiotic-resistant enterococci (ARE) are prevalent in food animals and are frequently recovered from foods of animal origin. ARE have been isolated from a range of foods including meat products, dairy products and RTE foods (Corpet, 1998; Teuber et al., 1999; Koluman et al., 2009; Riboldi et al., 2009). It is thought that resistance genes may therefore be frequently transferred from animals to humans via contaminated food (Heuer et al., 2006). While enterococci tend to be host-specific and deliberate introduction of enterococci from an animal host into a human host does not result in colonisation (Sørensen et al., 2001) a transfer of resistance or virulence genes can still occur in the human gastrointestinal tract (Lester et al., 2006). Therefore colonisation does not appear to be essential for transfer of resistance genes to occur.
Considering the potential for transfer of genes encoding antibiotic resistance and virulence factors between enterococci in food and humans, it would be prudent for food manufacturers to have a greater understanding of these organisms and methods for their control in food processing (Franz et al., 1999).

### 2.8.6 The use of phages in the control of enterococci

A major area of enterococcal phage related research is in phage therapy due to the increasing prevalence of enterococcal, including VRE, infections. Phage therapy has been investigated as a means of treating a number of different types of infection caused by *Enterococcus* spp. Oral infection associated with enterococci are common but are restricted to the root canal (Bachrach et al., 2003). The antimicrobial ability of *Enterococcus* phages was tested *in vitro* against *E. faecalis* infected root canals and dentinal tubules of human teeth by Paisano et al. (2004). At MOI of 1 and 10, no growth of *E. faecalis* was observed in the teeth after 3 hours of exposure. At lower MOI (0.1) mean growth was reduced by 98% compared to the control. Similarly, no *E. faecalis* was recovered from dentinal tubules following treatment with bacteriophage. The potential for phages to treat endodontic infection by *Enterococcus* spp. was also identified by Bachrach et al. (2003) who isolated phages specific for *E. faecalis* from human saliva.

Biswas et al. (2002) experimentally infected mice with $10^9$ CFU VRE to induce bacteraemia. All mice injected with $10^9$ CFU VRE died within 48 hours without treatment. A dose effect was observed in mice that were treated with a single intraperitoneal injection of phage ENB6. With higher MOI (3.0) 100% of the animals survived. As the phage dose decreased, the animals became critically ill and survival rates decreased. It was found that delaying phage treatment by up to 5 hours did not affect survival of the mice. Furthermore, although after delays of 18 hours and 24 hours led to increased morbidity and mortality, approximately 50% of the animals recovered. Phage therapy has been routinely carried out in cases of human illness for decades in Eastern Europe. However, published, peer-reviewed evidence of this practice is limited. One recent study by Letkiewicz et al. (2009) reported
using phages to successfully treat three patients with chronic bacterial prostatitis (CBP) caused by *E. faecalis*. Phage preparations were rectally administered twice daily for 28 to 33 days. Following the treatment, cultures taken from prostatic fluid were negative, urinary flow rates increased compared with pre-treatment and digital rectal examination revealed a normal prostate gland. While findings from these studies are promising, there is a need for a wide range of phages to overcome problems associated with narrow host specificity and the potential for host resistance. To our knowledge, no studies of phage biocontrol of enterococci in foods or on inert surfaces have been reported in the literature, indicating that this could be a viable avenue of research.

### 2.9 Potential applications for phage biocontrol of *E. coli* and *Enterococcus* spp.

#### 2.9.1 Surface biocontrol

The number of studies investigating phage biocontrol of pathogens on inert surfaces is comparatively lower than those focusing on phage biocontrol of pathogens in foods. While three papers investigating phage biocontrol of *E. coli* on hard surfaces have recently been published (Sharma *et al.*, 2005; Abuladze *et al.*, 2008; Viazis *et al.*, 2011a), further study is justified considering the growing burden of *E. coli*-associated disease. In contrast, there are no reported studies of phage biocontrol of *Enterococcus* spp. on surfaces. This is despite the fact that several studies have demonstrated the ability of *Enterococcus* spp. to survive on a variety of hard and soft surfaces for prolonged periods (Wendt *et al.*, 1998; Neely and Maley, 2000; Noskin *et al.*, 2000).

#### 2.9.2 Biocontrol of bacterial pathogens in milk

Milk, produced by mammals including cows, sheep, goats and camels, is consumed by humans in many countries around the world. In its natural state, milk is highly perishable as it provides an excellent medium for the growth and metabolism of microorganisms which are able to cause rapid spoilage (Singh and Bennett, 2002).
Milk can also support the growth of pathogens and there have been outbreaks associated with consumption of contaminated milk in Australia and worldwide (Kirk et al., 2010; Oliver et al., 2009).

### 2.9.2.1 Raw milk

Although pasteurization has been used extensively in industrialised countries for decades as a means of increasing milk shelf life and destroying pathogenic organisms (Singh and Bennett, 2002), there appears to be increasing interest in raw milk consumption in these countries, which is likely associated with some people’s desire to consume natural, unprocessed foods (Oliver et al., 2009). Proponents of raw milk consumption have advocated enhanced nutritional qualities and health benefits of raw milk, but this is not substantiated by reputable scientific evidence. In contrast, the risks associated with raw milk consumption have been well documented (Oliver et al., 2009). While Standard 4.2.4 of the Australia New Zealand Food Standards Code prescribes that all fluid milk products sold for human consumption within Australia must be pasteurized or equivalently heat treated (Food Standards Australia New Zealand, FSANZ, 2011), can be circumvented by marketing raw milk products as “bath milk”, or for pet or animal consumption (Oliver et al., 2009) (Figure 2.5).

![Product labels of two raw milk products marketed for cosmetic use. Both products were located in the refrigerated cabinet of a health food store next to pasteurized milk products for human consumption.](image-url)
Pathogenic *E. coli*, including STEC, have been detected in raw milk, and, furthermore, have been implicated in disease outbreaks caused by consumption of contaminated raw milk (Upton and Coia, 1994; Goh *et al.*, 2002; Baylis 2009). Similarly, sporadic cases of *E. coli* infection have occurred following consumption of raw milk on farms (Martin *et al.*, 1986; Trevena *et al.*, 1996; FSANZ, 2009b). As discussed in Section 2.8.4.1, enterococci are also commonly associated with raw milk. While there have been no reported incidents of enterococcal illness associated with milk consumption, potential exists for the transfer of virulence or antibiotic resistance genes from dairy derived strains to gut microbiota and therefore limiting these organisms in milk could be worthwhile (Franz *et al.*, 1999).

### 2.9.2.2 Pasteurized milk

Pasteurized milk has also been implicated in outbreaks of foodborne illness. Specifically, pathogens including *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. have all been implicated in pasteurized milk outbreaks, typically as a result of inadequate pasteurization or post-pasteurization contamination (PPC) (Boor and Murphy, 2002). There have been several cases in Australia where pasteurized milk products have been recalled by regulatory authorities due to contamination with *E. coli* (FSANZ 2006; FSANZ, 2009a). A recent case involved the state-wide recall of Woolworths Fresh Milk Lite in Victoria, Australia (Figure 2.6). *E. coli* is very sensitive to pasteurisation but can enter milk following post-pasteurization contamination.
Gram-positive organisms including *Enterococcus* spp. may be responsible for limiting the shelf life of pasteurized milk (Boor and Murphy, 2002). While Gram-positive rods including *Bacillus* spp. are the predominant microorganisms isolated from pasteurized milk following refrigerated storage, Gram-positive cocci including *Enterococcus* spp., have also been identified as potentially contributing to spoilage (Ternstrom et al., 1993). *Enterococcus* spp. are psychrotrophic, and are among the most heat resistant of the Gram positive cocci. They are also adaptable to many growth conditions and as such can survive and multiply in pasteurized milk during refrigeration (Giraffa, 2003).

### 2.9.3 Phage biocontrol of pathogens in milk

Several studies describe using phages to control pathogens including *Salmonella* and *Listeria* in different types of cheese made from pasteurized or raw milk (Modi et al., 2001; Whichard et al., 2003; Carlton et al., 2005). The reports concerning phage biocontrol of pathogens in fluid bovine milk predominantly focus on *S. aureus* (O’Flaherty et al., 2005b; Garcia et al., 2007; Martinez et al., 2008; Garcia et al., 2009). A new avenue of potential research could investigate using phages to control *E. coli* and *Enterococcus* spp. in milk, given their association.
2.10 Summary

This review summarised the main areas of bacteriophage research with a focus on those applications concerning control of pathogenic bacteria. Interest in phage therapy has regained momentum in the West due to the increased prevalence of infections caused by drug-resistant bacteria coupled with significant advancements in understanding of phage biology. Furthermore, interest in phage based biocontrol of pathogens in diverse fields including agriculture, aquaculture, and food production, continues to grow. There are several commercial phage based products in use in the United States for controlling *L. monocytogenes* in foods, *Xanthomonas* spp. on plants and *E. coli* O157:H7 in food animals.

The review also identified new areas for potential research into phage biocontrol. While several studies have recently been published concerning phage biocontrol of *E. coli* on surfaces and in foods, these have focused on the pathogen O157:H7. Furthermore, none have investigated controlling *E. coli* contamination in milk. *E. coli* has been implicated in outbreaks of disease associated with contaminated raw and pasteurized milk and there is potential for phages to be added to pasteurized milk as a hurdle step, or directly to raw milk, as a means of increasing the safety of these products.

Furthermore, phage biocontrol of *Enterococcus* spp. was identified as warranting further research, given the prevalence of enterococci, including ARE, in foods of animal origin, and the increasing burden of enterococcal-associated illness. While several studies have investigated enterococcal phages for therapeutic applications, there are currently no reports of phage-based biocontrol of enterococci.
CHAPTER 3

Materials and Methods
3.1 Equipment

Table 3.1
Equipment used in the current investigation

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioanalyzer</td>
<td>Agilent Technologies, Palo Alto, California, USA</td>
<td>Analysis of DNA fingerprints</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf South Pacific, North Ryde, NSW, Australia</td>
<td>Centrifugation of solutions</td>
</tr>
<tr>
<td>Finnpipette micropipettes</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
<td>Dispensing liquids</td>
</tr>
<tr>
<td>Gel Doc XR System, PC and Quantity One 1D software</td>
<td>Bio-Rad</td>
<td>Capturing images of UV gels</td>
</tr>
<tr>
<td>Laminar Flow Cabinet</td>
<td>Gelman Sciences Australia, Cheltenham, VIC, Australia</td>
<td>Drying plates, sterile work</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Eppendorf</td>
<td>Centrifugation of solutions</td>
</tr>
<tr>
<td>Mini-Sub cell GT electrophoresis gel tank</td>
<td>Bio-Rad</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>MyCycler™</td>
<td>Bio-Rad</td>
<td>PCR</td>
</tr>
<tr>
<td>Platform shaker</td>
<td>Bio-Rad</td>
<td>Mixing cultures</td>
</tr>
<tr>
<td>PowerPac Mini power supply</td>
<td>Bio-Rad</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Stomacher (Lab-blender 400)</td>
<td>FSE, Australia</td>
<td>Homogenisation of samples</td>
</tr>
<tr>
<td>UV light source</td>
<td>Integrated Sciences, Chatswood, NSW, Australia</td>
<td>Agarose gel visualisation</td>
</tr>
<tr>
<td>Vortex mixer</td>
<td>Ratek Instruments, North Ryde, NSW, Australia</td>
<td>Mixing solutions</td>
</tr>
<tr>
<td>Water bath</td>
<td>Labec, Marrickville, NSW, Australia</td>
<td>Incubation of cultures</td>
</tr>
</tbody>
</table>
3.2 Microbiological media

All dehydrated media bases were supplied by Oxoid (Australia), Becton Dickson or Merck (Australia). *Listeria* Brilliance Agar (Oxoid) was supplied as prepared plates. All media were prepared using distilled water (dH2O) and sterilised by autoclaving at 121°C for 16 minutes unless otherwise stated.

Table 3.2
Microbiological media used in this investigation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mass (g) per 1 L of water</th>
<th>Natural pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baird Parker Agar (BPA)</td>
<td>63.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td>Brain Heart Infusion (BHI)</td>
<td>37.0</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>BHI agar</td>
<td>47.0</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Buffered Peptone Water</td>
<td>20.0</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>0.1% Buffered Peptone Water (BPW)</td>
<td>2.0</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Chromocult® Coliform Agar</td>
<td>26.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7 ± 3.0</td>
</tr>
<tr>
<td>KF Streptococcus Agar (KFSA)</td>
<td>76.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td><em>Listeria</em> Brilliance Agar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate Count Agar (PCA)</td>
<td>23.5</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>Potato Dextrose Agar (PDA)</td>
<td>39.0</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>Tetrathionate Broth (TB)</td>
<td>46.0&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>n.a.</td>
</tr>
<tr>
<td>Tryptone Soy Broth (TSB)</td>
<td>30.0</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>Tryptone Soy Agar (TSA)</td>
<td>40.0</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>Xylose Lysine Desoxycholate (XLD)</td>
<td>53.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Agar</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Sterilised by autoclaving at 115°C for 10 minutes

<sup>b</sup>Following sterilisation, medium was cooled to 50°C and then 1 vial (5 mL) of 1% 2,3,5-

<sup>c</sup>Triphenyltetrazolium chloride was aseptically added

<sup>d</sup>Medium was boiled only (no autoclaving)

<sup>e</sup>Following sterilisation, medium was cooled to 45°C and then 20 mL of iodine-iodide solution was aseptically added

<sup>f</sup>Following sterilisation, medium was cooled to 50°C and then 50 mL of egg yolk tellurite emulsion was aseptically added
Soft agar overlays were prepared by adding 0.7% bacteriological agar to the appropriate broth media. The solution was then boiled in order to dissolve the agar and dispensed into small tubes prior to sterilisation. Overlays were re-autoclaved at 115°C for 10 minutes and cooled to 50°C for use in experiments.

### 3.3 Chemicals and buffers

#### 3.3.1 Commercial kits and solutions

<table>
<thead>
<tr>
<th>Chemical/solution/kit</th>
<th>Manufacturer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA reagents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol:Chloroform: Isoamyl alcohol</td>
<td>Invitrogen, Mulgrave, VIC, Australia</td>
<td>Isolation of phage DNA</td>
</tr>
<tr>
<td>Agarose</td>
<td>Promega, Alexandria, NSW, Australia</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Ethidium bromide (10 mg/mL)</td>
<td>Sigma, Castle Hill, NSW, Australia</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>GeneRuler™ DNA Ladder mix</td>
<td>Quantum Scientific, Murarrie, QLD, Australia</td>
<td>DNA marker</td>
</tr>
<tr>
<td>6x Loading Dye</td>
<td>Quantum Scientific</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>Sigma</td>
<td>DNA precipitation</td>
</tr>
<tr>
<td><strong>PCR reagents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GoTaq® Green</td>
<td>Promega</td>
<td>PCR</td>
</tr>
<tr>
<td><strong>Bacteria work</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROTECT Bead Storage</td>
<td>Technical Service Consultants</td>
<td>-70°C frozen storage of bacterial cultures</td>
</tr>
<tr>
<td>Remel ERIC RapID STR Biochemical identification</td>
<td>Oxoid, Basingstoke, UK</td>
<td>Speciation of <em>Enterococcus</em> Isolates</td>
</tr>
<tr>
<td>Staphytect</td>
<td>Oxoid</td>
<td>Confirmation of coagulase-positive Staphylococci</td>
</tr>
<tr>
<td>Microbact™ 12A/12B Identification System</td>
<td>Oxoid</td>
<td>Identification of <em>Salmonella</em></td>
</tr>
<tr>
<td>Chloroform</td>
<td>Sigma</td>
<td>Lysing of bacterial cells</td>
</tr>
</tbody>
</table>
3.3.2 Preparation of buffers

All chemicals used in the preparation of buffers were of analytical reagent (AR) grade and were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise specified. All buffers were prepared with Milli-Q water (Millipore). General use buffers and solutions were prepared according to Sambrook and Russell (2001). Stock solutions were appropriately diluted for use in experiments.

Table 3.4
Buffers and solutions

<table>
<thead>
<tr>
<th>Buffers and solutions</th>
<th>Composition (in 1 L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffered Saline (PBS) (pH 7.2)</td>
<td>3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.2</td>
</tr>
<tr>
<td>Saline</td>
<td>8.5 g NaCl</td>
</tr>
<tr>
<td>Suspension Medium (SM)</td>
<td>50 mM Tris-HCl, 100 mM NaCl, 80 mM MgSO4, 0.01% (w/v) gelatin, pH 7.5</td>
</tr>
<tr>
<td>50 x TAE</td>
<td>2 M Tris base, 6.5 M EDTA disodium salt, pH 8.0</td>
</tr>
<tr>
<td>TENS</td>
<td>50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 0.3% Sodium dodecyl sulphate (SDS)</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris, 1 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>2M ZnCl₂</td>
<td>272.6 g filter sterilized</td>
</tr>
<tr>
<td>20% PEG-8000</td>
<td>200 g PEG-8000</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>292.22 g NaCl</td>
</tr>
<tr>
<td>Tris-Glycine electrophoresis buffer</td>
<td>25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v)</td>
</tr>
</tbody>
</table>

3.3.3 Enzymes

All enzymes were supplied in lyophilised form. Restriction enzymes were reconstituted in nuclease free water at a concentration of 100 U/mL. All other
enzymes were prepared at stock concentrations listed in Table 3.5 and diluted appropriately for use in experiments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Manufacturer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNase I (10 mg/mL)</td>
<td>Invitrogen</td>
<td>Digestion of phage DNA</td>
</tr>
<tr>
<td>RNase A (100 mg/mL)</td>
<td>Invitrogen</td>
<td>Digestion of phage RNA</td>
</tr>
<tr>
<td>S1 Nuclease (20,000 U)</td>
<td>Invitrogen</td>
<td>Digestion of single-stranded DNA</td>
</tr>
<tr>
<td><strong>Proteinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinase K (20 mg/mL)</td>
<td>Invitrogen</td>
<td>Digestion of phage proteins</td>
</tr>
<tr>
<td><strong>Restriction endonucleases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HaeIII (GG↓CC )</td>
<td>Fermentas, Scoresby, VIC, Australia</td>
<td>Restriction endonuclease (RE) digestion of phage DNA</td>
</tr>
<tr>
<td>HhaI (GCG↓C)</td>
<td>Fermentas</td>
<td>RE digestion of phage DNA</td>
</tr>
<tr>
<td>EcoRI (G↓AATTC)</td>
<td>Promega</td>
<td>RE digestion of phage DNA</td>
</tr>
<tr>
<td>SspI (AAT↓ATT)</td>
<td>New England Biolabs, Ipswich, MA, USA</td>
<td>RE digestion of phage DNA</td>
</tr>
<tr>
<td>AluI (AG↓CT)</td>
<td>Fermentas</td>
<td>RE digestion of phage DNA</td>
</tr>
<tr>
<td>MboI (↓GATC)</td>
<td>Fermentas</td>
<td>RE digestion of phage DNA</td>
</tr>
<tr>
<td>EcoRV (GAT↓ATC )</td>
<td>Fermentas</td>
<td>RE digestion of phage DNA</td>
</tr>
</tbody>
</table>

### 3.4 Bacterial cultures

Table 3.6 and Table 3.7 list the range of bacterial organisms used throughout this study. All bacteria were grown aerobically at 37°C.
### Table 3.6

**E. coli** strains and characteristics

<table>
<thead>
<tr>
<th><strong>E. coli</strong></th>
<th>Relevant characteristics</th>
<th>Application in the current study</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>Laboratory strain</td>
<td>Primary host of phage</td>
<td>Swinburne University</td>
</tr>
<tr>
<td>K12</td>
<td>Laboratory strain</td>
<td>Primary host of phage</td>
<td>Swinburne University</td>
</tr>
<tr>
<td>G106</td>
<td>Laboratory strain</td>
<td>Primary host of phage</td>
<td>Monash University</td>
</tr>
<tr>
<td>G131</td>
<td>Laboratory strain</td>
<td>Primary host of phage</td>
<td>Monash University</td>
</tr>
<tr>
<td>HB101</td>
<td>Laboratory strain</td>
<td>Primary host of phage</td>
<td>Monash University</td>
</tr>
<tr>
<td>EDL 933</td>
<td>EHEC reference strain</td>
<td>Primary host of phage</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>O157:H7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘EH41’ O113:H21</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘EH38’ O111</td>
<td>non-motile</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
</tr>
<tr>
<td>‘EH43’ O130:H11</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘9922251’ O15</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘94156747’ O26:H11</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘EH48’ O5</td>
<td>non-motile</td>
<td>EHEC clinical isolate</td>
<td>Phage host range</td>
</tr>
<tr>
<td>2348/69</td>
<td>EPEC reference strain</td>
<td>Primary host of phage</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘11-1’ O111</td>
<td>EPEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘12-1’ O119</td>
<td>EPEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘C771’ O142:H6</td>
<td>EPEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘ABERDEEN’ O55:H6</td>
<td>EPEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
<tr>
<td>‘W1056’ O55:H7</td>
<td>EPEC clinical isolate</td>
<td>Phage host range</td>
<td>University of Melbourne</td>
</tr>
</tbody>
</table>
Table 3.7

Enterococcus strains and characteristics

<table>
<thead>
<tr>
<th>Enterococcus</th>
<th>Relevant characteristics</th>
<th>Application in the current study</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. faecalis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 39058</td>
<td>Laboratory strain</td>
<td>Phage host range</td>
<td>Swinburne University</td>
</tr>
<tr>
<td>M168600</td>
<td>vanA VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td>M193272</td>
<td>vanA VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td>M233165</td>
<td>vanA VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td>M255048</td>
<td>vanA VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td>M259849</td>
<td>vanA VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td>M252807</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Austin Hospital</td>
</tr>
<tr>
<td><strong>E. faecium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26480327</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>26483870</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>27196046</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>26460334</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>27206589</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>26472389</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>26472440</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>27188876</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>27187926</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
<tr>
<td>26477277</td>
<td>vanB VRE clinical isolate</td>
<td>Phage host range</td>
<td>Box Hill Hospital</td>
</tr>
</tbody>
</table>
3.4.1 Storage and maintenance of bacterial cultures

Agar slants were prepared for medium term storage of bacterial cultures. Overnight *E. coli* cultures were subcultured onto TSA slants and incubated at 37°C for 24 hours prior to storage at 4°C for up to six months. *Enterococcus* cultures were similarly stored using BHI slants. For long term storage of cultures, the Protect Bead storage system (Technical Service Consultants) was used which enabled frozen storage at -70°C for several years.

3.5 Preparation of raw materials for isolation of phages

3.5.1 Sewage

Samples of untreated sewage were collected from the Western Treatment (Werribee, Victoria) and the Eastern Treatment Plant (Bangholme, Victoria). Samples were transported on ice to the laboratory at Swinburne University. Three 20 mL aliquots of each sewage sample were centrifuged at 4 000 x g for 20 minutes. The supernatants were pooled and filtered (0.22 µm filter) and stored at 4°C until use.

3.5.2 Landfill leachate

A sample of landfill leachate was provided by Bass Coast Shire Council (Westernport, Victoria). Fifty millilitres of the sample was centrifuged at 4 000 x g for 20 minutes and filtered through a 0.22 µm filter and stored at 4°C until use.

3.5.3 Clinical faecal specimens

Human faecal specimens collected as part of another study approved by the Swinburne Human Research Ethics Committee were provided as 10% homogenates in PBS (pH 7.2). Samples were further diluted at a ratio of 1:1000 in PBS (pH 7.2), centrifuged at 5 000 x g for 15 minutes, filtered and stored as above.
3.5.4 Concentration of sewage and landfill leachate by freeze drying

Two hundred millilitre samples of sewage and landfill leachate were centrifuged at 4 000 x g for 20 minutes. The supernatants were transferred to round bottom flasks, which then attached to a freeze dryer overnight. Lyophilised samples were then reconstituted in a small volume (1 mL) of SM buffer and stored at 4°C until use.

3.6 Isolation of bacteriophages and preparation of stocks

3.6.1 Primary isolation of enterococcal phages

*Enterococcus* cultures were subcultured into fresh BHI broth and further incubated at 37°C with shaking for 4 hours. Following incubation, 100 µL of each exponential phase culture in addition to at least 100 µL of sewage, landfill leachate or faecal sample filtrate were added to molten BHI soft agar overlays. Overlays were then poured over BHI agar base plates and allowed to set before incubation at 37°C overnight. Plates were then inspected for plaque formation (Carey-Smith *et al.*, 2006).

3.6.2 Primary isolation of coliphages

The process outlined above was repeated using *E. coli* and TSB and TSA. In addition, bacterial hosts were pooled by adding three or four *E. coli* strains to a single overlay in an attempt to select for broader host range phages.

3.6.3 Serial purification of bacteriophage isolates

Several colonies of overnight host bacterial culture were inoculated into 10 mL BHI and incubated at 37°C with shaking for 3-4 hours. Meanwhile, a plaque obtained from a primary bacteriophage isolation experiment was picked using a sterile glass Pasteur pipette and the agar plug was resuspended in 1 mL SM. The suspension was mixed by vortex and incubated at room temperature for two hours, before serial dilution in SM from $10^{-1}$ to $10^{-8}$. The overlay method was then performed using one dilution of phage suspension and the appropriate bacterial host per overlay. Plates
were incubated at 37°C overnight and then inspected for plaque formation. This method was repeated four times successively, each time using a freshly picked, well-isolated plaque from a previous purification, to produce a pure plate lysate (Adams, 1959).

### 3.6.4 Preparation of high titre bacteriophage stocks

Following purification, a plaque was serially diluted ten-fold in SM buffer. For each dilution, three agar plates were overlaid with the appropriate host bacteria, before incubation at 37°C overnight. Following incubation, plates with just-confluent lysis were chosen to prepare stocks. To recover phages, 3 mL of SM was added to each plate, which were incubated at room temperature for one hour with regular swirling. The liquid was then decanted into a sterile 50 mL tube containing 20 mL SM buffer, and the soft agar overlay was added to the tube after scraping from the base-plate with a glass spreader. The suspension was mixed by vortex for one minute and then incubated at 37°C with shaking for 30 minutes. The suspension was subsequently centrifuged at 1 300 x $g$ for 15 minutes to remove agar and bacterial debris. The supernatant was transferred to a fresh, sterile tube and the centrifugation step was repeated. The supernatant was then filtered into a fresh sterile tube and stored at 4°C. The titre of bacteriophage stocks was determined by plaque assay before use in further experiments (Carey-Smith et al., 2007).

### 3.6.5 Plaque assays to determine titre of bacteriophage solutions

A ten-fold serial dilution of bacteriophage solution was performed in 900 µL aliquots of SM, from $10^{-1}$ to $10^{-9}$. The appropriate bacterial host was grown in broth to exponential phase and 100 µL was added to eight molten overlays. One-hundred microlitres of each bacteriophage dilution was added to an overlay. The overlays were poured onto agar base plates and allowed to set before incubation at 37°C overnight. Following incubation, a plate was selected that contained between 30 and 300 plaques, and the plaques counted to determine the plaque-forming units (PFU) per millilitre of original solution (Carey-Smith et al., 2007).
3.7 Biological characterisation of bacteriophage isolates

3.7.1 Chloroform sensitivity of bacteriophage isolates

Five-hundred microlitres of chloroform were added to an equal volume of high-titre bacteriophage sample, vortex mixed for 30 seconds and then centrifuged at 2 000 x g for 5 minutes. The aqueous phase was transferred to a fresh tube and again extracted with a 10% volume of chloroform. The solution was once more mixed by vortex and centrifuged. The aqueous phase was transferred to a fresh sterile tube and stored at 4°C until use.

One-hundred microlitres of exponential phase bacterial host culture was added to a molten overlay and poured over an agar base plate. Once the plate had set, 20 µL of chloroform extracted bacteriophage solution was spotted onto the surface and allowed to set with the aid of a laminar flow cabinet for approximately 30 minutes. A positive control plate was also prepared as above using a non-chloroform treated sample. Plates were incubated at 37°C overnight and inspected for plaque formation.

3.7.2 Host range of bacteriophage isolates

One-hundred microlitres of exponential phase bacterial host culture was added to a molten overlay and poured over an agar base plate. Once the plate had set, 20 µL of undiluted bacteriophage stock solution was spotted onto the surface and allowed to set with the aid of a laminar flow cabinet for approximately 30 minutes. Plates were incubated at 37°C overnight and inspected for plaque formation (Goodridge et al., 2003). Positive spot tests were confirmed by plaque assay using serially diluted phage solution.

3.7.3 Sensitivity of bacteriophage isolates to different pH

The effect of pH was examined by diluting phage preparations in SM adjusted to different pH levels (pH 2.2, 4, 6, 7, 9) with 1 N HCl or 1 N NaOH. Samples were taken after one hour of incubation at 37°C. Phage titres were determined by the overlay method (Tanji et al., 2005).
3.7.4 One-step growth curves of bacteriophage isolates

One-step growth curves were performed in order to determine the latent period and burst size of phage isolates. Host bacteria were grown in 10 mL of broth to exponential phase (OD$_{600}$ 0.4 to 0.5) and cells were harvested by centrifugation. The pellet was resuspended in 2.5 mL of fresh broth media (ca.10$^9$ CFU/mL). Bacteriophage was added at MOI of between 0.01 and 0.03 (Pajunen et al., 2000). The suspension was incubated for 5 minutes at RT to allow adsorption of phage. To synchronise infection, the mixture was then centrifuged and pelleted cells were resuspended in 10 mL of broth (TSB for *E. coli* and BHI for *Enterococcus*), and incubation was continued in a shaking water bath at 37°C. Samples, taken at five minute intervals, were immediately diluted and plated for phage titration. Plates were incubated at 37°C overnight and PFU/infected cell was plotted on a graph and the burst size and latent period were subsequently determined.

3.7.5 Purification of bacteriophages by PEG-precipitation

DNase I and RNase A were added to 10 mL high-titre bacteriophage stock solution to a final concentration of 10 µg and 1 µg/mL, respectively, and the solution was incubated at 37°C for 30 minutes. The mixture was subsequently centrifuged at 6 000 x g for 10 minutes at 4°C and the supernatant was transferred to a fresh tube. NaCl was added to the supernatant at a final concentration of 1 M and the solution was incubated at 4°C with rotation for 1 hour. The solution was centrifuged at 6 000 x g for 10 minutes at 4°C and the supernatant transferred to a fresh tube. PEG-8000 was added to the supernatant to a final concentration of 10% (v/v) and incubated at 4°C with rotation for at least 2 hours (Yamamoto et al., 1970).

Following incubation, the precipitate was pelleted by centrifugation at 6 000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 500 µL SM (without gelatin). The resuspended solution was then extracted with an equal volume of chloroform, vortex mixed for 30 seconds and then centrifuged at 2 000 x g for 5 minutes. The aqueous phase was transferred to a fresh tube and again extracted with a 10% volume of chloroform. The solution was once more mixed by vortex and
centrifuged. The aqueous phase was transferred to a fresh sterile tube and stored at 4°C until use (Sambrook and Russell, 2001).

3.7.6 Transmission electron microscopy of bacteriophage isolates

Phage solutions purified by PEG precipitation were pipetted onto the surface of grids and washed twice with dH₂O. The grids were then stained with 0.5% uranyl acetate for approximately 2 minutes. Excess stain was removed by gentle blotting on Whatman paper and then air dried. Grids were inspected with a Zeiss EM10 transmission electron microscope (Zeiss/LEO, Oberkochem, Germany).

3.8 Genetic characterisation of bacteriophage isolates

3.8.1 Extraction of bacteriophage nucleic acid

To 10mL bacteriophage stock, DNase I and RNase A were added to a final concentration of 10 µg and 1µg/mL respectively, and incubated at 37°C for 30 minutes. Following incubation, filter-sterilised 2 M ZnCl₂ was added at a ratio of 1:50 (v/v) and incubated at 30°C for 5 minutes. Phage particles were centrifuged at 4 000 x g for 5 minutes, and the pellet resuspended in 1 mL TENS. Proteinase K was added to a final concentration of 100 µg/mL and the solution incubated at 65°C for 30 minutes (Shivu et al., 2007).

The solution was further deproteinated by extraction with Phenol:Chloroform:Isoamyl Alcohol (Ph:CHCl₃:IAA) (25:24:1). An equal volume of Ph:CHCl₃:IAA was added to resuspended solution, mixed by vortexing for 30 seconds and centrifuged for 3 minutes at 10 000 x g. The upper aqueous layer was transferred to a new tube and the extraction was repeated. The aqueous phase was transferred to a fresh tube, and an equal volume of isopropanol was added. The solution was gently mixed by pipetting and the precipitate was collected by centrifugation (3 000 x g for 2 minutes) and washed twice with 70% ethanol. The bacteriophage DNA pellet was solubilised in 80 µl of sterile TE. DNA extracts were analysed by agarose gel electrophoresis (Shivu et al., 2007).
3.8.2 Nuclease digestion of bacteriophage DNA

Phage nucleic acid was digested with DNase I, RNase A and S1 nuclease in order to determine the nucleic acid type. 20 U of each enzyme was used to digest 100 ng of nucleic acid at 37°C for 1 hour. The digests were subsequently electrophoresed through 0.7% agarose gel (Shivu et al., 2007).

3.8.3 Restriction endonuclease analysis of bacteriophage DNA

Approximately 500 ng of bacteriophage DNA was digested with 10 U of restriction enzyme in the presence of the appropriate buffer. Restriction digests were incubated for a minimum of 2 hours at 37°C before analysis by agarose gel electrophoresis.

3.8.4 Random Amplification of Polymorphic DNA (RAPD) analysis of phage DNA

RAPD PCR was performed to generate fingerprints of phage DNA. Table 3.8 lists the primers used in RAPD PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>CCG CAG CCA A</td>
<td>Shivu et al., (2007)</td>
</tr>
<tr>
<td>P2</td>
<td>AAC GGG CAG A</td>
<td>Shivu et al., (2007)</td>
</tr>
</tbody>
</table>

Each 30 µL reaction contained 12.5 µL GoTaq Green Master Mix, 20 pmol/µL each primer, 100 ng DNA template and 10.5 µL nuclease-free water. RAPD PCR was performed as detailed in Table 3.9 and products were analysed following agarose gel electrophoresis.
Table 3.9

RAPD PCR conditions by primer

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycles</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P1</td>
<td>P2</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>94.0</td>
<td>94.0</td>
</tr>
<tr>
<td>Amplification</td>
<td>35</td>
<td>94.0</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.0</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.0</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
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<td>72.0</td>
<td>72.0</td>
</tr>
</tbody>
</table>

3.8.5 PCR of *E. coli* virulence factor *eaeA* in bacteriophage DNA

Coliphage DNA was tested for the presence of the *E. coli* pathogenic factor, enteropathogenic attachment and effacement (*eaeA*), by PCR. Table 3.10 describes the primers used in the PCR.

Table 3.10

Primers used in PCR of *eaeA*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>eaeA2</em></td>
<td>CGGTCGCGCACCAGGATTC</td>
<td>629</td>
<td>Heuvelink <em>et al.</em> (1995)</td>
</tr>
</tbody>
</table>

Table 3.11 outlines the PCR conditions used for amplification of *eaeA* in coliphage DNA as described by Heuvelink *et al.* (1995).
Table 3.11
PCR conditions for eaeA

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>94.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Amplification</td>
<td>35</td>
<td>94.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.0</td>
<td>3.0</td>
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<tr>
<td></td>
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<td>72.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Extension</td>
<td>1</td>
<td>72.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

3.8.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse DNA fragments generated by restriction digests and PCR. Samples were electrophoresed in a Bio-Rad Mini-Sub electrophoresis apparatus in 1×TAE. The percentage of agarose was adjusted according to the size of the DNA fragments being analysed. Ethidium bromide (final concentration of 0.5 μg/mL) was incorporated into agarose gels to allow detection of DNA bands. Electrophoresis was routinely performed at 120 V, 30-40 mAmps for 40 min. A DNA marker was included in each agarose gel.

3.9 Phage biocontrol of E. coli and Enterococcus sp. on surfaces

3.9.1 Preparation of test surfaces

All fabrics and plastics (100% cotton, 100% cotton terry, poly cotton, 100% polyester, and 100% polyethylene) were hand washed and cut to size (approximately 5cm). Glass slides and stainless steel coupons were similarly hand washed and air dried. All test surfaces were subsequently sterilized by autoclaving at 121°C for 15 minutes and allowed to dry before being aseptically transferred to sterile Petri dishes.
3.9.2 Preparation of bacterial hosts

Overnight broth cultures of host bacteria were centrifuged at 10 000 x g for two minutes. Bacterial pellets were washed twice and resuspended in PBS (pH 7.0). A small volume of the resuspended bacteria was then added to fresh PBS (7.0) and adjusted to a density equivalent to the 0.5 McFarland turbidity standard using a colorimeter. Solutions were then diluted 10-fold to an approximate concentration of 1.0 x 10^7 CFU/mL. Phage stocks were diluted in SM to the appropriate concentration for use in biocontrol studies.

3.9.3 Phage treatment of surfaces

Test surfaces were inoculated with approximately 1.0 x 10^6 CFU of test bacteria which were allowed to absorb. Surfaces were then inoculated with 100 µL of phage solution (final concentration of 10^6, 10^7, 10^8 or 10^9 PFU/mL) or an equivalent volume of PBS (control). Surfaces were incubated at ambient temperature (21±3°C) for between ten minutes and two hours.

3.9.4 Recovery of bacteria and phages from surfaces

To recover the bacteria, test surfaces were aseptically transferred to 50 mL Falcon tubes (BD, New Jersey, USA) containing 10 mL PBS and sterile glass beads (Viazis et al., 2011a). The contents were mixed and serially diluted in PBS. Dilutions were immediately surface plated onto the appropriate medium (KF streptococcus agar or TSA). Chloroform (1% [w/v]) was subsequently added to dilutions and plaque assays were then performed on BHI using the overlay method to determine phage titres. All plates were incubated at 37°C for 24-48 hours. Viable counts were subsequently determined. All experiments were conducted in triplicate.
3.10 Microbiological survey of raw milk

3.10.1 Raw milk sample collection and preparation

Raw cow’s milk samples were provided by two Victorian milk manufacturers. All of the milk was collected from dairy farms located in Victoria, Australia and transported to the manufacturers for processing. Staff from each manufacturer collected samples from bulk milk tankers immediately upon arrival at the facility, in sterile, 100 mL specimen containers. Samples were stored at 4°C until delivery on ice to Swinburne University of Technology. A total of 56 samples were provided, 24 from Manufacturer 1 and 32 from Manufacturer 2. In addition, ten samples of a raw “bath milk” were purchased from a local farmers market and transported on ice to Swinburne University of Technology. The bath milk was produced at a dairy farm in Victoria, Australia.

Samples were prepared and tested on the day of delivery. For microbiological analysis, 10 mL of each sample were added to 90 mL of 0.1% buffered peptone water (BPW) in sterile blender bags and homogenized in a Stomacher for two minutes. Samples were serially diluted in sterile 0.1% buffered peptone water.

3.10.2 Microbiological analysis of raw milk

3.10.2.1 Standard plate count (SPC)

One mL of the $10^1$ dilution of prepared sample was aseptically dispensed into the base of a sterile Petri dish. Approximately 15 mL molten Plate Count Agar (Oxoid) were then added to the dish and the contents were mixed thoroughly. This process was repeated using the $10^2$ and $10^3$ dilutions of each prepared sample. Once set, the plates were incubated at 37°C overnight.

3.10.2.2 Coliforms and *Escherichia coli*

A volume (0.1 mL) of the $10^1$ dilution of prepared sample was aseptically dispensed onto a Chromocult® coliform agar (Merck, NJ, USA) plate and spread evenly over
the entire surface using a sterile glass spreader. The plates were then incubated at 37°C for 24 hours.

3.10.2.3 *Enterococcus* spp.

A volume (0.1 mL) of the $10^{-1}$ dilution of prepared sample was aseptically dispensed onto a KF Streptococcus (Oxoid) plate and spread evenly over the entire surface using a sterile glass spreader. The plates were then incubated at 37°C for 48 hours. Isolates were confirmed by PCR (described below) and then identified to the species level using Remel ERIC RapID STR biochemical identification kits (Oxoid), which were used according to the manufacturer’s instructions.

3.10.2.4 Coagulase-positive *Staphylococcus* spp.

A volume (0.1 mL) of the $10^{-1}$ dilution of prepared sample was aseptically dispensed onto a Baird Parker Agar (Oxoid) plate and spread evenly over the entire surface using a sterile glass spreader. The plates were then incubated at 37°C for 48 hours. Presumptive coagulase-positive *Staphylococcus* colonies were confirmed using Staphytect Kit (Oxoid) which was used according to manufacturer’s instructions.

3.10.2.5 *Salmonella*

Twenty five grams of each neat sample were added to 225 mL of BPW, and incubated at 37°C for 18 hours. Ten millilitres were then added to 90 mL of Tetrathionate Broth USA (Oxoid). This suspension was incubated at 37°C for 18 hours. A loopful of the suspension was then streaked onto duplicate Xylose Lysine Desoxycholate (Oxoid) plates and incubated at 37°C for 48 hours. Presumptive *Salmonella* colonies were subcultured onto TSA and then confirmed using the Microbact™ 12A/12B Gram Negative Identification System (Oxoid) according to the manufacturer’s instructions.
3.10.2.6 *Listeria monocytogenes*

Twenty five millilitres of each neat sample were added to 225 mL BPW. The suspension was incubated at 37°C for 24 hours. A loopful of each enriched suspension was then streaked onto *Listeria* Brilliance Agar (Oxoid) and incubated at 37°C for 24 hours. Presumptive *L. monocytogenes* colonies were subcultured onto fresh TSA, incubated at 37°C overnight and then confirmed by PCR (section 3.10.4)

3.10.3 Isolation of bacterial DNA recovered from raw milk

Bacterial isolates were subcultured into TSB (Oxoid) and incubated overnight. The cultures were centrifuged at 10 000 x g for two minutes and the pellet was washed twice with PBS (pH 7.2), resuspended in 100 μL sterile distilled water and boiled for 10 minutes (Altalhi and Hassan, 2009). The suspension was then immediately placed on ice for a further five minutes before centrifugation at 8 000 x g for two minutes to pellet cellular debris. The supernatant was collected in a fresh tube and frozen at -18°C until use.

3.10.4 PCR

PCR was used to confirm presumptive *Listeria monocytogenes* colonies and *Enterococcus* spp. colonies. PCR was also used to detect antibiotic resistance markers *vanA* and *vanB* in confirmed *Enterococcus* isolates and toxin genes *stxl* and *stx2* in confirmed *E. coli* isolates. The PCR assays were employed as previously described (Altalhi and Hassan 2009; Brian *et al.*, 1992; Zhang *et al.*, 2009). The primer sequences and amplification conditions used for PCR are outlined in Table 3.12. PCR reactions were performed in a total volume of 25 μL, using 50 ng template DNA, 1 μL (10 pmol) forward primer, 1 μL (10 pmol) reverse primer and 25 μL GoTaq Green PCR master mix (200 μM each dNTP, 0.5 U *Taq* DNA polymerase and 1.5 mM MgCl₂) (Promega). Amplification was performed in a thermal cycler (Bio-Rad). The PCR products were resolved by 1.5% agarose gel electrophoresis.
### Table 3.12
Primer sequences, predicted sizes of PCR amplification products and PCR conditions

<table>
<thead>
<tr>
<th>Organism</th>
<th>PCR target</th>
<th>Primer sequences (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>PCR conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>hly</td>
<td>F:AGCACAACAAACTGAAGCAAAGGA R:ATTGTGATTCCTCTGTAAGCCATTTCGTCAT</td>
<td>596</td>
<td>1 cycle at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 7 min.</td>
<td>Zhang <em>et al.</em> (2009)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>16S rDNA</td>
<td>F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGCTACTCCCATTGT</td>
<td>144</td>
<td>1 cycle at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 15 s, annealing at 61°C for 20 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 7 min.</td>
<td>Malinen <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>vanA</em></td>
<td></td>
<td>F: TCTGCAATAGAGATAGGCGC R: GGAGTAGCTATCCAGCATT</td>
<td>377</td>
<td>2 cycles at 94°C for 2 min, annealing at 55 °C for 30 s and extension at 72 °C for 30 s; followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 4 min.</td>
<td>Lemcke and Bülte (2000)</td>
</tr>
<tr>
<td><em>vanB</em></td>
<td></td>
<td>F: GCTCCGAGCCTGATCGGACA R: ACGATCGCCATCCCTCTGCA</td>
<td>529</td>
<td>1 cycle at 94°C for 1 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min; followed by a final extension at 72°C for 5 min.</td>
<td>Lemcke and Bülte (2000)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>stx1</td>
<td>F: AAGATCGCCATCTCGTTGACTCTTCT R: TGCCATCTGCACTCGGATGCA</td>
<td>366</td>
<td>1 cycle at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 15 s; followed by a final extension at 72°C for 7 min.</td>
<td>Brian <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td>stx2</td>
<td>F: CGATCGTCACCTCAGCTTGGTCATCA R: GGATATTCTGCCACTCGGACACC</td>
<td>282</td>
<td>As above.</td>
<td>Brian <em>et al.</em> (1992)</td>
</tr>
</tbody>
</table>
3.11 Temperature abuse of raw milk

Triplicate samples of raw milk with initial low SPC were incubated in a domestic refrigerator (5 ± 4°C) for seven days. One milliliter aliquots of each sample were removed every 24 hours, plated on PCA and incubated at 37°C overnight. In addition, samples were tested for growth of coliforms, \textit{E. coli}, coagulase-positive \textit{Staphylococcus} spp. and \textit{Enterococcus} spp. every 24 hours during the study period.

3.12 Survey of pasteurized milk to determine prevalence of \textit{E. coli} and \textit{Enterococcus} spp.

Twenty full cream, pasteurized milk samples were tested for the presence of \textit{Enterococcus} spp. and \textit{E. coli}. One hundred microliter aliquots were dispensed onto KF Streptococcus Agar and Chromocult® Coliform Agar and a sterile spreader was used to distribute the samples evenly over the surface. All plates were incubated at 37°C. Chromocult® Coliform agar plates were incubated for 24 hours and KF Streptococcus agar plates were incubated for 48 hours.

3.13 Investigation of a pasteurized milk recall

3.13.1 Preparation of samples

Following the recall of a pasteurized milk product in Victoria, Australia a 3M™ Petrifilm™ plate (3M, St. Paul, MN, USA) containing presumptive \textit{E. coli} isolated from the implicated product was provided by the manufacturer. Twenty isolated colonies were subcultured onto Chromocult® coliform agar and incubated at 37°C overnight. Presumptive \textit{E. coli} colonies were subcultured into TSB and DNA was then isolated using the method described in 3.10.3
3.13.2 Enterobacterial repetitive intergenic consensus (ERIC) PCR

ERIC PCR was performed in order to generate DNA fingerprints of *E. coli* isolated from the recalled pasteurized milk using the protocol described by Duan *et al.* (2009). Two ERIC primers, ERIC1 (5’-ATGTAAGCTCCTGGGGATTCAC -3’) and ERIC2 (5’-AAGTAAGTGACTGGGGTGAGCG -3’), were used in each PCR reaction. The PCR reactions were performed in 25 µL solutions containing 25 pmol of each primer, GoTaq® Green master mix and 30 ng template DNA. PCR was performed in a thermocycler (Bio-Rad) using the conditions outlined in Table 3.13. PCR products were resolved by agarose gel electrophoresis before further analysis by Bioanalyzer (Agilent).

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>94.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Amplification</td>
<td>35</td>
<td>94.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Extension</td>
<td>1</td>
<td>72.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

3.13.3 Analysis of ERIC PCR products using Agilent 2100 Bioanalyzer

ERIC-PCR products were also analysed using the Agilent 2100 Bioanalyzer and DNA 7500 Kit (Agilent). The Bioanalyzer is an automated system that uses chip-based nucleic acid separation by capillary electrophoresis. Chips were prepared and loaded according to manufacturer’s instructions. Briefly, a chip was primed and loaded with gel-dye matrix (Agilent). One microliter of each PCR product was then added to a separate sample well, and the DNA marker (Agilent) was added to the marker well. The chip was then vortex mixed and loaded into the Bioanalyzer instrument. Electropherograms and virtual gels generated by the Bioanalyzer software were used to compare the DNA profiles of each isolate.
3.13.4 Construction of a dendrogram

FreeTree software (Hampl et al., 2001) was used to construct a dendrogram of DNA fingerprints obtained by ERIC PCR. Similarity analysis was performed using Jaccard coefficient. Clustering was performed using the unpaired group method of analysis (UPGMA). To test the robustness of the branches, 100 replicates of the data were analysed by bootstrapping. The reference tree was then imported to TreeView software.

3.14 Phage biocontrol of *E. coli* and *Enterococcus* sp. in milk

3.14.1 Preparation of milk samples

UHT milk was purchased in 100 mL tetra packs. Pasteurized milk was purchased in 1 L cartons which were transported on ice to the laboratory. Raw milk samples were collected from a local milk processing plant and transported on ice to the laboratory.

Pasteurized milk was tested for the presence of *E. coli* and *Enterococcus* spp. by plating on Chromocult and KF streptococcus agar. Raw milk samples were similarly tested for *E. coli* and *Enterococcus* in addition to SPC, coliforms and coagulase positive *Staphylococcus*. Three 10 mL samples of each milk were transferred to 50 mL Falcon tubes prior to inoculation with bacteria and infection with phage. Tubes were refrigerated until use.

3.14.2 Preparation of bacteria and phages

Enterococcal and *E. coli* hosts were prepared as described in Section 3.4. Phage stocks were appropriately diluted prior to addition to milk samples.
3.14.3 Phage biocontrol of Enterococcus sp. in UHT and pasteurized milk

Milk samples were inoculated with $1.0 \times 10^5$ CFU/mL of *E. faecalis* host and $1.0 \times 10^9$ PFU/mL of appropriate phage or an equivalent volume of PBS (control). Samples were incubated either at 25°C for 24 hours or at 10°C for 168 hours. 100 µL samples were removed from each milk sample at defined intervals. Samples were serially diluted in 0.1% BPW and dilutions were plated onto KF streptococcus agar and incubated at 37°C for 24 hours. Viable counts were subsequently determined. Chloroform (1% [v/v]) was then added to dilutions and phage titres were determined using the overlay method. Studies were conducted in triplicate.

3.14.4 Turbidimetric analysis of coliphage infection of *E. coli*

A 50 µL aliquot of overnight *E. coli* culture was inoculated into TSB and incubated with shaking at 37°C. When turbidity reached an OD$_{660}$ of 0.1, the culture was infected with phage or phage cocktail at an MOI of 10. Culture turbidity was monitored for 12 hours (Tanji *et al.*, 2005).

3.14.5 Phage biocontrol of *E. coli* in UHT milk

Milk samples were inoculated with $1.0 \times 10^5$ CFU/mL of *E. coli* host and $1.0 \times 10^9$ PFU/mL of appropriate phage, phage cocktail or an equivalent volume of PBS (control). Samples were incubated either at 25°C for 24 hours or in a domestic refrigerator ranging in temperature from 5-9°C for 168 hours. 100 µL samples were removed from each milk sample at defined intervals. Samples were serially diluted in 0.1% BPW and dilutions were plated onto TSA and incubated at 37°C for 24 hours. Viable counts were subsequently determined. Chloroform (1% [v/v]) was then added to dilutions and phage titres were determined using the overlay method. Studies were conducted in duplicate.
3.14.6 Phage biocontrol of *E. coli* in raw milk

Milk samples were inoculated and incubated as described in Section 3.14.5. Samples were plated on Chromocult coliform agar and incubated at 37°C overnight prior to determination of viable count. Phage titres were also monitored throughout the study as described in Section 3.14.5. Studies were conducted in duplicate. The pH of the milk samples was also monitored using pH indicator strips (Merck).

3.14.6.1 Raw milk monitoring

Additional samples of raw milk without added *E. coli* or phage were monitored for SPC, Coliforms, *E. coli*, *Enterococcus* spp. and coagulase positive *Staphylococcus* spp. over the period of the biocontrol studies conducted at 5-9°C.

3.15 Statistical methods

Comparison between means was carried out using paired-samples t-tests. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Version 18.0. Statistical significance was defined at a *p* value of less than or equal to 0.05.
CHAPTER 4

Isolation and characterisation of bacteriophages infecting
Enterococcus faecalis and Escherichia coli
4.1 Introduction

Bacteriophages are ubiquitous in nature and have been isolated from many environmental sources (Chibani-Chennoufi et al., 2004a). Isolation of phages from the environment is relatively easy but in order to determine their suitability for biocontrol, therapy or other applications, characterisation must be conducted. Characterisation of phages typically involves biological and genetic means. Primary characterisation is sometimes followed by complete genome sequencing where phages are to be used commercially (Carlton et al., 2005).

4.1.1 Enterococcal phages

Enterococcal phages have been isolated from many sources including sewage, fresh water, human faeces, human saliva and piggery effluent for a variety of purposes (Bachrach et al., 2003; Uchiyama et al., 2008; Letkiewicz et al., 2009; Mazaheri et al., 2010; Santiago-Rodriguez et al., 2010). Enterococcal phages have been proposed as an alternative indicator of faecal contamination in water sources (Santiago-Rodriguez et al., 2010) and several studies have investigated the ability of phages to control VRE infection in mice (Biswas et al., 2002) and in humans (Letkiewicz et al., 2009). However, no studies have described the characterisation of enterococcal phages for the purpose of biocontrol applications.

4.1.2 Coliphages

Coliphages are among the most studied of all bacteriophages. In particular, the T-phages have been integral to the development of molecular genetics, as well as having been extensively used as model systems to study their hosts and other viruses of plants and animals (Abedon, 2000). Coliphages have also been investigated as biocontrol agents in a range of applications predominantly related to food production and agriculture. Most biocontrol studies have focused on the control of the pathogen E. coli O157:H7 due to its ability to cause severe illness in humans (Kudva et al., 1999; O’Flynn et al., 2004; Sharma et al., 2005; Abuladze et al., 2008). Fewer studies have described the characterisation of coliphages for the biocontrol or
treatment of other pathogenic *E. coli* such as EPEC (Chibani-Chennoufi *et al.*, 2004b; Viscardi *et al.*, 2008).

### 4.1.3 Chapter aims

The major aims of this chapter were to:

i. Isolate and purify novel enterococcal phages, including VRE phages, from a variety of sources

ii. Isolate and purify coliphages from sewage using methods to select for broad host range

iii. Characterise selected phage isolates to determine their potential for use as biocontrol agents.

This chapter is divided into two sections. The first section describes the isolation and characterisation of novel enterococcal phages. The second section describes similar methods used in the isolation and characterisation of coliphages.
4.2 Results

4.2.1 Isolation and characterisation of enterococcal phages

4.2.1.1 Primary isolation of enterococcal phages

Primary isolation of enterococcal bacteriophages from environmental sources produced many plaques of different morphology. Plaque assays from sewage samples and landfill leachate resulted in a number of small and large plaque morphologies. Several turbid plaques were discarded because it was likely they were formed by temperate phages and these are not considered useful for biocontrol applications. Six clear plaques were chosen for purification based on differences in plaque morphology, source of isolation and primary host. No plaques were observed following plaque assays using faecal samples. Table 4.1 outlines the general characteristics of the six phages, hereafter referred to as ϕSUT1, ϕSUT2, ϕSUT3, ϕSUT4, ϕSUT5 and ϕSUT6.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>Source</th>
<th>Plaque diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ϕSUT1</td>
<td>E. faecalis</td>
<td>Sewage</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>ATCC 39058</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ϕSUT2</td>
<td>VRE M168600</td>
<td>Sewage</td>
<td>0.7</td>
</tr>
<tr>
<td>ϕSUT3</td>
<td>VRE M168600</td>
<td>Sewage</td>
<td>1.0</td>
</tr>
<tr>
<td>ϕSUT4</td>
<td>VRE M168600</td>
<td>Leachate</td>
<td>0.5</td>
</tr>
<tr>
<td>ϕSUT5</td>
<td>VRE M168600</td>
<td>Leachate</td>
<td>1.0</td>
</tr>
<tr>
<td>ϕSUT6</td>
<td>VRE M252807</td>
<td>Sewage</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Treatment of plate lysates with 1% (v/v) chloroform had no effect on the titres obtained, indicating that the ϕSUT phages did not contain lipids. Enveloped enterococcal phages have been described in the literature (Bachrach et al., 2003). Following serial purification of plaques from plate lysates, high titre bacteriophage stocks were prepared for use in characterisation experiments. Phages ϕSUT2 and
φSUT5 were discarded as they produced only low titres indicating poor efficiency of plating. The remaining phages consistently produced high titres (Table 4.2).

Table 4.2
Typical φSUT phage stock titres

<table>
<thead>
<tr>
<th>Phage</th>
<th>Titre (PFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φSUT1</td>
<td>3.5 X 10^{10}</td>
</tr>
<tr>
<td>φSUT3</td>
<td>2.6 X 10^{10}</td>
</tr>
<tr>
<td>φSUT4</td>
<td>3.4 X 10^{10}</td>
</tr>
<tr>
<td>φSUT6</td>
<td>5.9 X 10^{10}</td>
</tr>
</tbody>
</table>

4.2.1.2 Host range of φSUT phages

Host range studies were conducted using a range of vancomycin-resistant clinical isolates of both *E. faecalis* and *E. faecium* as well as Gram positive cocci belonging to the genera *Staphylococcus* and *Streptococcus*. Initial spot tests indicated that φSUT1 was the only phage able to infect a host in addition to the primary isolation host. A confirmatory plaque assay was subsequently performed using VRE (M252807) as the host. The plaque assay yielded a 2.0 log_{10} unit lower titre compared to the titre produced using the primary host, *E. faecalis*. Furthermore, the plaques produced on VRE (M252807) were smaller in diameter than those produced on *E. faecalis* (Figure 4.1). Thus it was determined that φSUT1 had a lower efficiency of plating on VRE (M252807) compared to its primary host, *E. faecalis* (ATCC 39058).
Enterococcal phages were readily isolated from sewage, as well as landfill leachate; the latter source has not been previously reported. Enterococci and other enteric bacteria are present in high concentrations in landfill leachate (Scarpino et al., 1980; Grisey et al., 2010) and it could therefore be a novel source for identification of further phages. Enterococcal phages have also been isolated from human saliva (Bachrach et al., 2003), piggery effluent (Mazaheri et al., 2010), and water sources from a range of geographic locations including Japan (Uchiyama et al., 2008) and Puerto Rico (Santiago-Rodriguez et al., 2010). No broad host range enterococcal phages were recovered from either sewage or leachate. This could be due to the method of isolation, the range of hosts used, or parameters such as incubation temperature. Santiago-Rodriguez et al. (2010) reported that a greater number of enterococcal phages were isolated from water samples when incubated at 22°C compared to 37°C, which is the temperature at which the current study was conducted.

Future enterococcal phage studies should undertake a more extensive screening process to increase the chances of isolating broad host range phages. Although genetically similar strains of VRE can be found in both hospital and urban wastewater, hospital wastewater has been shown to contain a higher prevalence of
VRE than urban wastewater. Furthermore, VRE strains isolated from hospitalised patients have been found to exhibit different antibiotic resistance patterns compared to those originating from environmental sources (Kotzamanidis et al., 2009). Therefore, screening for phages in hospital wastewater and patient faecal material should be investigated as more targeted sources of clinical VRE phages, while screening of other water sources may be preferable for isolation of phages of environmental enterococci.

4.2.1.3 φSUT phage morphology

Transmission electron microscopy revealed that all of the φSUT phages were tailed. Based on this information, the phages were classified into the order Caudovirales. All of the phages except for φSUT1 possessed isometric heads (Figure 4.2). φSUT1 was larger than the other phages and possessed an unusually long, elongated head. All phage tails appeared long and flexible, suggesting that they belonged to the family Siphoviridae. The phages φSUT3, φSUT4 and φSUT6 were further classified as belonging to the B1 morphotype due to their icosahedral capsids (Demuth et al., 1993). The average size of phage particles was determined by measuring at least five distinct particles of each phage (Table 4.3).

![Transmission electron micrographs of (A) φSUT1, (B) φSUT3, (C) φSUT4 and (D) φSUT6 respectively at 145,000x magnification.](image)
Enterococcal phages comprise a diverse range of morphologies, with tailed phages belonging to the *Myoviridae* and *Siphoviridae* commonly reported (Biswas et al., 2002; Uchiyama et al., 2008). However, in a study of enterococcal phages isolated from piggery effluent, Mazaheri et al. (2010) identified filamentous, polyhedral and pleomorphic enterococcal phages. In addition, Bachrach et al. (2003) observed small (70 nm diameter) enveloped, spherical enterococcal phages isolated from human saliva.

φSUT 3, 4 and 6 had comparable dimensions to the tailed phages (No. 8, 31, 42, 110) reported by Mazaheri et al. (2010) and to the group B enterophages described by Bonilla et al. (2010). φSUT1 most closely resembled phage no. 195 reported by Mazaheri et al. (2010), although its head was longer and narrower. However, the tailed phages reported by Santiago-Rodriguez et al. (2010) were much smaller particles than the φSUT phages, with an average head size of 12 nm in diameter and tail of 60 nm in length. This indicates the diversity of enterococcal phage morphologies even within the same family.

### 4.2.1.4 Analysis of φSUT phage nucleic acid

While the genome type of the phage isolates was unknown, nucleic acid isolation was attempted using a DNA protocol (Shivu et al., 2007). The extracts were electrophoresed in an ethidium bromide stained agarose gel and a band was detected for each phage. To confirm the presence of DNA, the nucleic acid extracts were

<table>
<thead>
<tr>
<th>Phage</th>
<th>Head width (nm)</th>
<th>Head length (nm)</th>
<th>Tail length (nm)</th>
<th>Tail width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φSUT 1</td>
<td>126</td>
<td>45</td>
<td>216</td>
<td>13.5</td>
</tr>
<tr>
<td>φSUT 3</td>
<td>63</td>
<td>63</td>
<td>180</td>
<td>10</td>
</tr>
<tr>
<td>φSUT 4</td>
<td>63</td>
<td>63</td>
<td>166</td>
<td>9</td>
</tr>
<tr>
<td>φSUT 6</td>
<td>63</td>
<td>63</td>
<td>198</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4.3

Average sizes of φSUT phage particles determined by electron microscopy
digested by DNase I. Undigested DNA and RNase A digested extracts were used as controls (Figure 4.3).

![Figure 4.3](image)

**Figure 4.3** Agarose gel (0.7%) depicting nuclease digestion of φSUT phage DNA. Lanes 1-3: undigested φSUT1 DNA, DNase digested φSUT1 DNA, RNase A digested φSUT1 DNA, respectively; lanes 4-6: undigested φSUT3 DNA, DNase digested φSUT3 DNA, RNase A digested φSUT3 DNA, respectively; lanes 7-9: undigested φSUT4 DNA, DNase digested φSUT4 DNA, RNase A digested φSUT4 DNA, respectively; lanes 10-12. Undigested φSUT6 DNA, DNase digested φSUT6 DNA, RNase A digested φSUT6 DNA, respectively; lanes M, molecular weight marker.

The DNA of the φSUT phages was digested with the restriction enzymes HaeIII and HhaI. Successful restriction (Figure 4.4) indicated that the φSUT phages possessed unmodified, double-stranded DNA (dsDNA) genomes. Double-stranded DNA genomes are consistent with genomes found within the Caudovirales (Voyles, 2002). Double-stranded DNA genomes have been commonly reported among the published enterococcal phages, including φEF24C (Uchiyama et al., 2008) and tailed phages reported by Mazaheri et al. (2010). However, enterococcal phages with ssDNA and ssRNA genomes have also been reported (Mazaheri et al. 2010). These genome types are typically found within the genera Inoviridae and Leviviridae respectively (Voyles, 2002).
Figure 4.4  Agarose gels (0.7% and 1.5%) showing electrophoretic patterns of (a) HaeIII digestion of phage DNA and (b) HhaI digestion of phage DNA. Lanes 1-4 represent restriction profiles for φSUT1, φSUT3, φSUT4 and φSUT6, respectively. Lanes M, molecular weight marker.

Methods of comparing the relatedness of dsDNA phages include pulse-field gel electrophoresis (PFGE), random fragment length polymorphism (RFLP) and
randomly amplified polymorphic DNA–PCR (RAPD-PCR). The two latter methods were attempted as part of this study. Comparison of the DNA fragments generated by HaeIII and HhaI digestion illustrated that the φSUT phages were genetically distinct. However, the pattern produced by both digests suggested φSUT3 and φSUT4 to be almost identical, indicating a close genetic relationship. This is supported by the fact that they infected solely the same host, despite the fact that they were isolated from different environmental sources. In contrast, the RFLPs produced by φSUT1 and φSUT6 DNA digested by both enzymes were clearly distinct.

### 4.2.1.5 RAPD analysis of φSUT phage DNA

While RAPD-PCR has been used successfully to distinguish between several phages of *Vibrio harveyi* (Shivu *et al.*, 2007), it did not produce a fingerprint for φSUT1, φSUT3 or φSUT4 and only one band was produced for φSUT6 (Figure 4.5). Potential alternatives such as random sequence analysis of the DNA fragments produced by restriction digests may be a more robust means of determining genetic relationships of the phages (Goodridge *et al.*, 2003).

![Agarose gel showing RAPD-PCR fingerprints of φSUT phages. Lanes 1-4; φSUT1, φSUT3, φSUT4, φSUT6, respectively. Lanes M, molecular weight marker.](image_url)

**Figure 4.5**
4.2.1.6 One-step growth curves

One step growth curves were used to determine the latent period and burst size of each φSUT phage (Table 4.4).

<table>
<thead>
<tr>
<th>Phage</th>
<th>Latent Period (min)</th>
<th>Burst size (PFU/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φSUT1</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>φSUT3</td>
<td>35</td>
<td>183</td>
</tr>
<tr>
<td>φSUT4</td>
<td>30</td>
<td>186</td>
</tr>
<tr>
<td>φSUT6</td>
<td>30</td>
<td>130</td>
</tr>
</tbody>
</table>

The phage latent period is defined by the timing of phage-induced host cell lysis (Abedon et al., 2001). The latent periods of the φSUT phages were within the range expected for tailed phages and the latent periods calculated for φSUT4 and φSUT6 were comparable to that of the enterococcal phage, φEF24C (Uchiyama et al., 2008).

While the burst sizes of φSUT3, φSUT4 and φSUT6 were larger than the median burst size of tailed phages (110-120 PFU/cell), the burst size of φSUT1 was smaller than expected. Other enterococcal phages have reported burst sizes ranging from 10 to 1000 PFU/cell (Bonilla et al., 2010).

Figure 4.6 illustrates the one-step growth curves determined for each of the φSUT phages propagated on their respective hosts at 37°C.
Figure 4.6  One-step growth curves of φSUT phages at 37°C.  (a) φSUT1 on *E. faecalis*; (b) φSUT3 on VRE (M168600); (c) φSUT4 on VRE (M168600) (d) φSUT on VRE (M252807). Shown are the PFU per infected cell at different time points.
4.2.1.7 Sensitivity of φSUT phages to pH

The isolated φSUT phages were intended for use as potential biocontrol agents on surfaces and in foods. In addition, given their ability to infect VRE, they could also be used in the treatment of enterococcal infections. In either case, it is possible that the phages might be exposed to low pH conditions either in the human gastrointestinal tract or in acidic foods. Therefore, their sensitivity to low pH was tested \textit{in vitro} (Figure 4.7).

![Figure 4.7](image)

\textbf{Figure 4.7} Stability of φSUT phages at different pH following exposure for one hour. Error bars represent 1 standard deviation. \( \phi \text{SUT1}, \phi \text{SUT3}, \phi \text{SUT4}, \phi \text{SUT6} \)

All of the phages were completely sensitive to pH 2.2 and pH 3.0. \( \phi \text{SUT1}, \phi \text{SUT3} \) and \( \phi \text{SUT6} \) titres remained stable after one hour of incubation at pH 4.0, pH 6.0, pH 7.0 and pH 9.0. The titre of \( \phi \text{SUT4} \) decreased by approximately 1.0 \( \log_{10} \) PFU/mL at pH 9.0 but was stable at pH 4.0, pH 6.0 and pH 7.0. No other studies of therapeutic enterococcal phages described their sensitivity to pH. This is likely because the phages were administered either rectally (Letkiewicz \textit{et al.}, 2009) or via i.p. injection (Biswas \textit{et al.}, 2002) rather than orally.
The results from the current study are supported by those reported by Verthé et al. (2004) for an Enterobacter-specific phage whose titre dropped below the detection limit immediately following exposure to pH 2.0 but was stable at pH 4.0, 6.0, 7.0 and 9.0.

These results suggest that φSUT phages would require protection from stomach acid if they were to be administered orally to treat infection. Several studies have reported success using antacids including NaHCO$_3$ and CaCO$_3$ to neutralize acid in vitro prior to addition of phages to improve phage viability (Slopek et al., 1983; Verthé et al., 2004; Tanji et al., 2005). Stanford et al. (2010) were also able to improve phage viability in the gastrointestinal tract of cattle by incorporating the phages into gelatin capsules.

### 4.2.1.8 Summary

The narrow host range of the φSUT phages isolated and characterised as part of this study suggests that they may have limited use as real world biocontrol agents. However, due to the paucity of literature surrounding enterococcal phages characterised for such applications, they may be useful for conducting preliminary studies into the feasibility of biocontrol of enterococci.
4.2.2 Isolation and characterisation of coliphages

4.2.2.1 Primary isolation of coliphages

In order to increase the probability of isolating coliphages with a broad host range, a range of strategies was employed. Firstly, several *E. coli* strains were combined for use in primary isolation of coliphages. Secondly, large volumes of sewage were centrifuged, lyophilised and resuspended in small volumes of SM buffer in an attempt to concentrate the phages present. Finally, sewage samples from treatment plants serving different geographic locations were used in primary isolation of phages.

The pooling of hosts did not increase the number of plaques isolated. Often, turbid plaques formed and it was necessary to perform a plaque assay using each individual host in order to determine the host range. There was no significant difference in the number of plaques isolated from sewage originating from the two treatment plants. Six plaques, designated фEC1, фEC5, фEC6, фEC9, фEC10 and фEC11, were chosen for further characterisation based on differences in plaque morphology.

As was observed with the фSUT phages, treatment of plate lysates with 1% (v/v) chloroform produced no effect on the titres obtained, indicating that the ФSUT phages did not contain any lipids. After serial purification of plaques from plate lysates, high titre bacteriophage stocks were produced for use in characterisation experiments.

Sewage and faecal material have long been recognised as the best sources for isolation of a diverse range of coliphages due to their association with *E. coli*. Historically, phages T2, T4 and T6 were likely isolated from faecal material or sewage (Abedon, 2000). F⁺ RNA coliphages which are genetically and morphologically different to the T-even phages are also frequently isolated from sewage and surface waters and are often used as indicators of animal or human faecal pollution in water (Cole *et al.*, 2003).
4.2.2.2 **Host range and plaque morphology of isolated coliphages**

The host range of the phage isolates was compared to the host range of T7. The phages were also tested against other enterobacteria including *Salmonella* sp. and *Shigella* sp., however, none of the isolates was able to infect these hosts. While an effort was made to select for broad host range coliphages, only three of the six isolates (фEC6, фEC9 and фEC11) were able to produce clear plaques on more than three hosts. These isolates were able to infect all of the non-pathogenic strains tested and at least one pathogenic strain. фEC9 had the broadest host range among the pathogens, infecting all but three. However, turbid plaques indicating only partial lysis were observed for several hosts (Table 4.5).

<table>
<thead>
<tr>
<th><strong>E. coli</strong></th>
<th>T7</th>
<th>фEC1</th>
<th>фEC5</th>
<th>фEC6</th>
<th>фEC9</th>
<th>фEC10</th>
<th>фEC11</th>
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<td>O157:H7</td>
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<td>O130:H11</td>
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<td>O26 H11</td>
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<td>O127:H6</td>
<td>+</td>
<td>-</td>
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<td>O111</td>
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<td>O119</td>
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<td>O142:H6</td>
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<tr>
<td>O55:H6</td>
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<td>O55:H7</td>
<td>-</td>
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<td>-</td>
<td>(+)</td>
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</tr>
</tbody>
</table>

(+) partial lysis

The ability of the phage isolates to be propagated on non-pathogenic hosts is a desirable feature for therapeutic or biocontrol candidates to avoid the potential for transfer of virulence or toxin genes to recipient cells via generalized transduction.
(Hagens and Loessner, 2010). Furthermore, it would simplify production and increase the safety of the phage preparation (Santos et al., 2010). Conversely, some researchers suggest that candidates for phage therapy should not infect non-pathogenic *E. coli* to reduce the effect on intestinal microbiota. However, a study by Chibani-Chennoufi et al. (2004b) demonstrated that their coliphages, while able to infect non-pathogenic *E. coli*, did not affect commensal *E. coli* in the gastrointestinal tract of mice. Interestingly, the phages were able to infect *E. coli* recovered from mice faeces. The authors suggested that the commensal *E. coli* are viable but non-growing in the gut lumen which does not allow for phage replication and are furthermore physically protected against phage infection in the mucin layer. They further hypothesised that the *E. coli* were able to resume active growth on laboratory media and therefore become susceptible to phage infection. Conversely, *E. coli* introduced into the gastrointestinal tract of mice following disruption to the indigenous flora remained susceptible to the phages in vivo. This study provided evidence that broad host-range phages such as the φEC phages isolated in this study may be effective in treating infection without detrimentally affecting the host microbiota.

Figure 4.8 illustrates the plaques produced by φEC6, φEC9 and φEC11 on a lawn of *E. coli* K12. All of the phages produced similar titres in the range of $10^{10}$ PFU/mL and produced the same sized plaques on each of the different hosts tested when propagated under the same conditions, indicating a relatively constant efficiency of plating.
4.2.2.3 Coliphage morphology

Transmission electron microscopy revealed that all of the phages belonged to the order Caudovirales (Figure 4.9). The average size of the phage particles was determined by measuring at least five distinct particles of each phage (Table 4.6). Based on morphology, φEC6 was classified into the family Siphoviridae given its isometric capsid and long, flexible tail (Voyles, 2002). φEC9 had an elongated head and contractile tail. This structure resembles the morphotype of the coliphage T4 (Goodridge et al., 2003). While a baseplate was evident, no tail fibres were observed in the micrographs. Based on measurements of the head and tail, φEC9 was classified into the Myoviridae. φEC11 belonged to the Podoviridae due to the presence of a very short tail. The capsid measurements were comparable to those of T7 (Brock, 1990).
FIGURE 4.9 Electron micrographs of coliphages (a) фEC6, (b) фEC9 and (c) фEC11. Scale bar represents 100 nm.

### Table 4.6

Average sizes of phage particles as observed by transmission electron microscopy

<table>
<thead>
<tr>
<th>Phage</th>
<th>Head length (nm)</th>
<th>Head width (nm)</th>
<th>Tail length (nm)</th>
<th>Tail width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>фEC6</td>
<td>61</td>
<td>61</td>
<td>133</td>
<td>12</td>
</tr>
<tr>
<td>фEC9</td>
<td>87</td>
<td>72</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>фEC11</td>
<td>58</td>
<td>58</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

Phages of the *Myoviridae* have been reported to possess a wider host range than phages belonging to the *Siphoviridae* among a range of bacterial hosts including *Listeria monocytogenes* (Loessner et al., 1997), *Staphylococcus* (Pantucek et al., 1998), *Vibrio cholerae* (Miller et al., 2003a) and also *E. coli* (Chibani-Chennoufi et al., 2004b). The results from the current study support this observation. фEC9 was the only phage out of the three isolates classified into the *Myoviridae* and was able to
infect all but three of the *E. coli* strains against which it was tested, including pathogenic strains belonging to a range of O serotypes.

### 4.2.2.4 DNA isolation and characterisation

DNA was isolated from the phages using the phenol:chloroform method described previously. The DNA extracts were initially confirmed by agarose gel electrophoresis (data not shown). Restriction digests were then performed using the endonucleases AluI, EcoRI, EcoRV, HaeIII, HhaI, MboI, RsaI and SspI. φEC6 and φEC11 could not be digested by any of the restriction enzymes used (data not shown) while φEC9 was digested by SspI only (Figure 4.9).

![Figure 4.10](image)

**Figure 4.10** Agarose gel (1.5%) illustrating electrophoretic pattern of φEC9 DNA. Lane 1, undigested (Control); Lane 2, digested with *SspI*; Lane M, molecular weight marker.

Further analysis revealed that φEC6 and φEC11 could be digested by DNase I but not RNase A or S1 nuclease, indicating that the DNA was double stranded despite being resistant to digestion by the restriction enzymes tested (Figure 4.10) (Shivu *et al.*, 2007). Double-stranded DNA genomes are consistent with bacteriophages belonging to the *Caudovirales* (Voyles, 2002).
Many bacteriophages possess genomes with modified DNA bases. These modifications enable phage DNA to avoid restriction by endonucleases upon entry into the host cell. For example, T4 DNA contains glucosylated hydroxymethyl cytosine instead of cytosine making it resistant to most restriction enzymes, with a few exceptions, including EcoRV and SspI (Carlson et al., 1994). φEC9 differed from T4 in that it was digested by SspI but not EcoRV. This was also observed by Chibani-Chennoufi et al., (2004b) for their T-even-like coliphages.

Many phages possess other antirestriction mechanisms which make their DNA resistant to host restriction endonucleases. These mechanisms include: inhibition of restriction enzymes by phage-encoded proteins, modification of DNA via self-methylation or activation of host methylase; or the absence of recognition sites for particular restriction enzymes (Kruger and Bickle, 1983; Sharp, 1986). These systems have been found commonly in coliphages and may account for the inability of φEC6 and φEC11 DNA to be cut by the restriction endonucleases tested. Further genetic analysis of the coliphages would be necessary should the phages prove effective biocontrol agents.
T4-like phages are considered to be good candidates for therapeutic applications as they are often able to infect a range of pathogenic *E. coli*, can be propagated on non-pathogenic hosts, and do not usually encode any virulence genes (Miller *et al.*, 2003b). However, O’Flynn *et al.* (2004) reported that their T4-like coliphage isolate, e41/c, was found by PCR to possess the virulence factor *eaeA* which they suggested had been acquired from its EHEC host. This finding highlights the importance of using non-pathogenic hosts to propagate phages destined for biocontrol or therapy. None of the coliphages from the current study were found to possess *eaeA* (Figure 4.11).

**Figure 4.12** Agarose gel (0.7%) depicting amplicons generated by PCR of *E. coli* virulence factor, *eaeA*: Lane 1, фEC6 DNA; Lane 2, фEC9 DNA; Lane 3, фEC11 DNA, Lane 4, negative control, Lane 5, positive control (*E. coli* O157:H7) Lane M, DNA ladder.
4.2.2.5 One-step growth curves

One step growth curves were used to determine the length of the latent period and the burst size of each phage (Table 4.7).

<table>
<thead>
<tr>
<th>Phage</th>
<th>Latent Period (min)</th>
<th>Burst size (PFU/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φEC6</td>
<td>20</td>
<td>113</td>
</tr>
<tr>
<td>φEC9</td>
<td>30</td>
<td>62</td>
</tr>
<tr>
<td>φEC11</td>
<td>30</td>
<td>80</td>
</tr>
</tbody>
</table>

The latent periods of φEC9 and φEC11 were longer than has been reported for the T-even phages, whose latent periods range from 21 to 25.5 minutes. However, they were within the range typical of tailed phages (Ackermann and DuBow, 1987). Similarly, the burst sizes of these phages were smaller than the T even phages as well as the median burst size of tailed phages. The latent period and burst size of φEC6 were comparable to T2, whose latent period and burst size are 21 minutes and 120 PFU/cell, respectively (Brock, 1990).

The latent period and burst sizes of coliphages reported in the literature vary significantly. While greater burst sizes are typically associated with longer latent periods, Goodridge et al. (2003) reported a burst size of 38 for their coliphage, AR1, despite a longer than average latent period of 40 minutes (Abedon et al., 2001). The opposite was found in the current study, with φEC6 producing the greatest burst size despite having the shortest latent period of the phages studied.

The one-step growth curves for each φEC phage are depicted graphically in Figure 4.13.
Figure 4.13  One-step growth curves of coliphages on *E. coli* K12 at 37°C. Shown are the PFU per infected cell at different time points. (a) ϕEC6; (b) ϕEC9; (c) ϕEC11
4.2.2.6 Stability of coliphages at different pH values

The coliphages had a more variable tolerance to pH compared to the φSUT enterococcal phages. While φEC6 and φEC11 were not recoverable following exposure to pH 2.2 for one hour, φEC9 survived with only a small reduction in titre. In addition, φEC9 was recovered with no loss in titre following exposure to pH 3.0, while φEC6 was reduced by approximately 6.0 log_{10} units and φEC11 was not recovered. There was no significant difference in titres produced by φEC6 or φEC9 following exposure to pH 4.0 to pH 9.0. φEC11 was stable from pH 6.0 to pH 9.0 but a significant ($p < 0.05$) reduction in titre was observed following exposure to pH 4.0 (Figure 4.13).

The results observed for φEC6 and φEC11 are in agreement with those reported for other lytic phages of enterobacteria including Enterobacter aerogenes and E. coli as well as phages of Lactococcus garvieae, whereby the phage titres were reduced at pH lower than 3.0 (Nakai et al., 1999; Leverentz et al., 2001; Verthé et al., 2004; Tanji et al., 2005). However, the ability of phages to tolerate pH as low as 2.2, as φEC9 was able, has been less commonly reported.

![Graph representing Log_{10} PFU/mL coliphage recovered following exposure to different pH values for one hour. φEC6; φEC9; φEC11.](image-url)
Many studies that have investigated phages as therapeutic candidates have assessed their ability to survive acidic conditions such as would be encountered in the gastrointestinal tract. Several *in vitro* studies of different phages have found low pH to be a barrier to oral administration for therapy. Tanji *et al.* (2005) observed a 50% loss in viability of coliphages following exposure to pH 3.0 *in vitro* after 10 minutes of incubation. The phage titres remained stable for one hour thereafter. The experiment was carried out at 37°C with shaking. Under similar conditions, Verthé *et al.* (2004) observed that their *Enterobacter*-phage was immediately inactivated when exposed to gastric juice at pH 2.0. At 25°C, Nakai *et al.* (1999) observed a decrease in *Lactococcus*-phage titre within 30 minutes of incubation in buffer at pH 3.0 while no phage was recovered following incubation at pH 2.5. Of these studies, Verthe *et al.* (2004) and Tanji *et al.* (2005) were able to improve the viability of their phages in low pH buffers by addition of antacids.

However, an *in vivo* study of newly isolated coliphages by Chibani-Chennoufi *et al.* (2004b) found that the phages were able to passage through the gastrointestinal tract of mice unprotected. The T4-like phages were administered orally to mice and were subsequently recovered from the mice faeces at approximately the same titre as was administered. This indicated that the phages were able to survive transit through the gastrointestinal tract. The pylorus-proximal region of the stomach in mice has a mean pH value of 2.2. This finding was confirmed by a subsequent study using axenic mice which demonstrated that the phages were able to survive passage through the gastrointestinal tract in the absence of commensal *E. coli* which could be used for amplification. These results support the finding of the current study that φEC9 was able to survive at pH 2.2 for one hour.

The effect of pH on phages in biocontrol studies has also been measured indirectly. Leverentz *et al.* (2001) studied the effect of a phage preparation in reducing *Salmonella* Enteritidis on honeydew melon and apple slices. A reduced efficacy of the phage treatment was observed on the apple slices in addition to a reduction in phage titre compared to the treatment on honeydew. The authors suggested that the acidic pH of the apple slices could have contributed to the loss of phage viability.
Similar effects were observed for a phage preparation used in the biocontrol of *Listeria monocytogenes* on apples slices (Leverentz *et al.*, 2003). The phages were also tested in liquid culture for their ability to reduce *L. monocytogenes* at different pH. In the absence of phage, *Listeria* showed minimal growth below pH 4.5, increasing growth between pH 5 and 6 and optimum growth between 6 and 8. In the presence of the phages, *Listeria* was inhibited at neutral pH from (6.5 to 8.0) but growth was observed between pH 4.0 and pH 5.0 indicating that the phages were not active at this pH range.

EHEC has been demonstrated to survive in low pH foods such as yoghurt and fermented meats (Massa *et al.*, 1997). Therefore, the ability of the φEC coliphages to survive under acidic conditions unprotected would be advantageous if they were to be used as biocontrol agents in these foods. The methods that have been used to improve phage viability at low pH in therapeutic applications would not be practical for food based applications.
4.3 Chapter summary

A range of enterococcal phages and coliphages were isolated from environmental sources. Selected isolates were characterised in order to determine their suitability for use in biocontrol studies. All of the isolated phages belonged to the order *Caudovirales*.

4.3.1 φSUT enterococcal phages

The enterococcal phages had a very narrow host range, with φSUT1 being the only isolate able to infect more than one host. However, the efficiency of plating on the secondary host was low. While all of the φSUT phages were classified into the *Siphoviridae*, φSUT3, φSUT4 and φSUT6 belonged to the B1 morphotype due to the presence of icosahedral capsids. The enterococcal phages reported in the literature are morphologically diverse. Each of the φSUT phages possessed unmodified dsDNA genomes. HaeIII and HhaI restriction profiles indicated that φSUT3 and φSUT4 were closely related while φSUT1 and φSUT6 were genetically distinct. RAPD analysis did not produce DNA fingerprints for the phages.

One-step growth curves indicated that the φSUT phages had similar latent periods to other enterococcal phages reported in the literature (Uchiyama *et al.*, 2008). The average burst sizes produced by φSUT3 and φSUT4 were similar at 180 and 183 per infected cell respectively. φSUT6 produced a burst size of 130 while the burst size of φSUT1 was relatively small at 68 PFU/cell.

The ability of φSUT phages to persist during exposure to a range of pH was measured. As expected, none of the phages survived following incubation at pH 2.2 or pH 3.0 for one hour. φSUT1, φSUT3 and φSUT6 survived without significant loss in titre when incubated at pH 4.0, 6.0, 7.0 or 9.0 for one hour. φSUT4 was stable at pH 4.0, pH 6.0 and pH 7.0 but decreased at pH 9.0.
4.3.2 **φEC coliphages**

Coliphages φEC6, φEC9 and φEC11 were chosen for characterization based on differences in plaque morphology and host range. These phages had a broader host range than was observed for the φSUT phages. φEC9 had the broadest host range, and was able to infect 83% of the *E. coli* strains tested including pathogens belonging to different serotypes. All of the phages infected both non-pathogenic and pathogenic *E. coli* strains.

Morphological analysis of the phage isolates revealed them to belong to different families within the order *Caudovirales*. φEC6 was classified into the *Siphoviridae* due to the presence of a long, flexible tail and icosahedral capsid. The morphology of φEC9 was similar to T4, with an elongated head and contractile tail, indicating it belonged to the *Myoviridae*. φEC11 appeared to possess a very small tail and an icosahedral capsid and was therefore grouped into the *Podoviridae*. The genomes of the φEC phages consisted of dsDNA, which is consistent with the genomes of phages of the *Caudovirales*. Of the restriction enzymes tested, φEC9 was restricted only by SspI. This indicated that the DNA of φEC9 was modified. φEC6 and φEC11 were resistant to restriction by all of the enzymes tested which suggested that their DNA was also modified but by different mechanisms. PCR revealed that none of the phages possessed the *E. coli* virulence factor *eaeA*.

The latent periods and burst sizes of each of the phages were determined by one-step growth curves. φEC6 had the shortest latent period (20 minutes) and the highest burst size (113 per infected cell). φEC9 and φEC11 both had a latent period of 30 minutes. Their burst sizes varied at 62 and 80 PFU per infected cell respectively, which is smaller than has been reported for the T-phages (Brock, 1990). However, the coliphages LG1 and AR1, described by Goodridge *et al.* (2003) had longer latent periods and smaller burst sizes than φEC9 and φEC11.

The phages were exposed to buffers set to different pH in order to determine the effect on viability. φEC9 was the most tolerant, with no observable reduction in titre following incubation for 1 hour at pH 3.0, pH 4.0, pH 6.0, pH 7.0 or pH 9.0 and only
a small loss in titre at pH 2.2. φEC6 was stable from pH 4.0 to pH 9.0, but its titre was reduced by 6.0 log_{10} units following one hour of incubation at pH 3.0 and was not recoverable following exposure to pH 2.2. φEC11 was the most sensitive of the phages to low pH. It was not recoverable following exposure to pH 2.2 or pH 3.0, and the titre was reduced by 5.0 log_{10} units following one hour of incubation at pH 4.0. No effect on titre was observed following incubation at pH 6.0 to pH 9.0.

4.3.3 Overall summary

Enterococcal phages were isolated and characterised in order to determine their suitability as biocontrol agents. Given the narrow host range of the φSUT phages, it is unlikely that they would be suitable real-world biocontrol candidates. However, they may be useful for primary proof-of-concept studies given the paucity of literature relating to phage biocontrol of enterococci. Furthermore, the methods used to isolate and characterise the enterococcal phages were optimised in order to isolate coliphages with a broader host range. These coliphages were able to infect a range of *E. coli* strains including pathogens. Primary characterisation of the coliphage isolates confirmed that they are morphologically and genetically distinct from one another. Additional characterisation of these coliphage isolates would be necessary if further study reveals them to be good biocontrol agents. Such characterisation could include sequencing to determine homology to known coliphages as well as to rule out the presence of common virulence factors.
Chapter 5

Bacteriophage biocontrol of *Enterococcus faecalis* and *Escherichia coli* on surfaces
5.1 Introduction

5.1.1 Enterococci

Enterococci are common environmental contaminants in hospitals and have been demonstrated to survive on fomites, including hard and porous surfaces, for extended periods (Wendt et al., 1998; Neely and Maley, 2000). Furthermore, infections caused by vancomycin-resistant enterococci (VRE) are a cause of continuing concern. While education and improved monitoring of cleaning procedures have been shown to decrease VRE contamination of surfaces in hospitals (Hota et al., 2009), novel targeted cleaning methods should also be considered to help reduce enterococcal-associated morbidity and mortality. A potential alternative to conventional disinfection is the use of lytic bacteriophages as biocontrol agents against enterococcal contamination of surfaces. While bacteriophages have been used to control Pseudomonas sp. and Staphylococcus sp. on medical devices such as catheters (Curtin and Donlan, 2006; Fu et al., 2010), there are no reports describing the use of phages to reduce bacteria on soft surfaces commonly found in hospitals.

5.1.2 E. coli

Overuse of chemical sanitizers has led to increased resistance among foodborne pathogens including E. coli (Davidson and Harrison, 2002; Mokgatla et al., 1998). In addition, many chemical sanitizers may be corrosive or toxic and are therefore unsuitable for use on surfaces that come into direct contact with food (Viazis et al., 2011a). Numerous studies have investigated phage-based preparations to control pathogenic bacteria including E. coli and L. monocytogenes on surfaces (Roy et al., 1993; Hibma et al., 1997; Sharma et al., 2005; Abuladze et al., 2008; Viazis et al., 2011a). Furthermore, while several studies have targeted E. coli contamination of surfaces commonly found in food processing environments including stainless steel, ceramic and glass, these studies have predominantly focused on serotype O157 (Sharma et al., 2005; Abuladze et al., 2008; Viazis et al., 2011a).
5.1.3 Chapter aims

This chapter aimed to determine the ability of the φSUT phages and φEC phages characterised in Chapter Four to control their respective hosts on a range of surfaces.

Specifically, the aims were to:

i. Determine the optimum contact time for effective biocontrol of enterococci using φSUT phages on surfaces

ii. Investigate the capacity of the φSUT phages to reduce the numbers of their respective hosts on soft surfaces and glass

iii. Determine whether φSUT phage cocktails were more effective than individual phages in reducing enterococci on surfaces

iv. Assess the effects of phage concentration and contact time on the ability φEC phage cocktails to reduce four strains of *E. coli* strains on stainless steel and glass
5.2 Results and Discussion

5.2.1 φSUT phage biocontrol of Enterococcus on surfaces

The surfaces tested in this study comprised a combination of natural, synthetic and blended fabrics commonly used in hospitals for a variety of applications as well as one hard surface (glass).

5.2.1.1 Determining the optimum contact time for phage treatment

In order to determine the optimum contact time for phage treatment, preliminary experiments were conducted using φSUT1 treatment of E. faecalis on glass (Figure 5.1). Phage-treated glass and controls were incubated at ambient temperature for between 10 minutes and 120 minutes.

![Figure 5.1](image)

Figure 5.1 Viable counts of E. faecalis (ATCC 39058) recovered from glass treated with φSUT1 for varying lengths of time. E. faecalis control; φSUT1 treated. Error bars represent one standard deviation.

Figure 5.1 illustrates that φSUT1 phage treatment on glass was time-dependent. While a complete reduction in E. faecalis was not achieved at any time point, the greatest reduction was observed after 120 minutes of incubation. In the current study, a high concentration of phage was used (10⁹ PFU/mL) and the surfaces were
incubated at ambient temperature. Based on this information, all subsequent φSUT phage biocontrol studies involved treatment of materials for 120 minutes.

5.2.1.2 φSUT1 biocontrol of *E. faecalis* on surfaces

φSUT1 significantly reduced *E. faecalis* (ATCC 39058) on all surfaces. *E. faecalis* (ATCC 39058) was not recovered from terry cotton treated with φSUT1 while a 4.5 log₁₀ unit reduction was observed on φSUT1-treated cotton (Figure 5.2). It is possible that the greater surface area of the terry cotton enabled more even distribution of target cells and phage compared to plain cotton which may have contributed to the greater efficacy observed.

![Figure 5.2](image)

**Figure 5.2** Viable counts of *E. faecalis* (ATCC 39058) recovered from (a) cotton and (b) terry cotton treated with ΦSUT1 or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). **E. faecalis** control; φSUT1 treated. Error bars represent one standard deviation.

The effect of φSUT1 on reducing *E. faecalis* (ATCC 39058) on polyester and poly cotton (a blend of 60% cotton and 40% polyester) was comparable. A 4.4 log₁₀ unit reduction was observed on both surfaces (Figure 5.3).
On polyethylene, φSUT1 reduced *E. faecalis* (ATCC 39058) by 4.47 log$_{10}$ unit after 120 minutes (Figure 5.4). The phage treatment was least effective on glass, achieving only a 3.71 log$_{10}$ unit reduction of *E. faecalis*, which was comparable to the effect observed in the preliminary time study (Figure 5.1). It also had a large standard deviation indicating potential difficulty in recovering the bacteria from the glass slides consistently.
Results from the current study showed that φSUT1 was able to significantly reduce *E. faecalis* on all surfaces tested. The titre of φSUT1 was also found to be stable during all studies.

### 5.2.1.3 φSUT phage biocontrol of (VRE M252807) on surfaces

The enterococcal phages φSUT1 and φSUT6 were used in the following experiments. The phages were tested individually and in combination. The study parameters were identical to the φSUT1 biocontrol studies described above.

![Figure 5.5](image)

**Figure 5.5** Viable counts of VRE (M252807) recovered (a) cotton and (b) terry cotton treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). 

- Blue: VRE (M252807) Control; Black: φSUT1; Green: φSUT6; Pink: φSUT1 & φSUT6.
- Error bars represent one standard deviation.

On cotton, the reduction in VRE (M252807) achieved by any phage was less than 1 log$_{10}$ CFU/mL (Figure 5.5). On terry cotton, the phage combination produced the greatest reduction in VRE (M252807) of 1.0 log$_{10}$ unit. Figure 5.6 illustrates the effect of the φSUT phages in reducing VRE (M252807) on poly cotton and polyester.
Figure 5.6  Viable counts of VRE (M252807) recovered (a) poly cotton and (b) polyester treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). VRE (M252807) Control; φSUT1; φSUT6; φSUT1 & φSUT6. Error bars represent one standard deviation.

A similar pattern was observed on poly cotton and polyester. Reductions in VRE (M252807) were less than 1 log$_{10}$ unit and the phages used in combination were the most effective. Similarly, φSUT6 produced the smallest reductions. The φSUT phages were least effective on glass of all surfaces tested (Figure 5.7).

Figure 5.7  Viable counts of VRE (M252807) recovered (a) polyethylene and (b) glass treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). VRE (M252807) Control; φSUT1; φSUT6; φSUT1 & φSUT6. Error bars represent one standard deviation.
While φSUT1 produced significant reductions in *E. faecalis* on the same surfaces, this was not observed when the surfaces were contaminated with VRE (M252807). Results of preliminary characterisation studies, reported in Chapter Four, revealed that φSUT1 had a lower efficiency of plating on VRE (M252807) compared to *E. faecalis*. However, φSUT1 was more effective than φSUT6, indicating that φSUT6 is unlikely to make a good biocontrol candidate.

### 5.2.1.4 φSUT phage biocontrol of VRE (M168600) on surfaces

The remaining φSUT phages, φSUT3 and φSUT4, were similarly tested for their ability to control VRE (M168600) on the surfaces used in the previous studies.

![Graph](image)

**Figure 5.8** Viable counts of VRE (M168600) recovered (a) cotton and (b) terry cotton treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). □ VRE (M168600) Control; ■ φSUT3; ▼ φSUT4; ▲ φSUT3 & φSUT4. Error bars represent one standard deviation.

Interestingly, the phage combination achieved poorer results on cotton and terry cotton compared to φSUT3 and φSUT4 used individually (Figure 5.8). φSUT4 produced reductions in VRE (M168600) of greater than 1.2 log<sub>10</sub> units on both surfaces. Similarly, the phage cocktail was also less effective than the individual phages in reducing VRE (M168600) on poly cotton and polyester (Figure 5.9).
Chapter 5  Bacteriophage biocontrol of *E. faecalis* and *E. coli* on surfaces

Figure 5.9  Viable counts of VRE (M168600) recovered (a) poly cotton and (b) polyester treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). VRE (M168600) Control; φSUT3; φSUT4; φSUT3 & φSUT4. Error bars represent one standard deviation.

Glass was the only surface on which the phage cocktail was more effective than φSUT3 and φSUT4 used individually. The phage titres were stable in all studies.

Figure 5.10  Viable counts of VRE (M168600) recovered (a) polyethylene and (b) glass treated with φSUT phages or PBS (control) for 120 minutes at ambient temperature (21 ± 3°C). VRE (M168600) Control; φSUT3; φSUT4; φSUT3 & φSUT4. Error bars represent one standard deviation.
Subculture of selected bacterial colonies recovered from surfaces and subsequent re-infection with φSUT phages revealed that they remained sensitive to infection. It is probable that the phages did not possess sufficient lytic ability against the hosts on surfaces, or limited diffusion may have impeded contact between the phages and their target cells (Guenther et al., 2009). However, the sensitivity of the recovered bacteria to infection suggested that the surviving cells were not bacteriophage insensitive mutants (BIMs) (O’Flynn et al., 2004) and should not hinder the efficacy of the treatment (Abuladze et al., 2008).

A potential limitation of the current study is that the bacterial cells were grown to exponential phase prior to inoculation of surfaces. Given that enterococci are able to survive on surfaces for extended periods (Wendt et al., 1998) it is likely that they may be in stationary phase and/or in a starved state. Bacteria are more susceptible to phage infection when actively growing (Abedon, 2009), and further research should determine the effect of the φSUT phages on stationary phase enterococci on surfaces.

It has been well established that phage cocktails are often more effective in lysing bacteria than single or narrow host range phages and can also delay the appearance of phage resistant host cells (Tanji et al., 2005; Callaway et al., 2008; Niu et al., 2009). For example, Roy et al. (1993) found that a phage cocktail containing three bacteriophages was able to produce significantly greater reductions in growth of Listeria compared to a single broad host range Listeria phage suspension ($p < 0.05$). However, the phage cocktails used in the current study produced varied results. The suspension containing φSUT1 and φSUT6 was able to significantly ($p < 0.05$) reduce the recoverable VRE (M252807) on glass, cotton and poly cotton, compared with treatments containing the single phages. Furthermore, while not statistically significant, the phage cocktail also produced greater reductions in VRE (M252807) on all other surfaces compared to the single phage treatments. In contrast, surface biocontrol studies using the phages φSUT3 and φSUT4 in combination saw improved efficacy on glass, but higher viable counts on all other surfaces compared to treatments using either phage separately. However, the viable counts were lower than the controls. These results, and results of the phage characterisation studies detailed in Chapter Four which showed φSUT3 and φSUT4 to be morphologically
Chapter 5  Bacteriophage biocontrol of E. faecalis and E. coli on surfaces

and genetically similar, indicates that phages used in a cocktail should be significantly different from one another so that they do not compete for the same receptors and are not susceptible to the same host mutations.

Although phage biocontrol of nosocomial pathogens on soft surfaces has not been previously reported, O’Flaherty et al. (2005a) described the lytic effect of Bacteriophage K against a range of methicillin resistant Staphylococcus aureus strains in vitro. An in situ hand washing study found that a hand washing solution enriched with phage K achieved a 100-fold greater reduction in staphylococci on human skin compared to a phage-free solution. A similar study could be conducted using φSUT phages or other VRE-specific phages to reduce the microbial load on hands of health care workers and patients and therefore reduce the risk of VRE transmission.

Another potential application is the use of phage in combination with chemical sanitizers in a hurdle approach to disinfection. In a surface study by Roy et al. (1993), phages were combined with a quaternary ammonium compound solution to disinfect stainless steel and polypropylene cylinders that had been inoculated with Listeria sp. It was found that the combined approach produced a total (5 log₁₀) reduction using a reduced concentration of quaternary ammonium compound (40 ppm) compared to the quaternary ammonium compound alone (50 ppm) (Roy et al., 1993). This approach has also been investigated for the biocontrol of pathogens in foods. Leverentz et al. (2003) and Martinez et al. (2008) used phage preparations combined with nisin to reduce L. monocytogenes contamination of fruit slices and S. aureus contamination in pasteurized milk, respectively. The combined approach produced a synergistic effect in both cases. Similarly, Viazis et al. (2011b) used bacteriophages combined with trans-cinnamaldehyde to reduce E. coli O157:H7 contamination of leafy vegetables. The combination resulted in a greater and more rapid log₁₀ reduction in E. coli O157:H7 compared to individual treatments.

The preliminary results from this “proof of concept” study indicated that phage biocontrol of enterococci on hospital surfaces can be effective and further exploration is warranted. However, given the variable efficiency of the phages used
in this study, future research should focus on isolating and characterising phages with a broader host range among enterococcal nosocomial pathogens.

5.2.2 φEC biocontrol of *E. coli* on surfaces

A similar study was undertaken using the φEC coliphages φEC6, φEC9 and φEC11, characterised in Chapter Four. The phages were tested at different concentrations ($10^6$, $10^7$ and $10^8$ PFU/mL) for their ability to control various strains of *E. coli* on glass and stainless steel. In this study, the phages were combined in cocktails. The cocktail used to treat *E. coli* G106, K12 and O127:H6 comprised the phages φEC6, φEC9 and φEC11. The cocktail used to treat *E. coli* O5:NM contained φEC6 and φEC9 only as *E. coli* O5:NM was found to be insensitive to φEC11. After addition of phage, surfaces were incubated at ambient temperature for 10 minutes and 60 minutes prior to recovery of bacteria.

5.2.2.1 φEC biocontrol of *E. coli* G106 on surfaces

When the highest concentration of phage cocktail was used, *E. coli* G106 was completely eliminated on both glass and stainless steel regardless of contact time (Figure 5.11). At lower phage concentrations, the reduction in *E. coli* G106 was more pronounced after 60 minutes than after 10 minutes. There was little difference in the effect of the two lowest concentrations of phage cocktail ($10^6$ and $10^7$ PFU/mL) on recovery of *E. coli* G106 from stainless steel after 60 minutes. Both treatments were able to reduce *E. coli* G106 by approximately 0.8 log units. In contrast, the effect of the phage on glass was dose-dependent, with the treatment containing $10^7$ PFU/mL achieving a greater reduction in *E. coli* G106 than the lowest concentration (0.87 and 0.53 log$_{10}$ units respectively, after 60 minutes).
Chapter 5  Bacteriophage biocontrol of *E. faecalis* and *E. coli* on surfaces

Figure 5.11  Viable counts of *E. coli* G106 recovered from surfaces treated with φEC phage cocktail or PBS (control) at ambient temperature (21 ± 3°C). Control; 10^6 PFU/mL; 10^7 PFU/mL; 10^8 PFU/mL. Error bars represent one standard deviation

5.2.2.2  φEC  biocontrol of *E. coli* K12 on surfaces

A similar effect was observed when the phage cocktail was used to disinfect surfaces contaminated with *E. coli* K12, with the highest concentration of phage cocktail completely eliminating the bacteria (Figure 5.12). While there was no discernible difference between the control and the two lowest concentrations of phage on stainless steel after 10 minutes, the phage treatment containing 10^7 PFU/mL reduced *E. coli* K12 by 0.69 log_{10} units after 10 minutes on glass. However, this was not significant (p > 0.05)
Figure 5.12  Viable counts of *E. coli* K12 recovered from surfaces treated with φEC phage cocktail or PBS (control) at ambient temperature (21 ± 3°C).  

Control; 10^6 PFU/mL; 10^7 PFU/mL; 10^8 PFU/mL. Error bars represent one standard deviation

5.2.2.3  φEC biocontrol of *E. coli* O127:H6 on surfaces

The highest concentration of phage cocktail (10^8 PFU/mL) similarly eliminated *E. coli* O127:H6 on both surfaces irrespective of contact time. In contrast to the other strains, when 10^7 PFU/mL of φEC phage cocktail was applied to glass and stainless steel, a significant (*p < 0.05*) reduction in *E. coli* O127:H6 was observed after 60
minutes of incubation (1.70 and 1.48 log_{10} units respectively). Reductions produced by 10^6 PFU/mL phage cocktail were less than 1 log_{10} unit.

![Graph showing viable counts of E. coli O127:H6 recovered from surfaces treated with φEC phage cocktail or PBS (control) at ambient temperature (21 ± 3°C).](image)

**Figure 5.13** Viable counts of *E. coli* O127:H6 recovered from surfaces treated with φEC phage cocktail or PBS (control) at ambient temperature (21 ± 3°C). Blue: Control; Red: 10^6 PFU/mL; Green: 10^7 PFU/mL; Black: 10^8 PFU/mL. Error bars represent one standard deviation.

### 5.2.2.4 φEC biocontrol of *E. coli* O5:NM on surfaces

On *E. coli* O5:NM contaminated surfaces, the phage cocktail containing φEC6 and φEC9 was able to eliminate host cells when the highest concentration of phage was used after both 10 and 60 minutes of incubation. In contrast to the other hosts, the
cocktail achieved the same results when $10^7$ PFU/mL phage were used, but only after 60 minutes of incubation. Furthermore, a significant reduction ($p < 0.05$) was achieved on glass treated with $10^7$ PFU/mL phage cocktail after 10 minutes of incubation.

![Figure 5.14](image)

**Figure 5.14** Viable counts of *E. coli* O5:NM recovered from surfaces treated with φEC phage cocktail or PBS (control) at ambient temperature (21 ± 3°C). | Control | $10^6$ PFU/mL | $10^7$ PFU/mL | $10^8$ PFU/mL. Error bars represent one standard deviation.

In most cases, the phage treatment was dose-dependent, with the reduction in *E. coli* increasing with phage concentration. Similarly, phage treatment generally improved with contact time, except for the highest concentration of phage which was equally
effective at both time points. Viazis et al. (2011a) also reported greater efficiency of a phage cocktail in reducing *E. coli* O157:H7 on stainless steel, ceramic tiles and high density polyethylene with increasing phage MOI and incubation time. At lower MOI (1 and 10), a greater log reduction in *E. coli* O157 was observed following 24 hours of incubation compared to both 1 hour and 10 minutes. At the highest MOI, the effect was comparable regardless of contact time. The authors also reported that phage efficacy increased with temperature, with the greatest reductions in *E. coli* observed at the highest temperature (37°C). The current study was conducted at ambient (21 ± 3°C) only.

The results of the current study are also comparable to those reported by Abuladze et al. (2008) who similarly used a phage cocktail, ECP-100, to reduce *E. coli* O157:H7 on glass slides and gypsum boards. Phage cocktail was applied to surfaces contaminated with *E. coli* and surfaces were then incubated for 5 minutes at ambient temperature prior to recovery of the bacteria. The authors reported significant (*p* < 0.05) reductions in *E. coli* O157:H7 recovered from glass slides treated with 10⁸ and 10⁹ PFU/mL ECP-100. Even higher reductions were achieved when 10¹⁰ PFU/mL of the phage preparation was used. Although it has been repeatedly demonstrated that using high concentrations of phages results in greater reductions in target cells on surfaces (Abuladze et al., 2008; Viazis et al., 2011a), it is possible that lower concentrations could be applied for longer periods of time to achieve comparable results. This may be preferable in situations where it is impractical to prepare high concentrations or large volumes of phage.

Both Abuladze et al. (2008) and Viazis et al. (2011a) acknowledged that the normal lytic cycle of a bacteriophage takes, on average, between 20 and 40 minutes and that the significant reduction in *E. coli* observed in the biocontrol studies incubated for 10 minutes or less was unlikely to be the endpoint of the lytic process and rather the result of initial adsorption of the phages to the target cells. This is also likely true for the effects reported in the current study.

*E. coli* may proliferate on surfaces in nutrient-rich food processing environments making them highly susceptible to phage attack (Viazis et al., 2011a). While the
levels of bacteria used in the current study were much higher than would likely be encountered naturally, they are comparable to the levels used in similar studies (Abuladze et al., 2008; Viazis et al., 2011a). Further study should evaluate whether a host-threshold exists which would impact on φEC phage efficacy. In some cases, there is a requirement for a minimum number of host cells to be present before replication of phages will occur (Hudson et al., 2005). Minimum host-thresholds have been reported for phages infecting *Pseudomonas* in milk (Ellis et al., 1973) and *Salmonella* in chickens (Berchieri et al., 1991).

Furthermore, *E. coli* may become persistent in food processing environments due to attachment to surfaces or formation of biofilms. Bacteria attached to surfaces or enmeshed in biofilms may be more difficult to remove than actively growing planktonic cells (Sharma et al., 2005). For example, *Salmonella* in biofilms has been demonstrated to be more resistant to hypochlorite than planktonic cells (Joseph et al., 2001). Sharma et al. (2005) reported that bacteriophage KH1 was able to reduce *E. coli* O157:H7 attached to stainless steel coupons by 1.2 log CFU/coupon after one day of incubation at 4°C. However, no reduction was observed when KH1 was used to treat *E. coli* O157:H7 in biofilms.

Several studies have reported the use of bacteriophages to control *E. coli* O157:H7 on surfaces but this is the first which has focused on a range of *E. coli*, including EPEC and EHEC, other than the O157 serotype. Further study should be conducted to determine the ability of the φEC phage cocktail to perform at low temperature and in the presence of fewer host cells.
5.3 Summary

5.3.1 φSUT phage biocontrol of enterococci on surfaces

Preliminary studies revealed that φSUT1 treatment on glass was more effective in reducing *E. faecalis* with increasing contact time. φSUT1 reduced *E. faecalis* by at least 4 log\(_{10}\) units on all surfaces except glass and a total (5.47 log\(_{10}\) units) reduction was observed on terry cotton. φSUT1 was less effective in reducing VRE (M252807) on surfaces. Reductions of less than 1 log\(_{10}\) unit were observed on all surfaces. However, φSUT1 was slightly more effective than φSUT6 in reducing VRE (M252807). When the phages were combined, the cocktail was as at least as effective as φSUT1. However, reductions were still less than 1 log\(_{10}\) unit.

φSUT3 and φSUT4 were used alone and in combination to treat surfaces contaminated with VRE (M168600). φSUT4 was more effective than φSUT3 in reducing VRE (M168600) on all surfaces except polyester. The phage cocktail was more effective than either individual phage only on glass. On the other surfaces, the cocktail was less effective than each of the individual phages in reducing VRE (M168600).

5.3.2 φEC phage biocontrol of *E. coli* on stainless steel and glass

A cocktail containing the φEC phages was tested at different concentrations for its ability to reduce four strains of *E. coli* on stainless steel and glass. Treated surfaces and controls were incubated at ambient temperature for either 10 minutes or 60 minutes prior to recovery of bacteria. When the highest concentration of phage was used, all *E. coli* strains were completely eliminated on stainless steel and glass regardless of contact time. When the two lowest concentrations of phage cocktail were used on surfaces contaminated with *E. coli* G106, K12 or O127:H6, the effect was time- and dose-dependent. On *E. coli* O5:NM contaminated surfaces, the cocktails containing 10\(^7\) PFU/mL and 10\(^8\) PFU/mL were equally effective after 60 minutes of incubation.
5.3.3 Overall summary

While φSUT1 and the φEC phage cocktails were successful in eliminating or reducing their respective hosts on surfaces under optimum conditions, further studies should investigate the effect of the phages under sub-optimum conditions that may be more likely encountered in real world applications.
CHAPTER 6

Investigation of milk as a potential application for phage biocontrol
6.1 Introduction

6.1.1 Milk and foodborne illness

Milk is recognised as a vehicle for foodborne infection and has been implicated in disease outbreaks both in its raw and pasteurized forms. Furthermore, both *E. coli* and *E. faecalis* have been frequently isolated from many raw and pasteurized dairy products including milk. While *E. coli* is easily killed by pasteurization, it can enter milk by means of post-pasteurization contamination (PPC) or faults in the pasteurization process (Boor and Murphy, 2002). Recently, a pasteurized milk product was recalled in Australia due to contamination with *E. coli* (FSANZ, 2009a). In contrast, *Enterococcus* spp. are among the most thermotolerant of the non-sporeforming bacteria and may survive pasteurization (Giraffa, 2003). Alternatively, enterococci may also enter milk by means of PPC. While there have been no reported incidents of enterococcal illness associated with milk consumption, the potential for transfer of virulence or antibiotic resistance genes to gut microbiota suggests that limiting these organisms in milk would be worthwhile (Franz *et al*., 1999).

Although pasteurization has been used extensively in industrialised countries for decades as a means of increasing milk shelf life and destroying pathogenic organisms (Singh and Bennett, 2002), there appears to be increasing interest in raw milk consumption in these countries (Oliver *et al*., 2009). While the Australia New Zealand Food Standards Code requires all fluid milk products sold for human consumption to be pasteurized or equivalently heat treated (FSANZ, 2011), evidence from other countries suggests that raw milk is commonly consumed by dairy farming families and workers due to ease of access and convenience (Shiferaw *et al*., 2000). There is also anecdotal evidence to indicate that raw milk products marketed as pet food or “bath milk” are being consumed by humans (Oliver *et al*., 2009; FSANZ, 2009b). Furthermore, consumption of raw cow’s milk on school camps or during farm visits has been implicated in eight outbreaks of illness between 1998 and 2003 in Australia (FSANZ, 2009b). Given the perceived consumer demand for raw milk products, FSANZ is currently reviewing the requirements in the Australia New Zealand Food Standards Code for the sale of raw milk products through Proposal...
P1007 – Primary Production and Processing Requirements for Dairy (Raw Milk) Products (FSANZ, 2009b).

6.1.2 Consumer knowledge of food safety

Regulatory efforts to reduce the potential for foodborne illness have focused on the application of food safety systems during food production, processing and retail storage (Kennedy et al., 2005). While these efforts are necessary and worthwhile, poor food handling practices among consumers can often lead to foodborne illness, nullifying these stringent food safety efforts made during earlier stages of the food chain (Jay et al., 1999; Kennedy et al., 2005) Studies have highlighted that the mean temperature of domestic refrigerators is often higher than the recommended 5°C (Flynn et al., 1992; Kennedy et al., 2005) and that many consumers are unaware of recommended refrigeration temperatures for high risk foods (Kennedy et al., 2005). Temperature abuse of milk products may therefore lead to proliferation of potential foodborne pathogens, including *E. coli* and *E. faecalis*, during storage in the home.

6.1.3 Chapter aims

This chapter is primarily concerned with determining the prevalence of *Enterococcus* spp. and *E. coli* in Australian raw and pasteurized milk, and furthermore, whether phage biocontrol may be beneficial in limiting the growth of these organisms in milk. Secondary aims are to survey the microbiota of raw milk produced in Victoria, Australia, as well as study the effects of mild temperature abuse on raw milk microbiota. Finally, an investigation was conducted to determine the genetic diversity of *E. coli* isolated from a pasteurized milk product involved in a recall using rep-PCR based DNA fingerprinting.
6.2 Results and Discussion

6.2.1 Raw milk survey

Raw milk samples were obtained from three sources: one large dairy manufacturer (source 1), one medium sized dairy manufacturer (source 2) and one retailer (sold as bath milk) (source 3). The distribution of organisms recovered from raw milk samples varied considerably among the three sources (Table 6.1). \textit{Enterococcus} spp. was the most frequently isolated organism and was recovered from 85% of samples overall and from 100% of samples originating from source 1.

Coagulase positive \textit{Staphylococcus} was the only other organism to be detected in milk samples from all three sources. Coagulase positive \textit{Staphylococcus} commonly causes contagious mastitis in cows, and is isolated in 27.4 to 42% of raw milk samples (Oliver et al., 2009). Furthermore, \textit{S. aureus} can produce 16 enterotoxins capable of causing disease in humans. A study by Srinivasan et al. (2006) evaluated \textit{S. aureus} isolated from milk of cows with mastitis for the prevalence of enterotoxin gene sequences. Of 78 isolates, 73 (93.6%) were positive for one or more of the enterotoxin genes. While testing for the presence of enterotoxin gene sequences was not conducted in the current study, it is possible that these genes may have been present given the high prevalence of coagulase-positive \textit{Staphylococcus} spp. (73%) recovered. However, toxin production was unlikely to occur in the milk given the low levels encountered (Table 6.2).

According to Oliver et al. (2009), \textit{L. monocytogenes} is one of the most frequently isolated pathogens from bulk tank raw milk, with isolation rates ranging from 2.8% to 7.0%. In the current study, however, \textit{L. monocytogenes} was not recovered from any sample. While several presumptive colonies were qualitatively verified by OBIS (Oxoid) to be \textit{L. monocytogenes}, these were found to be false positives when confirmed by PCR of the \textit{hly} gene (data not shown).

\textit{Salmonella} is another pathogen that is commonly isolated from bulk tank raw milk (0-11% samples) (Oliver et al., 2009). The detection rate in the current study was 5% (n=3) and all positive samples originated from source 1. Furthermore, all samples
that tested positive for *Salmonella* spp. had an SPC of greater than $3.0 \times 10^5$ CFU/mL and faecal coliforms in excess of $3.0 \times 10^3$ CFU/mL. While the *Salmonella* isolates were not enumerated in the current study, its presence is concerning for proponents of raw milk consumption, as all *Salmonella* spp. are considered to be potentially pathogenic to humans and infection may result from ingestion of as few as 100 cells (D’Aoust, 1985).

An unpublished survey of raw cow’s milk conducted in Western Australia (FSANZ, 2009b) reported similar findings to those observed for raw milk originating from source 1 in the current study. For example, a high prevalence of *E. coli*, coliforms and *S. aureus* were reported while the prevalence of *Salmonella* was also comparable at approximately 8%. Similarly, no EHEC or *Listeria monocytogenes* were reported (FSANZ, 2009b).

### Table 6.1

Incidence of bacteria in raw milk, by source

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source 1</th>
<th>Source 2</th>
<th>Source3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase positive</td>
<td>30 (94)</td>
<td>14 (59)</td>
<td>4 (40)</td>
<td>48 (73)</td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>29 (91)</td>
<td>8 (33)</td>
<td>n.d.</td>
<td>37 (56)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>32 (100)</td>
<td>18 (75)</td>
<td>6 (60)</td>
<td>56 (85)</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>32 (100)</td>
<td>15 (63)</td>
<td>n.d.</td>
<td>47 (71)</td>
</tr>
<tr>
<td><em>monocytogenes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>3 (9)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3 (5)</td>
</tr>
</tbody>
</table>

n.a. not detected
Table 6.2
Mean viable counts and standard deviations calculated for organisms recovered from raw milk samples

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mean viable count Log_{10} CFU/mL (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source 1</td>
</tr>
<tr>
<td>Coagulase positive</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td>2.05 (1.11)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.94 (1.01)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>2.30 (0.89)</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>2.85 (0.89)</td>
</tr>
<tr>
<td>Standard Plate Count</td>
<td>5.54 (1.30)</td>
</tr>
</tbody>
</table>

Figure 6.1 illustrates the significant variation in bacterial viable counts for each source of bulk tank raw milk. Overall, milk samples from source 3 had the lowest viable counts for all organisms tested while milk samples from source 1 had the greatest variation in viable counts as well as the highest mean viable counts.

The SPC is an indicator of milk quality and of herd health and sanitation practices (Oliver *et al.*, 2009). SPC exceeding 10,000 CFU/mL may indicate poor cleaning of milking systems, as milk residues on equipment surfaces are able to support the growth and multiplication of microorganisms and the formation of biofilms, which can lead to subsequent contamination of milk products (Oliver *et al.*, 2009). High SPC may also indicate the failure to rapidly cool milk to below 4.0°C after milking. In the current study, only 52% of samples tested had SPC below 10,000 CFU/mL (Figure 6.1). However, the majority of samples with high SPC originated from source 1 (n=29) whereas only 12% (n=3) of samples from source 2 and none of the samples from source 3 had an SPC above this limit. The median SPC was also below this limit at 3.9 log_{10} CFU/mL.
The sale of raw cow’s milk for human consumption is currently prohibited in Australia and, as such, there are no prescribed Australian standards regarding limits for SPC. In the USA there are several states that allow the sale of raw milk for human consumption. The microbial standards for raw milk vary among states but the maximum SPC allowed by any state is ≤50,000 CFU/mL (Oliver et al., 2009). The results from the current study indicated that 33% of samples tested (n=22) exceeded the highest SPC allowable by US regulations. All samples originated from source 1.

Similarly, while there are no Australian Standards regarding limits for coliforms in raw milk for retail sale, the highest limit in the US for any state that permits the sale of raw cow’s milk is < 100 CFU/mL. According to this standard, 47% (n= 31) of raw milk samples tested in this study exceed this upper limit.
Figure 6.1  Viable counts obtained from raw milk samples according to percentage of samples within 1 log CFU/mL range. • < 1.0 log CFU/mL; □ 1.0 log CFU/mL; ▼ 2.0 log CFU/mL; ▲ 3.0 log CFU/mL; ▽ 4.0 log CFU/mL; ○ 5.0 log CFU/mL
The results from this microbiological survey provide an indication of the variable quality of raw milk produced in Australia which may have implications for the current review of Australian legislation concerning local manufacture of raw milk cheeses.

6.2.1.1 *E. coli* in raw milk survey

*E. coli* was detected in 37 raw milk samples in the current study, and of these, 66% had a viable count of < 100 CFU/mL. While most *E. coli* is considered harmless, pathogenic strains exist, including the shiga-toxin producing *E. coli*. PCR of *E. coli* isolates was unable to detect the presence of *Shiga* toxin genes, *stx1* or *stx2* (Figure 6.2).

![Agarose gels depicting amplicons generated by PCR of positive controls: Lane 1, stx1 (Enterohaemorrhagic E. coli O157:H7); Lane 2, stx2 (Enterohaemorrhagic E. coli); Lane M, DNA ladder (GeneRuler, Fermentas).](image)

**Figure 6.2** Agarose gels depicting amplicons generated by PCR of positive controls: Lane 1, stx1 (Enterohaemorrhagic *E. coli* O157:H7); Lane 2, stx2 (Enterohaemorrhagic *E. coli*); Lane M, DNA ladder (GeneRuler, Fermentas).

Detection rates of STEC in raw milk vary. Altalhi and Hassan (2009) detected STEC in 9.1% of raw milk samples, while other authors have reported much lower rates, ranging from 0.4% (Quinto and Cepeda, 1997) to 3.9% (Klie *et al*., 1997). While no STEC was detected in the current study, there are several other virulence factors that *E. coli* can possess which contribute to human illness, including *eaeA, cnf1, cnf2, fyuA, lutA* and *traT* which were not tested for. Furthermore, the method used for isolation and enumeration of *E. coli* in the current study relied on direct plating of
milk samples on chromogenic media, on which many pathogenic *E. coli* do not produce typical colonies (Feng, 2001). As only typical colonies were selected for PCR, shiga toxin producing *E. coli* may have been overlooked during the screening process.

Raw milk is a common vehicle for foodborne *E. coli* infection. Gillespie et al. (2003) reviewed foodborne outbreak surveillance data from England and Wales (1992-2003) and identified *E. coli* O157 as the causative agent of 33% of reported outbreaks attributed to consumption of milk products. Furthermore, *E. coli* O157 was more commonly associated with milkborne outbreaks compared with other outbreaks of foodborne origin (Gillespie et al., 2003).

In addition to its role in foodborne outbreaks, a study by Schlegelova et al. (2002) identified a high prevalence of antibiotic resistance among dairy isolates of Gram negative bacilli including *E. coli*. Examination of the resistance profiles of 26 isolates of *E. coli* from bulk tank milk found twelve strains to be resistant to three or more antibiotics. Moreover, another study found that the PFGE fingerprints and antibiograms of *Salmonella* and *E. coli* O157:H7 isolated from dairy cattle faeces and bulk tank milk were indistinguishable, indicating that the dairy environment and infected cattle are a direct source of drug resistant pathogens in bulk tank milk (Oliver et al., unpublished data). The presence of antibiotic-resistant *E. coli* in bulk tank milk may lead to colonisation of the gut in humans and further dissemination in the community, making infections more difficult to treat (van den Bogaard and Stobberingh, 1999; Anderson et al., 2003; Sturenburg and Mack, 2003).

### 6.2.1.2 Enterococcus in raw milk survey

Although there was considerable variation in the distribution of organisms in the raw milk samples, the most frequently recovered organism(s) from raw milk samples originating from all sources were *Enterococcus* spp. (Table 6.1). The mean enterococcal viable count was 1.56 log$_{10}$ CFU/mL.
PCR of *Enterococcus* 16S rDNA-targeted primers was conducted to confirm that colonies isolated on KF *Streptococcus* media belonged to the genus *Enterococcus*. Figure 6.3 illustrates the amplicons produced for selected *Enterococcus* isolates and a positive control.

![Figure 6.3](image)

**Figure 6.3** Agarose gels depicting amplicons generated by PCR of *Enterococcus* 16S rDNA-targeted primers Lane M, molecular weight marker; Lane 1, Positive control (*E. faecalis*); Lane 2, Negative control; Lane 3-13, representative *Enterococcus* isolates from raw milk.

Following confirmation by PCR, selected isolates were identified to the species level using Remel ERIC RapID™ STR kit (Oxoid) (Table 6.3). The most frequently identified species was *E. faecalis*, followed by *E. faecium*, which is consistent with findings reported by others (Citak *et al.*, 2005; Franciosi *et al.*, 2009).

**Table 6.3**

*Enterococcus* species identified biochemically by Remel ERIC RapID

<table>
<thead>
<tr>
<th><em>Enterococcus</em> species</th>
<th>% isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>75</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>15</td>
</tr>
<tr>
<td><em>E. casseifulavus</em></td>
<td>5</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>5</td>
</tr>
</tbody>
</table>
Many studies have investigated the prevalence of *Enterococcus* spp. in raw milk cheeses (Gelsomino *et al*., 2003; Giraffa *et al*., 1997). However, there are fewer that consider the prevalence of *Enterococcus* spp. in raw milk itself. In one study, Franciosi *et al.* (2009) determined the range of *Enterococcus* spp. in raw milk samples to be between $3.5-5.3 \log_{10} \text{CFU/mL}$. While *Enterococcus* spp. was isolated from 85% of samples in the current study, the mean viable counts were considerably lower than the levels reported by Franciosi *et al.* (2009). Although *Enterococcus* is considered to be an important opportunistic nosocomial pathogen, there are no prescribed limits for *Enterococcus* spp. in foods. However, due to their presence in human faeces, enterococci are not GRAS.

Confirmed *Enterococcus* isolates were also subjected to PCR to detect the presence of vancomycin resistance genes. The genetic targets *vanA* and *vanB* were selected for PCR as they are the most common vancomycin resistance genes found in *E. faecalis* and *E. faecium* (Appleman *et al*., 2004). However, these genes were not detected in any sample except for the positive controls (Figure 6.4). While several studies have shown high rates of vancomycin resistance among *Enterococcus* isolated from raw milk using phenotypic methods such as minimum inhibitory concentration (MIC) and agar diffusion assay (ADA) (Choi *et al*., 2002; Citak *et al*., 2005), genetic evidence of vancomycin resistance among isolates is limited. For example, Choi *et al.* (2002) isolated 19 strains of VRE from raw milk samples over a six month period using MIC assays. However, PCR analysis was able to detect the presence of vancomycin resistance genes in only five of the isolates. Therefore, PCR may not be the most reliable method for detection of VRE and should be used in combination with traditional phenotypic methods.
Figure 6.4  Agarose gel depicting PCR amplification of positive controls for \textit{vanA} (VRE M168600) (lane 1) and \textit{vanB} (VRE M252807) (lane 2). 40 Enterococcus isolates were also assayed with negative results.

6.2.2 Evaluation of the effect of mild temperature abuse on raw milk

Five raw milk samples from source 1 with initial low SPC (~3.0 \(\log_{10}\) CFU/mL) were incubated in a domestic refrigerator. The temperature was measured using a long-probe thermometer (Ratek) placed on a middle shelf next to the milk samples. The temperature was monitored daily and ranged from 5.4 to 9.2°C, consistent with mild temperature abuse conditions that may be encountered in the home (Koutsoumanis \textit{et al.}, 2010). The mean temperature, calculated from the daily readings, was 7.1°C. Figure 6.5 illustrates the increase in viable counts over the experimental period. The mean SPC and coliforms increased significantly \((p < 0.01)\), more than doubling over the test period. Enterococci also increased significantly \((p < 0.05)\) over 7 days of incubation. While \textit{E. coli} and coagulase positive staphylococci did not increase during the study, the organisms were recoverable after 7 days. This indicated that the bacteria were able to survive but not replicate. The pH was monitored by indicator strips (Merck) and was found to decrease from pH 6.5 to 6.0 after 96 hours at which level it remained stable until the end of the study.
The current study found that SPC and coliforms significantly increased when stored at slightly elevated temperatures. Koutsoumanis et al. (2010) reviewed the results of nine survey studies of domestic refrigerators conducted in the United Kingdom, France, Ireland, and Greece and calculated the weighted mean temperature of 1,171 refrigerators tested to be 6.4°C and determined that 64.1% of the refrigerators operated at a temperature of > 5°C. In addition, it was found that the door and top shelf of domestic refrigerators had a higher mean temperature than the inner shelves and that 51% and 25% of respondents usually stored pasteurized milk in the door shelf and top shelf, respectively. Furthermore, in a study of mean temperatures of high risk foods in domestic homes, Marklinder et al. (2004) identified the mean temperature of milk to be 6.9°C and that 31% of samples tested were above 8°C.

Psychrotrophic and cold adapted strains of bacterial pathogens may be able to survive and grow during storage of milk at low temperature. For example, psychrotrophic strains of B. cereus isolated from pasteurized milk were able to grow from 7°C to 37°C (Te Giffel et al., 1997). Furthermore, E. coli O157:H7 has also
been shown to grow in experimentally inoculated pasteurized and raw milk during incubation at 8°C (Wang et al., 1997). Therefore, pathogens if present, may be able to grow in milk to levels sufficient to cause disease if stored at even slightly elevated temperatures.

Milk with a low initial SPC was chosen for the current study and no obvious signs of spoilage were observed during the study period. Using milk with a higher initial SPC may have resulted in spoilage prior to completion of the study. Although milk samples were tested initially for pathogens including *L. monocytogenes* and *Salmonella*, these were not tested for during the remainder of the study. While it is possible that these organisms, if present at low levels, may have been able to proliferate undetected, it is unlikely as enrichment steps conducted in the initial study failed to detect the organisms.

The raw milk used in this study was full cream milk that had not been homogenised. The fat and protein contents may influence the numbers and types of organisms that are recovered from milk. Similarly, unpasteurized milk is also susceptible to seasonal variations which may also affect the type and number of organisms recovered (Salmerón et al., 2002; Pangloli et al., 2008; Zhou et al., 2008). Therefore, it would be beneficial to repeat this study to investigate these physical and seasonal variables.

Although the incubation conditions were designed to mimic those that might be found within the home of consumers, it is likely that the milk samples were exposed to fewer fluctuations in temperature than would be encountered at home. For example, the milk samples in the current study were removed from the refrigerator only for sampling, at which point they were placed on ice, and the refrigerator door was not otherwise opened. It is assumed that domestic refrigerators would be opened more frequently which has been shown to cause increases in refrigerator air temperature (James and Evans, 1992).
6.2.3 Investigation of the prevalence of *Enterococcus* spp. and *E. coli* in pasteurized milk products

In the current study, no *Enterococcus* spp. were isolated from the 20 samples of pasteurized milk tested. However, it is possible that *Enterococcus* spp. could be present at low numbers and therefore enrichment may be necessary for their detection. Most *Enterococcus* species are able to grow at temperatures ranging from 10 to 45°C and many are able to survive for 30 minutes at 60°C (Ogier and Serror, 2008). Previous studies have reported the isolation of *Enterococcus* spp., including antimicrobial resistant enterococci, from pasteurized milk. El Zubeir *et al.* (2008) isolated *E. faecalis* from 3.9% of pasteurized milk samples and *E. faecium* from 2.6% of samples. In a study of the antimicrobial resistance patterns of *Enterococcus* spp. isolated from poultry and pasteurized milk, Fracalanzza *et al.* (2007) recovered 127 isolates from pasteurized milk, including *Enterococcus faecalis* (62.6%), *E. casseiflavus* (17.3%), *E. durans* (6.5%), *E. gallinarum* (3.0%), *E. gilvus* (2.4%), *E. faecium* (2.0%), *E. hirae* (1.4%), and *E. sulfureus* (1.0%).

*E. coli* was not detected in any of the pasteurized milk samples, which was expected because *E. coli* is routinely tested for by milk processors. However a number of *E. coli* isolates originating from pasteurized milk implicated in a product recall were provided by the regulatory authority for further investigation.

6.2.4 Investigation of the genetic diversity of *E. coli* isolated from pasteurized milk involved in a recall

Approximately 100 *E. coli* isolates were provided by the regulatory authority. Therefore, a viable count from the original sample could not be calculated. None of the *E. coli* isolates tested positive for *stx1*, *stx2* or *eaeA* genes following PCR (data not shown). Subsequently, ERIC PCR was used to create DNA fingerprints of selected isolates in order to determine the diversity of strains present and provide information to the manufacturer to help identify how and/or where the contamination occurred.
DNA fingerprinting has been used to trace the source of bacterial contaminants during production of dairy products and the use of ERIC PCR may be used similarly for the tracking of *E. coli* during milk production (Svensson *et al.*, 1999; Banykó & Vyletělová, 2009). Agarose gel electrophoresis was used to compare the fingerprints (Figure 6.6). Many of the DNA profiles shared common bands at approximately 300, 400 and 1300 bp. However, the resolution of the gels made it difficult to distinguish between similar patterns. The samples were therefore subjected to analysis using the Bioanalyzer (Agilent). The resulting computer generated gel image is illustrated in Figure 6.7

![Figure 6.6](image_url)

**Figure 6.6** Agarose gel depicting representative DNA fingerprints following ERIC PCR of *E. coli* DNA isolated from pasteurized milk. Lane 1-19: *E. coli* samples. Lane 20-21: Controls; *E. coli* (ATCC 25922) and EHEC O157:H7, respectively. Lane M: DNA marker.
Figure 6.7  Representative DNA fingerprints generated by Bioanalyzer (Agilent) following ERIC PCR of *E. coli* DNA isolated from pasteurized milk. Lane 1-19: *E. coli* samples. Lane 20-21: Controls; *E. coli* (ATCC 25922) and EHEC O157:H7 respectively. Lane M: DNA marker. Green boxes represent different profiles. Blue boxes represent controls.
Based on visual analysis of the Bioanalyzer gel image, nine distinct fingerprints could be discerned from the 19 samples analysed, in addition to the fingerprints generated for the controls. This diversity among the contaminants ruled out the possibility of a clonal contamination. A possible explanation for the diversity of the *E. coli* found is that the pasteurized product had been contaminated by raw product following pasteurization.

In order to further delineate the genetic relationships between the isolates, a dendrogram was constructed. The fingerprints were categorised into 6 clusters (A, B, C, D, E, and F) (Figure 6.8). Isolates with less than 80% similarity were considered distinct. Cluster C contained the largest number of samples. Interestingly, no samples from the recall were categorised into the same cluster as *E. coli* O157:H7 and only one sample was grouped into Cluster F with the ATCC strain.

![Figure 6.8](image)

**Figure 6.8** Dendrogram representing genetic relationships between *E. coli* isolates from pasteurized milk based on ERIC-PCR fingerprints.
Although pasteurization is a very effective means of ensuring milk is safe for consumption, faults in the process or contamination after pasteurization can lead to outbreaks of foodborne illness, particularly in the absence of competitive microbiota (Oliver et al., 2005). In the current study, a pasteurized milk product was involved in a state-wide recall due to contamination with \textit{E. coli}. ERIC PCR of selected isolates revealed broad genetic diversity, indicative of multiple contaminants. It is likely that the diversity was even broader than reported as only one fifth of isolates were subjected to PCR. There are several other examples of pasteurized milk being recalled and/or implicated in outbreaks of foodborne illness (Lecos, 1986; Oliver et al., 2005; FSANZ, 2006; FSANZ, 2009b). It is therefore reasonable to suggest that phage biocontrol may be applicable to pasteurized as well as raw milk.
6.3 Summary

While the microbiota of raw milk has been studied extensively worldwide, there is scant published data regarding the quality of raw milk produced in Australia. This study found Australian raw milk to contain pathogens including *Salmonella* spp., as well as indicators of faecal contamination including coliforms, *E. coli* and *Enterococcus* species. There was considerable variation in the microbiological quality of raw milk obtained from three sources indicating that a wider study of Australian milk over time could be beneficial.

It has been widely established that consumer handling of high risk food products contributes to enteric disease (Kennedy *et al.*, 2005). Furthermore, the variable temperature of domestic refrigerators suggests that raw milk products could be subjected to mild temperature abuse (Marklinder *et al.*, 2004). Therefore, this study evaluated the effect of mild temperature abuse on raw milk. It was found that SPC, coliforms and enterococci increased significantly over the seven day test period, and that the potential pathogens coagulase-positive staphylococci and *E. coli* were able to survive.

No *E. coli* or *Enterococcus* spp. were recovered from the survey of pasteurized milk product. However, *E. coli* isolates implicated in a pasteurized milk recall were provided by the state government regulatory body for further investigation. PCR did not detect shiga toxin genes *stx1* or *stx2*. ERIC-PCR was performed in order to determine whether the isolates were clonal or genetically distinct. Results indicated a genetically diverse contamination incident. A dendrogram was constructed to further delineate the genetic relationship between the isolates. Six clusters were identified and designated A, B, C, D, E and F. Cluster C contained the largest number of isolates.

Given the incidence of *E. coli* and enterococci in raw milk in the current study, investigation of the ability of phages to control these organisms in raw milk is justified. Although neither organism was isolated from pasteurized milk in the current study, several other studies have recovered *E. coli* and enterococci from
pasteurized milk (Goh et al., 2002; Fracalanzza et al., 2007; El Zubeir et al., 2007; Zanella et al., 2010). Therefore, a more comprehensive survey could provide additional information. Nonetheless, phage biocontrol of these organisms in pasteurized milk may also be worthwhile.
CHAPTER 7

Phage biocontrol of *Enterococcus faecalis* and *Escherichia coli* in milk
7.1 Introduction

Bacteriophages have long been negatively associated with the dairy industry. Of the approximately 500 million tons of milk produced globally each year, an estimated one third is processed into fermented products (Brussow, 2001). The presence of dairy bacteriophages in milk leads to interruption of the fermentation of lactose by starter cultures. This can cause delays in production and alteration of product quality. Dairy bacteriophages are among the best characterised of the phages due to their potential to cause huge economic losses (Brussow, 2001). Despite this negative association, in recent years there has been increasing interest shown in using bacteriophages to control bacterial pathogens in dairy products. Given the high host-specificity of phages in general, the risk to starter cultures is remote.

Different types of milk have been implicated in cases of illness caused by particular pathogens. Phages have been investigated to control *Listeria monocytogenes* contamination of chocolate flavoured milk (Guenther et al., 2009), *Cronobacter sakazakii* contamination of reconstituted powdered infant formula (Kim et al., 2007) and *Staphylococcus aureus* contamination of pasteurized and raw milk (O’Flaherty et al., 2005a; Garcia et al., 2009) due to the association of these pathogens and milk types to incidence of illness.

7.1.1 Chapter aims

The aims of this chapter were to:

i. Determine the ability of the bacteriophages isolated and characterised in Chapter Four to reduce *E. faecalis* and *E. coli* in a range of experimentally inoculated milk including UHT, pasteurized and raw milk

ii. Assess the effect of temperature and milk fat content on the efficacy of the phage treatment

iii. Investigate the effect of milk on phage viability
7.2 Results and Discussion

ϕSUT1 and ϕSUT4 were selected for investigation as biocontrol agents in milk. The remaining phages, ϕSUT3 and ϕSUT6 were not considered due to their poor efficacy in reducing their respective hosts on surfaces as reported in Chapter Five.

7.2.1 Phage biocontrol of *E. faecalis* in UHT milk

Initial experiments were conducted using UHT milk in order to assess the behaviour of the phages and bacterial hosts in milk in the absence of competitive microbiota. Figures 7.1 and 7.2 illustrate the effect of ϕSUT4 in reducing VRE (M168600) in full cream UHT and skim UHT milk, respectively.

**Figure 7.1** ϕSUT4 biocontrol of VRE (M168600) full cream UHT milk. VRE (M168600); VRE (M168600) + ϕSUT4, ϕSUT4 titre. Error bars represent one standard deviation.
The growth pattern of VRE (M168600) in the full cream UHT milk control did not differ significantly ($p > 0.05$) to the growth observed in the skim UHT milk control at either 10 or 25°C. VRE (M168600) in both control milk samples increased by $\sim 2.8\ \log_{10}$ units within 72 hours of incubation at 10°C and by $\sim 3.7\ \log_{10}$ units after 24 hours at 25°C. $\phi$SUT4 was more effective in reducing VRE (M168600) in both milk samples at 10°C than at 25°C.
While ϕSUT4 was able to prevent VRE (M168600) from increasing for the first 48 hours of incubation at 10°C, the level increased to within 1.5 $\log_{10}$ units of the control in full cream UHT milk and 1.0 $\log_{10}$ units of the control in skim UHT milk at the end of the study. At 25°C, the difference between the control and ϕSUT4-treated milk samples was less than 1.0 $\log_{10}$ unit after 24 hours.

Figures 7.3 and 7.4 illustrate the effect of ϕSUT1 in reducing *E. faecalis* in full cream UHT milk and skim UHT milk, respectively.

**Figure 7.3**  ϕSUT1 biocontrol of *E. faecalis* in UHT full cream milk. ■ *E. faecalis*; □ ϕSUT1 + *E. faecalis*; --×-- ϕSUT1 titre. Error bars represent one standard deviation.
ϕSUT1 reduced *E. faecalis* to below the level of detection within three hours of incubation in both full cream and skim UHT milk at 25°C. However, following incubation for nine hours, the level of *E. faecalis* increased to approximately 4.0 log_{10} CFU/mL in both skim and full cream UHT milk. In both cases, the viable count of *E. faecalis* in ϕSUT1-treated milk was still significantly lower (*p* < 0.01) than the controls after 24 hours. There was no significant difference (*p* > 0.05) in growth rate.
of *E. faecalis* in the skim UHT milk control and full cream UHT milk control at 25°C.

At 10°C, the reduction in *E. faecalis* was more pronounced in skim UHT milk than in full cream UHT milk while the growth pattern of *E. faecalis* in the controls was not significantly different (*p* > 0.05). In both φSUT1 treated milk samples, *E. faecalis* was significantly reduced (*p* < 0.001). The titre of φSUT1 increased comparably in full cream and skim UHT milk after 24 hours of incubation from 7.5 log<sub>10</sub> PFU/mL to approximately 8.6 log<sub>10</sub> PFU/mL. At 25°C, no significant (*p* > 0.05) change in phage titre was observed in either full cream or skim milk. In contrast, φSUT4 increased in both milk samples at 10°C and 25°C, by ~1.0 log<sub>10</sub> PFU/mL and ~2.0 log<sub>10</sub> PFU/mL, respectively.

In both studies of φSUT4 and φSUT1 biocontrol, the initial inoculum of *Enterococcus* sp. used was higher than would likely be encountered in pasteurized milk and higher than has been reported for other phage biocontrol studies in milk targeting different hosts (Garcia *et al*., 2009; Guenther *et al*., 2009). However, it is within the range that would be expected to be found in raw milk (Franciosi *et al*., 2009). Furthermore, the concentration of bacteria used in the current study is comparable to the concentration of bacteria used in a similar study investigating the effect of phages on *Salmonella* Enteritidis during manufacture and storage of cheddar cheese (Modi *et al*., 2001).

Garcia *et al*. (2009) used two dairy-derived phages, φA72 and φH5, as antimicrobial agents to control *S. aureus* in whole fat UHT and pasteurized milk incubated at 37°C. While the individual phages inhibited the growth of *S. aureus* in UHT milk compared to the untreated control, a cocktail containing both phages was significantly more efficient. However, a complete clearance of *S. aureus* in UHT milk was not achieved. Similarly, the phage cocktail inhibited but did not eliminate *S. aureus* in pasteurized milk, probably due to the very low phage/cell ratio used. In comparison to the current study, φSUT4 was unable to significantly inhibit VRE (M168600) in either UHT milk or pasteurized milk incubated at 25°C while φSUT1 significantly reduced *E. faecalis* in both milk samples under the same conditions.
Kim *et al.* (2007) similarly used two phages, ESP 732-1 and ESP 1-3, to reduce *Cronobacter sakazakii* in reconstituted infant formula. *C. sakazakii* is an opportunistic pathogen which can cause serious illness in neonates and infants and infant formula has been implicated in cases of *Cronobacter*-related illness (Kim *et al.*, 2007). After contamination, *Cronobacter* spp. can persist in dried infant formula for up to 2.5 years. At concentrations of $10^8$ and $10^9$ PFU/mL, ESP-1 was able to completely eradicate *C. sakazakii* in reconstituted infant formula over time at 24°C. At 12°C, the highest concentration of phage was able to achieve a 2.42 log unit reduction compared with the control. The other phage, ESP 732-1, was able to completely eradicate *C. sakazakii* at 37°C, 24°C and 12°C when $10^9$ PFU/mL were used. In contrast to the current study, the length of incubation was limited to 40 hours at 12°C, 16 hours at 24°C and 10 hours at 37°C. This is likely because infant formula is consumed soon after reconstitution whereas UHT milk has a longer shelf life if refrigerated after opening. However, the results are comparable to those observed for φSUT1 biocontrol of *E. faecalis* in UHT skim milk.
7.2.2 Phage biocontrol of *E. faecalis* in pasteurized milk

Subsequent experiments were conducted using commercially pasteurized full cream and skim milk. While most vegetative cells are destroyed by this process, thermotolerant bacteria, sporeformers and heat-stable enzymes can survive. These studies were carried out at 10°C only.

![Graph](image)

**Figure 7.5** ϕSUT4 biocontrol of VRE (M168600) at 10°C in (a) pasteurized full cream milk and (b) pasteurized skim milk. □ VRE (M168600); ■ VRE (M168600) + ϕSUT4, -- - - - - ϕSUT4 titre. Error bars represent one standard deviation.
Despite initial reductions in φSUT4-treated pasteurized milk, VRE (M168600) began to increase after 24 hours of incubation at 10°C. Interestingly, VRE (M168600) grew more rapidly and to a higher concentration in pasteurized milk than in UHT milk. However, the φSUT4 titre did not increase during the experiment.

Figure 7.6  φSUT1 biocontrol of *E. faecalis* at 10°C in (a) pasteurized full cream milk and (b) pasteurized skim milk. ■ *E. faecalis*; ■ φSUT1 + *E. faecalis*; ---φSUT1 titre. Error bars represent one standard deviation.
In contrast to VRE (M168600), *E. faecalis* decreased by approximately $0.4 \log_{10}$ CFU/mL in the pasteurized full cream milk control and by $0.1 \log_{10}$ CFU/mL in the pasteurized skim milk control over the course of the study. The reductions in *E. faecalis* in φSUT1-treated pasteurized milk samples were approximately $2.0 \log_{10}$ units indicating that biocontrol using this phage may still be effective when the host is not actively growing. Furthermore, while the φSUT1 titre did not increase, its stability over 5 days at 10°C indicated that it was not inactivated in the milk. Phage stability in milk incubated at low temperature was also reported by Guenther *et al.* (2009). In their study, phages survived in pasteurized chocolate milk contaminated with *L. monocytogenes* for 6 days at 6°C. In the same study, *L. monocytogenes* in phage-treated chocolate milk was reduced to below the limit of detection within 2 days of incubation at 6°C (Guenther *et al.*, 2009).

Many phages are thermotolerant and can survive pasteurization temperatures. Survival of thermotolerant phages during pasteurization is thought to be a major entry point for problematic phages in dairy plants (Hudson *et al.*, 2005). Garcia *et al.* (2009) also reported that φA72 and φH5 could withstand incubation at 72°C for 15 seconds in UHT milk. Considering the reported ability of some phages to withstand high temperature, it is possible that phages to be used for biocontrol could be added to milk prior to pasteurisation. Further study should therefore determine whether φSUT1 and φSUT4 are similarly able to tolerate high temperature.

Given the inability of either φSUT1 or φSUT4 to completely eliminate its host in pasteurised milk, a further study using raw milk was not attempted.
7.2.3 Phage biocontrol of *E. coli* in UHT milk

As with the *Enterococcus* biocontrol studies, the coliphages were first tested for their ability to control *E. coli* in UHT milk. The strains of *E. coli* used were laboratory strains G106 and K12 in addition to two pathogenic strains, *E. coli* O127:H6 (EPEC) and O5:NM (EHEC). While the enterococci studies were incubated in a refrigerated water bath regulated by a thermostat at 10°C, the *E. coli* biocontrol studies were incubated in a domestic refrigerator with a variable temperature ranging from 5.0 to 9.2°C. This was intended to better simulate conditions that may be encountered during commercial and domestic handling and storage of milk. Furthermore, φEC phages were tested for their ability to suppress growth of the different strains of *E. coli* in TSB before the milk studies were conducted.

7.2.3.1 Turbidimetric analysis of φEC phage infection of *E. coli*

The second set of phages isolated and characterised in Chapter Four were used as biocontrol agents against strains of *E. coli* in milk. These phages, φEC6, φEC9 and φEC11, were found to effectively control several strains of *E. coli* for at least eight hours when tested under aerobic conditions in TSB at 37°C (Figure 7.7).
Figure 7.7  E. coli cell lysis by φEC phages. Cultures were incubated aerobically with shaking at 37°C. Phage was added at MOI of 10 when the OD\textsubscript{660} reached 0.1.

In E. coli K12, G106 and O127:H6 cultures, the phage cocktail containing φEC6, φEC9 and φEC11, was able to delay a rise in turbidity for at least 24 hours. Interestingly, φEC11 was as effective as the cocktail after 24 hours of incubation, whereas φEC6 and φEC9, when used individually, were able to initially suppress the growth of E. coli before an increase in turbidity was observed after between eight
and 12 hours. After 24 hours of incubation, the turbidity of all phage-infected cultures remained lower than uninfected controls.

In the *E. coli* O5:NM culture, ϕEC6 and ϕEC9, when used individually, were as effective as the cocktail in delaying an increase in turbidity for eight hours. The poorer performance of this cocktail compared to the cocktail used to infect the other *E. coli* strains is likely because it contained only two phages. Furthermore, uninfected *E. coli* O5:NM grew to a greater turbidity during the study compared to the other *E. coli* strains. This may have contributed to the more rapid emergence of resistant cells.

In a similar study, Tanji *et al.* (2005) observed that when their coliphages, SP15, SP21 and SP22, were individually added to *E. coli* O157:H7 liquid culture, cell growth resumed following 4-6 hours of incubation at 37°C. When two phages were used, turbidity did not increase until up to 30 hours following infection and a cocktail of the three phages suppressed *E. coli* O157:H7 for 70 hours following infection of the culture. The authors suggested that the cocktail was able to delay the emergence of resistant bacterial cells. When three phages were used in combination, the results achieved in the current study were comparable to those reported by Tanji *et al.* (2005). However, the current study was performed over 24 hours only, so it is unclear whether further incubation would have resulted in an increase in turbidity of phage cocktail-treated cultures within 70 hours. The individual phages used in the current study were able to suppress the growth of *E. coli* for at least 8 hours, compared to 4-6 hours reported by Tanji *et al.* (2005). In contrast, the cocktail containing ϕEC6 and ϕEC9 suppressed *E. coli* O5:NM for just eight hours compared to 30 hours reported by Tanji *et al.* (2005) for their two-phage cocktail.

This study confirmed that the three phages were able to suppress growth of different strains of *E. coli* for varying lengths of time in liquid culture under optimal conditions. The phages were subsequently tested in milk at lower temperatures, as detailed in the following sections.
7.2.3.2 Phage biocontrol of *E. coli* K12 in UHT milk

Figure 7.8 illustrates the effect of the φEC phages in reducing *E. coli* K12 in full cream and skim UHT milk incubated at 5-9°C and 25°C.

*Figure 7.8* Phage biocontrol of *E. coli* K12 in (a) UHT full cream milk and (b) UHT skim milk at 5-9°C and 25°C. *E. coli* K12 (control); *E. coli* K12 + φEC6; *E. coli* K12 + φEC9; *E. coli* K12 + φEC11; *E. coli* K12 + φEC phage cocktail. Error bars represent one standard deviation.
At low temperature, *E. coli* K12 decreased slightly (~0.15 log_{10} CFU/mL) in both the skim and full cream UHT milk controls. However, *E. coli* K12 in all phage-treated milk samples decreased to below the level of detection within 24 hours of incubation, regardless of which phage was used. At 25°C, *E. coli* K12 increased in both control milk samples by greater than 4.0 log_{10} CFU/mL. While *E. coli* K12 was eliminated in skim and full cream UHT milk treated with φEC6, φEC11 and the phage cocktail within three hours, it took six hours to achieve the same result in milk treated with φEC9. Furthermore, *E. coli* K12 in both milk samples treated with φEC11 increased to above the level of the control after 24 hours despite an initial reduction. This is in contrast to the effect observed in the turbidimetric study described in section 7.2.3.1, in which φEC11 was as effective as the phage cocktail in reducing *E. coli* K12 in TSB incubated at 37°C for 24 hours.

### 7.2.3.3 Phage biocontrol of *E. coli* G106 in UHT milk

The effect of the φEC phages in reducing *E. coli* G106 in full cream and skim UHT milk (Figure 7.9) was more variable than was observed for *E. coli* K12. The phages were more effective in controlling *E. coli* G106 in both UHT milk samples incubated at 25°C than at 5-9°C. All of the phage treatments were able to eliminate *E. coli* G106 within 3 hours of incubation at 25°C.

The phage cocktail was effective in eliminating *E. coli* G106 in full cream and skim UHT milk at low temperature within 24 hours and at 25°C within 3 hours. φEC9 also achieved a complete reduction of *E. coli* G106 at low temperature, although not until 48 hours of incubation in skim milk and 72 hours in full cream milk. φEC11 produced approximately ~1 log_{10} unit reductions in both full cream and skim UHT milk while *E. coli* G106 in φEC6-treated milk increased in line with the control. Furthermore, *E. coli* G106 increased in both full cream and skim UHT milk controls at low temperature (by 1.8 and 2.4 log_{10} unit CFU respectively), whereas *E. coli* K12 decreased slightly under the same conditions. This may have contributed to the differences observed in the effect of the phages on the different hosts.
Chapter 7  Phage biocontrol of *E. faecalis* and *E. coli* in milk

(a) Full cream UHT milk

![Graph showing phage biocontrol of E. coli G106 in full cream UHT milk at 5-9°C and 25°C.](image1)

(b) Skim UHT milk

![Graph showing phage biocontrol of E. coli G106 in skim UHT milk at 5-9°C and 25°C.](image2)

**Figure 7.9** Phage biocontrol of *E. coli* G106 in UHT skim milk at 5-9°C and 25°C.  
- *E. coli* G106 (control);  
- *E. coli* G106 + φEC6;  
- *E. coli* G106 + φEC9;  
- *E. coli* G106 + φEC11;  
- *E. coli* G106 + φEC phage cocktail. Error bars represent one standard deviation.
### 7.2.3.4 Phage biocontrol of EPEC in UHT milk

The φEC phages were also tested for their ability to control enteropathogenic *E. coli* O127:H6 (Figure 7.10).

**Figure 7.10** Phage biocontrol of *E. coli* O127:H6 (EPEC) in UHT skim milk at 5-9°C and 25°C.  
- **Blue line**: EPEC (control);  
- **Red line**: EPEC + φEC6;  
- **Orange line**: EPEC + φEC9;  
- **Purple line**: EPEC + φEC11;  
- **Green line**: EPEC + φEC phage cocktail. Error bars represent one standard deviation.
Although \( \Phi EC9 \) was able to suppress the growth of \( E. coli \) O127:H6 in TSB, it was unable to effect reductions in UHT milk either at low temperature or 25°C. The remaining phages and the phage cocktail were able to completely eliminate EPEC at both temperatures. However, elimination in \( \Phi EC6 \)-treated milk was not achieved until 48 hours post-inoculation, compared to 24 hours for \( \Phi EC11 \) and the phage cocktail. In the control milk samples, \( E. coli \) O127:H6 increased by approximately 0.7 \( \log_{10} \) CFU over 168 hours at 5-9°C and by 3.6 \( \log_{10} \) CFU/mL over 24 hours at 25°C.

### 7.2.3.5 Phage biocontrol of \( E. coli \) O5:NM in UHT milk

The phage cocktail was able to eliminate \( E. coli \) O5:NM, at both temperatures and in both milk samples (Figure 7.11). While \( \Phi EC6 \) and \( \Phi EC9 \) were able to eliminate \( E. coli \) O5:NM at 25°C, the individual phages were less effective at low temperature. The increase in \( E. coli \) O5:NM in control milk samples incubated at 25°C was approximately 4.2 \( \log_{10} \) units, which was comparable to the increase observed in \( E. coli \) G106 controls. At 5-9°C, the increase in \( E. coli \) O5:NM was less than 1 \( \log_{10} \) unit. \( E. coli \) O5:NM is associated with dairy cattle and has been isolated from dairy products, including cheese, as well as from the faeces of people suffering from haemorrhagic colitis (Sandhu et al., 1997; Pradel et al., 2008). Given its association with dairy products it may be better adapted to surviving in milk compared to laboratory strains.
Chapter 7  Phage biocontrol of *E. faecalis* and *E. coli* in milk

(a) Full cream UHT milk

(b) Skim UHT milk

Figure 7.11  Phage biocontrol of *E. coli* O5:NM (EHEC) in UHT skim milk at 5-9°C and 25°C.  
EHEC (control);  EHEC + φEC6;  EPEC + φEC9;  EHEC + φEC phage cocktail.  Error bars represent one standard deviation.
7.2.3.6 Phage stability in UHT milk

Phage titres were monitored in full cream UHT milk throughout the course of the biocontrol studies (Figures 7.12 and 7.13).

Figure 7.12 Phage titres in full cream UHT milk determined over the course of the phage biocontrol studies described in 7.2.3. Phage cocktail (total phage).
The changes in the φEC phage titres were non-significant during incubation at 25°C indicating that they were not inactivated in the milk.

At 5-9°C, the behaviour of the phages in full cream UHT milk containing *E. coli* K12 and G106 was comparable. Specifically, the titres of φEC9 and φEC11 decreased by up to 2 log\(_{10}\) PFU/mL while the titre of the phage cocktail and φEC6 remained stable. Reductions in phage titres of less than 1 log\(_{10}\) PFU/mL were observed in milk containing *E. coli* O127:H6 and O5:NM.

**Figure 7.13** Phage titres in full cream UHT milk determined over the course of the phage biocontrol studies described in 7.2.3. ·····φEC6; ·····φEC9; ·····φEC11; ·····Phage cocktail (total phage).
In the current studies, the phage cocktail was able to reduce all four strains of *E. coli* to below the detection limit and was effective even when one of the phages comprising the cocktail was completely ineffective, or when individual phages did not produce either total or sustained reductions. The superior efficacy of coliphage cocktails in eliminating *E. coli* in vitro compared to individual phages has been reported by several authors (Tanji *et al*., 2005; Callaway *et al*., 2008) and the same effect was observed in a study of phage biocontrol of *S. aureus* in full cream UHT milk (Garcia *et al*., 2009).

Phage biocontrol has been shown to be more effective in liquid foods than in solid foods. Guenther *et al.* (2009) found that a bacteriophage cocktail was able to eliminate *L. monocytogenes* populations in chocolate milk and mozzarella cheese brine incubated at 6°C over 6 days. The same phage preparation produced significant reductions in *L. monocytogenes* in solid foods such as smoked salmon, cabbage and sliced turkey, but the organism was still detected following 6 days of incubation. The author suggested that the large and uneven surface area of the solid foods may have limited the distribution of phage particles and that bacteria embedded in food matrices may have been shielded from diffusing phage. On solid foods, diffusion is limited and a greater concentration of phages may be necessary to achieve the same result as in liquid foods. However, liquid foods may also present unique barriers to phage biocontrol. For example, components of milk, such as proteins or fat globules may obstruct contact between phages and their target cells resulting in inactivation of the phages (O’Flaherty *et al*., 2005b; Garcia *et al*., 2009). In any circumstance, the phages must possess sufficient lytic ability to perform in the food. For example, a phage cocktail containing two phages was unable to completely clear *S. aureus* populations in UHT or pasteurized milk (Garcia *et al.* 2009) and this was similarly observed for the φSUT phage biocontrol studies described earlier in this chapter. Additional factors contributing to lower phage efficacy in milk include low phage/cell densities and the potential emergence of resistant cells (Garcia *et al*., 2009).
There is uncertainty surrounding the mode of action of phage biocontrol applications in food systems (Monk et al., 2010). Phage propagation may occur only when the bacterial host is actively growing and a host threshold must be reached before an increase in phage titre is observed (Cairns et al., 2009). In contrast, when a high concentration of phages is administered, such as the dose administered in the current study, bacterial cell death may still occur without active replication of the phages. This process is known as “lysis from without”, and occurs when a large number of phage particles attach to the surface of a bacterial cell causing disruption to the membrane (Callaway et al., 2008). Abedon (2009) suggested that it may be preferable for biocontrol studies to aim to achieve passive treatment (lysis without the requirement for phage replication) whereas Callaway et al. (2008) reported the detrimental effect of using too many phages. It is unknown whether the reductions in E. coli observed in the current study were the result of infection or lysis from without. However, many studies have used comparable concentrations of phages and reported similar findings to those of the current study (Abuladze et al., 2008; Guenther et al., 2009). Furthermore, some studies have reported a dose-dependent effect of phages in which higher concentrations have increased the log reductions in target cells in foods and on surfaces (Abuladze et al., 2008; Guenther et al., 2009; Viazis et al., 2011a). This was also observed in Chapter 5 when the log reductions in E. coli on surfaces increased with phage concentration. In addition to MOI, time and temperature also contribute to efficacy of phages in biocontrol applications (Viazis et al., 2011a). Irrespective of the mode of action, the φEC phages investigated in the current study were effective in controlling their target hosts in UHT milk. Based on these findings, further investigation in raw milk was undertaken.
7.2.4 Phage biocontrol of *E. coli* in raw milk

A sample of non-homogenised, full cream, raw milk with a low SPC and no detectable *E. coli* was used for this set of experiments (Table 7.1).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Viable count (Log$_{10}$ CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>2.45</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>n.d.</td>
</tr>
<tr>
<td>Coliforms</td>
<td>1.0</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>n.d.</td>
</tr>
<tr>
<td>Enterococci</td>
<td>1.0</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>n.d.</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not detected

In the UHT milk studies described in Section 7.2.3, the phage cocktails were at least as effective as the individual phages. Therefore, only phage cocktails were used in the raw milk studies. Furthermore, a time-course assay was performed during the study to monitor the growth of selected microbiota present in the milk (Figure 7.14). The pH of the milk was also monitored, and was observed to decrease from 6.5 to 6.0 after 96 hours.
The SPC of the raw milk increased in line with the results obtained for temperature abused milk studied in section 6.2.1. Coliforms and enterococci also increased, while coagulase positive staphylococci remained stable. The number and type of microbiota present will likely affect the efficacy of the phages, either by acting as competition to the introduced \textit{E. coli} target cells and inhibiting growth, or by impeding the phages from making contact with the target cells (Hudson \textit{et al.}, 2005). Similarly, it is possible that indigenous phages, if present, may also affect the ability of the introduced phages to infect the host population.

### 7.2.4.1 Phage biocontrol of \textit{E. coli} G106 in raw milk

At both 5-9°C and 25°C, the phage cocktail eradicated \textit{E. coli} G106 within the period prior to the first sampling point (Figure 7.15). No \textit{E. coli} G106 was recoverable from any phage-treated milk at any sampling point during the study. The titre of the phage cocktail remained constant throughout the study at both temperatures. Interestingly, the level of \textit{E. coli} in control milk samples also decreased to below the detection limit within 144 hours at 5-9°C and within nine hours at 25°C.
Figure 7.15  Phage biocontrol of *E. coli* G106 in raw milk. *E. coli* G106 (control); *E. coli* G106 + ϕEC phage cocktail; ϕEC phage cocktail titre. Error bars represent one standard deviation.
7.2.4.2 Phage biocontrol of *E. coli* K12 in raw milk

Similar results were observed for *E. coli* K12 in raw milk. The phage cocktail eradicated *E. coli* K12 at both temperatures within the period preceding the first sampling point. However, at 25°C, *E. coli* K12 in the control milk was not eliminated until 12 hours post-inoculation, compared with 9 hours for *E. coli* G106.

**Figure 7.16** Phage biocontrol of *E. coli* K12 in raw milk. ■ *E. coli* K12 (control); ■ *E. coli* K12 + φEC phage cocktail; ••••• φEC phage cocktail titre. Error bars represent one standard deviation.
7.2.4.3 Phage biocontrol of enteropathogenic *E. coli* O127:H6 in raw milk

The phage cocktail proved as effective in eliminating *E. coli* O127:H6 as for the non-pathogenic strains at both temperatures (Figure 7.17). Although at temperatures between 5-9°C, the decline in EPEC in the control milk was comparable to the non-pathogenic *E. coli*, at 25°C, it was not eliminated until up to 24 hours of incubation.

**Figure 7.17** Phage biocontrol of *E. coli* O127:H6 (EPEC) in raw milk. EPEC (control); EPEC + φEC phage cocktail; φEC phage cocktail titre. Error bars represent one standard deviation.
7.2.4.4 Phage biocontrol of enterohaemorrhagic *E. coli* O5:NM in raw milk

*E. coli* O5:NM was the only strain of *E. coli* that persisted in raw milk for the duration of the study at either temperature (Figure 7.18). At 25°C, *E. coli* O5:NM increased by approximately $1.2 \log_{10} \text{CFU/mL}$ after 24 hours while at 5-9°C, no significant change ($p > 0.05$) was observed.

**Figure 7.18** Phage biocontrol of *E. coli* O5:NM (EHEC) in raw milk. EHEC (control); EHEC + φEC phage cocktail; φEC phage cocktail titre. Error bars represent one standard deviation.
While the phage cocktail comparably eliminated *E. coli* G106, K12 and O27:H6 in raw milk regardless of incubation temperature, regrowth of *E. coli* O5:NM in phage-treated milk occurred following nine hours of incubation at 25°C and after 144 hours of incubation at 5-9°C. The phage cocktail used in that study comprised only two phages, compared to the three phages used for the other *E. coli* strains. Furthermore, while the phage cocktail eliminated EHEC in UHT milk incubated at 5-9°C, the individual phages were unable to achieve sustained reductions. In addition, in raw milk studies using other *E. coli* strains, the host in the control also died out during study, making the probability of *E. coli* recurring in the corresponding phage-treated milk unlikely.

The results from the current raw milk study are promising given the less encouraging findings reported by O’Flaherty *et al.* (2005b) for phage biocontrol of *S. aureus* in raw milk. Bacteriophage K was able to eliminate *S. aureus* in heat-treated milk but not raw. In a similar study, Garcia *et al.* (2009) used phages φA5 and φA72 to reduce *S. aureus* in full cream and semi-skimmed raw milk incubated at 37°C. *S. aureus* in phage-treated milk samples increased during the study but to significantly lower levels (*p* < 0.05) than in the untreated milk samples. The concentration of the host bacteria and phage were lower and probably more realistic than the concentrations used in the current study. The authors acknowledged that lower host cell densities require greater concentrations of phage for sufficient infection to occur and that the antimicrobial activity of the phages may have been reduced due to the slower growth of *S. aureus* in raw milk compared to heat treated milk as a result of the presence of competitive microbiota. Furthermore, φA5 and φA72 were temperate phages which may also have contributed to the lower efficacy in inhibiting *S. aureus*. In another study by the same researchers, lytic mutants of φA5 and φA72 were found to be effective in eliminating *S. aureus* in UHT milk and during curd production using pasteurized milk (Garcia *et al.*, 2007). However, the efficacy of these lytic mutants in raw milk was not investigated.

In the current study, phage titres remained stable. O’Flaherty *et al.* (2005b) found that phage K was inhibited in raw milk due to agglutination of bacterial cells with fat globules which prevented phage attachment. Similarly, Garcia *et al.* (2009) reported
that the titre of фA5 and фA72 decreased by 1 log₁₀ unit after 10 hours of incubation in raw milk suggesting possible partial inactivation. However, phages of other bacteria have been successful in inhibiting their hosts in raw milk products without significant loss in titre. For example, Modi et al. (2001) were able to recover Salmonella-specific phages from raw milk and raw milk cheese with no loss in titre.

The composition and microbiota of raw milk varies greatly depending on the breed of cattle, geographic location, season and milking and handling procedures (Oliver, 2005). These factors may contribute to the efficacy of phage biocontrol and a more extensive study should be undertaken to determine their effect on the reproducibility of the results of this study. The concentration of microbiota in the current study was relatively low and therefore the effect of a high microbial load should also be assessed. Wilkinson (2001) described a mathematical model in an attempt to explain the mixed success reported by others in using phages to eliminate pathogenic bacteria in the gastrointestinal tract. He theorised that phage efficiency in the gastrointestinal system was impeded by the high density of non-target species (decoys). Only when the host was present in higher numbers than the decoys would the phages be predicted to reach the same levels as in the absence of competitive organisms. The results from the current study are in agreement with this model. The concentration of *E. coli* initially added to the milk samples was approximately 500-fold higher than the SPC calculated at the beginning of the study. All phage-treated milk samples showed a reduction in *E. coli* within 24 hours of inoculation at 5-9°C.

While most *E. coli* did not survive in the raw milk controls, the phage cocktail was able to eliminate all strains of *E. coli* apart from O5:NM within 24 hours suggesting that phage biocontrol may be useful where raw milk is to be consumed within 24 hours of production. Future research should focus on characterising phages able to infect dairy isolates of *E. coli* which may be better adapted to surviving in raw milk and would serve as more reliable controls. Garcia et al. (2009) also suggested that the origin of phages should be taken into account when designing preparations for biopreservation or therapeutic purposes.
7.3 Chapter summary

7.3.1 Phage biocontrol of enterococci in milk

The enterococcal phages фSUT1 and фSUT4 were selected for study as biocontrol agents to control strains of *E. faecalis* in UHT and pasteurized milk. The two phages varied considerably in their ability to reduce their respective hosts in each type of milk. фSUT1 reduced *E. faecalis* to below the level of detection within three hours of incubation in both full cream and skim UHT milk at 25°C. However, following incubation for nine hours, *E. faecalis* increased, albeit to a significantly lower concentration than the control after 24 hours. At 10°C, фSUT1 significantly reduced, but did not eliminate, *E. faecalis* over 168 hours in both skim and full cream UHT milk. Conversely, фSUT4 was more effective in reducing VRE (M168600) in both skim and full cream UHT milk at 10°C than at 25°C. However, while фSUT4 was able to prevent VRE (M168600) from increasing for the first 48 hours of incubation at 10°C, the level increased to within 1.5 log_{10} units of the control in full cream UHT milk and 1.0 log_{10} units of the control in skim UHT milk at the end of the study. At 25°C, the difference between the control and фSUT4-treated milk samples was less than 1.0 log_{10} units after 24 hours.

Despite initial reductions in фSUT4-treated pasteurized milk, VRE (M168600) began to increase after 24 hours of incubation at 10°C. Interestingly, VRE (M168600) grew more rapidly and to a higher concentration in pasteurized milk than in UHT milk. In contrast *E. faecalis* in the control milk decreased during the study. Both phages were more effective in controlling their respective hosts in UHT milk compared to pasteurized milk.

All phage titres increased in UHT milk inoculated with enterococci and incubated at 25°C and remained stable at 10°C. In pasteurized milk, no significant changes in phage titres were observed. The phages were not tested for their ability to control *Enterococcus* in raw milk because they were unable to achieve significant reductions in pasteurized milk.
7.3.1 Phage biocontrol of *E. coli* in milk

The three coliphages, φEC6, φEC9 and φEC11, were tested for their ability to lyse *E. coli* strains in TSB at 37°C with shaking under aerobic conditions. For all strains infected with the phages, culture turbidity did not increase significantly until after at least 8 hours of incubation. The phages were also tested in cocktails. Cocktails containing all three phages were used to infect *E. coli* K12, G106, and O127:H6. Culture turbidity did not increase for the duration of the 24 hour study. A cocktail containing φEC6 and φEC9 only was used to infect *E. coli* O5:NM but was unable to delay culture turbidity for longer than was achieved by the phages when used individually.

The phages were also tested for their ability to control the same strains of *E. coli* in full cream UHT milk and skim UHT milk at 5-9°C and at 25°C. In all experiments, the phage cocktails eliminated all strains of *E. coli* whereas the individual phages varied in their effectiveness. The phage titres remained relatively stable during the 25°C studies while φEC9 and φEC11 decreased by up to 2.0 log\(_{10}\) units in *E. coli* K12 and *E. coli* G106 studies at 5-9°C. Given their superior effectiveness in reducing *E. coli* in UHT milk over the individual phages, cocktails only were tested when the study was repeated using raw milk. A sample of full cream, non-homogenised milk was first tested for SPC, *E. coli*, coliforms, staphylococci and enterococci. The sample was negative for *E. coli* and had a low SPC. Over the duration of the phage biocontrol study, the SPC, coliforms and enterococci increased steadily while coagulase positive staphylococci remained stable.

The phage cocktail eliminated *E. coli* K12, G106 and O127:H6 in raw milk in both the 5-9°C and 25°C study. In milk contaminated with *E. coli* O5:NM, the phage cocktail reduced the number of bacteria to below the level of detection. However, elimination was not achieved as regrowth of *E. coli* O5:NM occurred at both 5-9°C and 25°C. The results of the raw milk studies were promising but further investigations should be carried out to determine the effect of differences in milk composition and microbiota on the efficacy of the phages.
CHAPTER 8

Conclusion
8.1 Introduction

The preceding chapters described the studies undertaken in order to address the major aims of the project, which were to:

- isolate and characterise phages infecting *Enterococcus* spp. and *E. coli*; and
- assess the ability of these phages to control their hosts on surfaces and in milk under a range of conditions

This chapter provides an overview of the major findings of this thesis as well as identifying the scope for further research.

8.2 Summary of findings

8.2.1 Chapter Four

Four enterococcal phages were isolated from sewage and landfill leachate. The phages were designated φSUT1, φSUT3, φSUT4 and φSUT6. Biological characterisation revealed that the phages possessed a very narrow host range, with φSUT1 the only isolate able to infect more than one host. The phages were found by electron microscopy to be tailed phages resembling the *Siphoviridae*. φSUT3, φSUT4 and φSUT6 possessed isometric capsids while φSUT1 had an elongated capsid and longer tail. Each of the φSUT phages possessed unmodified dsDNA genomes. Restriction profiles illustrated a close genetic relationship between φSUT3 and φSUT4 whereas φSUT1 and φSUT6 were distinct.

One-step growth curves indicated that the φSUT phages had similar latent periods to other enterococcal phages reported in the literature (Uchiyama et al., 2008). The average burst sizes produced by φSUT3 and φSUT4 were similar at 180 and 183 per infected cell, respectively. φSUT6 produced a burst size of 130 while the burst size of φSUT1 was relatively small at 68 PFU/cell. The ability of φSUT phages to persist during exposure to a range of pH was also measured. None of the phages were recovered following incubation at pH 2.2 or pH 3.0 for one hour but all survived without significant loss in titre when incubated at pH 4.0, 6.0 and 7.0.
Given the narrow host range of the φSUT phages, it was considered unlikely that they would be suitable real-world biocontrol candidates. However, they were considered useful for primary proof-of-concept studies given the paucity of literature relating to phage biocontrol of enterococci.

Several coliphages were also isolated from sewage. Selected isolates, designated φEC6, φEC9 and φEC11, were chosen for characterization based on differences in plaque morphology and host range. These phages had a broader host range compared to the enterococcal phages, including pathogenic strains belonging to different serotypes. All three phages were also able to infect a range of non-pathogenic *E. coli* strains. Primary characterisation of the coliphage isolates confirmed that they were morphologically and genetically distinct from one another. Morphological analysis of the phage isolates revealed them to belong to different families within the order *Caudovirales*. φEC6 was classified into the *Siphoviridae*, φEC9 into the *Myoviridae* and φEC11 into the *Podoviridae*, according to tail structure. Genetic analysis of the phages indicated modified dsDNA genomes as well as the absence of *E. coli* virulence factor *eaeA*.

φEC6 was found by one-step growth curve to have the shortest latent period (20 minutes) and the highest burst size (113 per infected cell). φEC9 and φEC11 both had a latent period of 30 minutes. Their average burst sizes varied at 62 and 80 PFU per infected cell, respectively. The sensitivity of the coliphages to pH was more varied than was observed for the enterococcal phages. φEC9 was the most tolerant, with no observable reduction in titre following incubation for 1 hour at pH 3.0, pH 4.0, pH 6.0, pH 7.0 or pH 9.0 and only a small loss in titre at pH 2.2. While φEC6 was stable from pH 4.0 to pH 9.0, φEC11 was the most sensitive of the phages to low pH, with a significant reduction in titre observed following incubation at pH 4.0.

### 8.2.2 Chapter 5

The φSUT enterococcal phages and φEC coliphages isolated in Chapter 4 were used to control *E. faecalis* and *E. coli* contamination on different surfaces. φSUT1 reduced *E. faecalis* by at least 4 log₁₀ unit on all surfaces but was less effective in
reducing VRE (M252807) on the same surfaces. The reductions in VRE (M252807) were less than $1 \log_{10}$ unit on all surfaces. However, this was slightly higher than the reductions achieved by $\phi$SUT6. When the phages were combined, the cocktail was as at least as effective as $\phi$SUT1, but reductions remained less than $1 \log_{10}$ unit. The remaining enterococcal phages, $\phi$SUT3 and $\phi$SUT4, were similarly used to disinfect surfaces contaminated with VRE (M168600). $\phi$SUT4 was more effective than $\phi$SUT3 on all surfaces except polyester. Interestingly, the phage cocktail was less effective than $\phi$SUT4 on all surfaces except glass.

A similar study was performed using a cocktail containing the $\phi$EC phages to control four strains of $E. coli$ on stainless steel and glass. Surfaces contaminated with $E. coli$ and treated with phage cocktail were incubated at ambient temperature for either 10 minutes or 60 minutes. When the highest concentration of phage was used ($10^8$ PFU), all $E. coli$ strains were eliminated on stainless steel and glass regardless of contact time. The two lowest applications of phage cocktail ($10^6$ and $10^7$ PFU) produced reductions in $E. coli$ G106, K12 and O127:H6 on both surfaces which were time and dose dependent. On surfaces contaminated with $E. coli$ O5:NM, the cocktails containing $10^7$ PFU and $10^8$ PFU were equally effective in eliminating $E. coli$ after 60 minutes of incubation.

8.2.3 Chapter 6

While the microbiota of raw milk has been studied extensively worldwide, there is little published information regarding the quality of raw milk produced in Australia. This study found Australian raw milk to contain pathogens including $Salmonella$ spp., as well as indicators of faecal contamination including coliforms, $E. coli$ and $Enterococcus$ species. There was considerable variation in the microbiological quality of raw milk obtained from three sources indicating that a wider study of Australian milk over time could be beneficial. Mild temperature abuse of raw milk resulted in significant increases in SPC, coliforms and $Enterococcus$ spp. over seven days while coagulase positive $Staphylococcus$ and $E. coli$ survived without significant reduction in viable count. Given the incidence of $E. coli$ and enterococci
in raw milk in the current study, an investigation of the ability of phages to control these organisms in raw milk was justified.

Although neither *E. coli* nor *Enterococcus* sp. were identified in the pasteurized milk survey, phage biocontrol of these organisms could still be worthwhile. Many studies have isolated *Enterococcus* spp. from pasteurized milk and at the time of the current study, a pasteurized milk product was recalled by the Victorian state regulatory authority in Australia due to *E. coli* contamination. Isolates recovered from the implicated product were found by ERIC-PCR to be genetically diverse, suggesting that phages with a broad host range would be necessary for effective control of *E. coli* in milk.

### 8.2.4 Chapter 7

Due to their superior efficacy compared to the other enterococcal phages in reducing their respective hosts on surfaces (Chapter 5), φSUT1 and φSUT4 were selected for study as biocontrol agents to control strains of *E. faecalis* in UHT and pasteurized milk. The two phages varied considerably in their ability to reduce their respective hosts in each type of milk. φSUT1 initially reduced *E. faecalis* to below the level of detection in full cream and skim UHT milk at 25°C before an increase was observed after 9 hours. At 10°C, *E. faecalis* was significantly reduced in φSUT1-treated UHT milk over 168 hours. On the other hand, φSUT4 was more effective in reducing VRE (M168600) in UHT milk at 10°C than at 25°C. However, while φSUT4 was able to prevent VRE (M168600) from increasing for the first 48 hours of incubation at 10°C, the level increased to within 1.5 log_{10} units of the control in full cream UHT milk and 1.0 log_{10} units of the control in skim UHT milk at the end of the study. At 25°C, the difference between the control and φSUT4-treated milk samples was less than 1.0 log_{10} units after 24 hours.

Despite initial reductions in φSUT4-treated pasteurized milk, VRE (M168600) began to increase after 24 hours of incubation at 10°C. Interestingly, VRE (M168600) grew more rapidly and to a higher concentration in pasteurized milk than in UHT milk. In contrast, *E. faecalis* in the control milk decreased during the study.
Both phages were more effective in controlling their respective hosts in UHT milk compared to pasteurized milk. All phage titres increased in UHT milk incubated at 25°C and remained stable at 10°C. In pasteurized milk, no significant changes in phage titres were observed.

The coliphages фEC6, фEC9 and фEC11 were used individually and in a cocktail to control four strains of *E. coli* in UHT milk incubated at 5-9°C and at 25°C. While the individual phages varied in their effectiveness, the cocktails eliminated all four *E. coli* strains at both temperatures. The phage titres remained stable during the 25°C studies while фEC9 and фEC11 decreased by up to 2 log_{10} units in *E. coli* K12 and *E. coli* G106 studies at 5-9°C. A subsequent study used the фEC phage cocktails to reduce *E. coli* contamination in raw milk. A sample of full cream, non-homogenised milk with a low SPC and no detectable *E. coli* was used in the study. The phage cocktail eliminated *E. coli* K12, G106 and O127:H6 in raw milk within 6 hours at 25°C and within 24 hours at 5-9°C. In milk contaminated with *E. coli* O5:NM, the phage cocktail achieved apparent elimination of bacteria during the initial stages but *E. coli* grew at both 5-9°C and 25°C over time. Interestingly, *E. coli* K12, *E. coli* G106 and *E. coli* O127:H6 in raw milk controls also declined to below the level of detection within 24 hours at 25°C and after 144 hours at 5-9°C. In contrast, *E. coli* O5:NM increased by 1.2 log_{10} CFU/mL after 24 hours in raw milk incubated at 25°C and remained stable over 168 hours in raw milk incubated at 5-9°C. Phage titres did not decrease in *E. coli*-contaminated raw milk incubated at either temperature.

### 8.3 Scope for further research

#### 8.3.1 Additional characterisation of phages

Additional characterisation of enterococcal phage φSUT1 and coliphages φEC6, φEC9 and φEC11 should be considered given their effectiveness in controlling their respective hosts in different applications. Furthermore, the coliphages identified in this study should also be tested further for their lytic ability against a greater range of *E. coli* hosts including those isolated from milk and other dairy products. Comprehensive characterisation, including genome sequencing, can be used to
confirm identity and relation to known phages, as well as rule out the presence of undesirable elements such as bacterial toxins or lysogenic components (Monk et al., 2010). Carlton et al. (2005) sequenced the genome of a phage, P100, which was used to control *Listeria monocytogenes* in foods. Bioinformatic analyses confirmed that the phage did not possess any proteins homologous to those known to be toxins, pathogenicity factors or antibiotic resistance determinants. The authors also conducted oral toxicity studies in rats to determine whether the phages could be safely ingested without causing abnormal histological changes. The safety of bacteriophages has also been demonstrated in human studies (Chibani-Chennoufi et al., 2004b; Bruttin and Brussow, 2005). Furthermore, a commercial phage product used to control *Listeria monocytogenes* in foods was given GRAS status (Hagens and Loessner, 2010).

### 8.3.2 Evaluation of phages under sub-optimum conditions

While φSUT1 and the φEC phage cocktails were successful in reducing their respective hosts on surfaces under optimum conditions, further studies should investigate the effect of the phages under different conditions to determine their efficacy in realistic situations. Most phage biocontrol studies, including the current study, have reported using target cells that were first grown to mid-exponential phase (Abuladze et al., 2008; Viazis et al., 2011a). Bacteria are more susceptible to phage infection while actively growing (Abedon, 2009) and using stationary phase target cells could result in lower phage efficacy. Host cell concentration is another important consideration given that low host densities require higher phage concentrations to achieve significant infection (Greer, 2005; Hagens and Loessner, 2010). This is particularly important on surfaces where diffusion of phages may be limited. Further studies should determine the lowest limit at which the phages described in the current study are effective.

A more extensive study into the effect of incubation temperature should also be undertaken in both surface and food biocontrol studies. The surface biocontrol studies reported in this thesis were conducted at ambient temperature only. Surfaces in food processing environments may also be exposed to cold temperatures such as
those found in walk-in refrigerators. Viazis et al. (2011a) reported a significant decrease in phage efficacy when phage-treated surfaces were incubated at 4°C compared to ambient temperature. Furthermore, bacteria can have a greater capacity to form biofilms when exposed to low temperature (Chmielewski and Frank 2003) which may also affect phage biocontrol efforts. Sharma et al. (2005) reported that bacteriophage KH1 was able to reduce *E. coli* O157:H7 attached to stainless steel coupons by 1.2 log CFU/coupon after one day of incubation at 4°C. However, no reduction was observed when KH1 was used to treat *E. coli* O157:H7 in biofilms. Similarly, in food biocontrol studies, phage efficacy has been reported to be reduced by incubation at low temperature (Leverentz et al., 2001; Viazis et al., 2011b). In the milk biocontrol studies described in this thesis, the phages were tested at low temperatures designed to simulate mild temperature abuse. However, further study should determine the effect of the phages under lower temperature conditions (< 5°C) rather than just abuse conditions (Hagens and Loessner, 2010).

In surface biocontrol studies, the effect of the presence of organic matter and non-target host cells should also be studied. While in the current study skim milk was first added to surfaces contaminated with *E. coli*, the surfaces inoculated with *E. faecalis* were clean. The skim milk appeared to have no effect on phage efficacy which was also reported by Abuladze et al. (2008). Similarly, the presence of non-host bacteria may affect the ability of the phages to adsorb to the intended host (Wilkinson, 2001). A final consideration that deserves further investigation is whether the φEC phage cocktail is capable of controlling more than one strain of *E. coli* at a time on surfaces and in milk. The genetic diversity of *E. coli* isolated from pasteurized milk (Chapter 6) suggests that phages may need to infect multiple strains in order to be effective. Other studies have reported using phage cocktails to reduce more than one strain of *E. coli* O157:H7, but not strains belonging to different serotypes (Abuladze et al., 2008; Viazis et al., 2011a).

### 8.3.3 Phages in hurdle treatments

Phages have been combined with chemical sanitisers to disinfect surfaces (Roy et al., 1993) and bacteriocins to reduce bacterial contamination in foods (Leverentz et al.,
2003; Martinez et al., 2008). Such hurdle approaches have been shown in some circumstances to produce a synergistic effect (Greer, 2005). For example, when melon and apple slices artificially contaminated with *L. monocytogenes* were treated with a phage cocktail, bacterial numbers were reduced by 2.0–4.6 log_{10} units on honeydew melons and by 0.4 log_{10} units on apples. Nisin in the absence of phage produced reductions of 3.2 log_{10} units on honeydew and up to 2.0 log_{10} units on apples. However, when the two treatments were combined, the solution reduced *L. monocytogenes* populations by up to 5.7 log_{10} units on honeydew melon slices and by up to 2.3 log_{10} units on apples compared to the control. The differences in treatment efficacy were due to the lower pH of apple slices compared to the melon slices.

In a similar study, a phage cocktail was combined with nisin to reduce *S. aureus* contamination in pasteurized milk (Martinez et al., 2008). A synergistic effect was observed when nisin was used in combination with the phages. However, a nisin-adapted isolate was obtained which also exhibited partial resistance to the phages used. When the adapted isolate reverted to the nisin-sensitive phenotype, phage susceptibility was similarly restored. Conversely, phage insensitive mutants did not exhibit nisin-resistance. The authors suggested that changes in the bacterial cell surface linked with nisin resistance could have interfered with phage attachment.

A surface disinfection study by Roy et al. (1993) investigated three phages, 2671, H387, and H38, as a means of disinfecting stainless-steel and polypropylene contaminated with *L. monocytogenes*. The study also compared the effectiveness of the phage cocktail to a chemical disinfectant (a quaternary ammonium compound, QUATAL), and to a solution containing both phage and QUATAL. The phage solution containing 8.0 log_{10} units PFU/mL was as efficient as a 20 ppm solution of QUATAL in disinfecting the contaminated surfaces. Furthermore, a synergistic effect was observed when the surfaces were disinfected with the combined treatment containing the phages suspended in QUATAL. The phages could maintain their infectivity when exposed to various concentrations of QUATAL (1 to 50 ppm) for up to 4 hours.
Based on these findings, it would be worthwhile to determine whether the phages characterised in this study are able to survive contact with chemical sanitizers or bacteriocins, and furthermore, whether the combined approach could be used to control pathogens on surfaces and in milk.

8.4 Final discussion

While the number of publications relating to phage biocontrol has been increasing over recent years, the progression to commercial development of phage-based products has been limited. Table 8.1 lists some of the phage biocontrol products that are currently available and their intended applications.

Table 8.1
Commercial phage based products

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgriPhage™</td>
<td>Targets bacterial spot on crops</td>
<td>Omnilytics</td>
</tr>
<tr>
<td>Biotecter</td>
<td>Animal feed for control of <em>Salmonella</em> in poultry</td>
<td>CheilJedang Corporation</td>
</tr>
<tr>
<td>EcoShield™</td>
<td>Targets <em>E. coli</em> O157:H7 contamination in foods and food processing facilities</td>
<td>Intralytix</td>
</tr>
<tr>
<td>ListShield™</td>
<td>Targets <em>L. monocytogenes</em> contamination in foods and food processing facilities</td>
<td>Intralytix</td>
</tr>
<tr>
<td>Listex™ P100</td>
<td>A food processing aid that targets <em>L. monocytogenes</em> strains on food products</td>
<td>EBI Food Safety</td>
</tr>
</tbody>
</table>

Adapted from Monk *et al.* (2010)

The biological nature of phages and their host specificity make some of the issues relating to their development, commercialisation and regulation unique. Host specificity is often touted as an advantage of phages over traditional antimicrobial drugs and disinfectants. However, the specificity of phages may also limit their
ability to be widely used as biocontrol agents in foods. Unlike therapeutic applications, where phages can be matched to a specific cause of infection on a case-by-case basis, phage biocontrol products will require frequent adjustment and incorporation of new phages to keep up with changes in the bacterial flora (Hagens and Loessner, 2010). Therefore, while production of phages is relatively inexpensive, the cost effectiveness may be reduced when the need for continual modification is taken into account.

Many studies have demonstrated that phages can effectively control bacteria that have been experimentally inoculated onto small areas of foods and surfaces. However, advancement to “in field” studies assessing the efficiency of phages under practical conditions is less commonly reported (Greer, 2005). There are a number of issues relating to scale-up of phage solutions and application to realistic situations. The mode and timing of application are important considerations (Hagens and Loessner, 2010) with many laboratory studies simply using pipettes to add small volumes of phages to food or surfaces shortly after bacterial inoculation. While spraying or washing foods and surfaces with phage-based solutions have also been proven effective, these require large volumes of phage and result in substantial waste production. Furthermore, addition of phages in large volume to liquid foods such as milk will result in dilution of phage concentration and product (Hagens and Loessner, 2010).

To the author’s knowledge, no phage products designed for food-based applications have yet been approved for use in Australia and it is unknown how local regulators will respond to this technology. Given that the majority of phage research and development is undertaken by small companies (Monk et al., 2010), there may be a reluctance to invest in this technology in Australia due to the uncertainty regarding regulation. An additional consideration is the attitude of the local food industry and consumers towards the use of phages in the production of food (Hagens and Loessner, 2010). Anecdotal evidence from the local dairy industry suggests that producers would be reluctant to adopt this technology (D. Eddy, personal communication).
Given the increasing burden of bacterial disease, the emergence of new pathogens and the increasing resistance of bacteria to conventional antimicrobials, continued research into novel biopreservation strategies such as phage biocontrol is vital. While the issues highlighted above add to the challenges associated with such research, the success reported in this and other studies provides cause for optimism.

### 8.5 Conclusion

The results from the current study provide encouraging evidence that phages may be used successfully to control *E. faecalis* and *E. coli* on surfaces and in milk. Further study should be carried out to determine the effectiveness of the phages in a wider range of conditions.
References


References


FSANZ (2011) Australia New Zealand Food Standards Code, Standard 4.2.4, FSANZ, Canberra.


References


Publications arising from this thesis

A.1 Book chapter


A.2 Refereed articles


A.3 Conference presentations

A.3.1 Oral presentation


A.3.2 Poster presentation