A Functional Genomics Approach for Improved Bacteriophage Cocktail Design

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Abstract

The ability of virulent bacteriophages to lyse and kill bacteria has dramatic impacts on bacterial evolution, fitness, and population structures. In addition, they also have great potential for use in therapy as well as biocides in food processing and agriculture. For successful bacteriophage applications, in depth knowledge of their interactions with a susceptible host is required to ensure optimal effectiveness. This should include knowledge of host susceptibility and resistance factors which can be used to select combinations of phages, which when used together, kill bacteria more efficiently. In this study, we conduct an extensive case study of a raw pet food producer to investigate microbial pathogens present within the factory environment and understand stages of production where bacteriophages could be used to reduce microbial pathogen presence. We present the isolation and in-depth characterisation of twelve bacteriophages which infect *Salmonella* enterica strains isolated from pet food products. Utilising a high throughput functional genomic screen, we better understand how bacteriophages interact with a susceptible host and select a combination of bacteriophages which is ten times more effective at killing *Salmonella.* Testing the use of a phage cocktail containing these phages, we demonstrate its efficacy at reducing *Salmonella* within a food matrix in conditions consistent with culture-based food surveillance testing. A better understanding for the use of bacteriophages will help inform their use, ensure efficacy, and help to reduce the burden of bacterial diseases by ensuring a safe supply of food.

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1. Introduction

1.1. Introduction to *Salmonella*

Foodborne Disease (FBD) is an ongoing problem posed to global health care systems. FBD is defined as an illness caused by the consumption of contaminated food products. In 2010, FBD was estimated to result in 600 million illnesses and 420,000 deaths worldwide (Havelaar et al., 2015). Over half of these deaths could be attributed to infections caused by non-typhoidal *Salmonella*. Although mortality in high income countries is low, the European Food standards agency (EFSA) has estimated that the total economic cost of *Salmonella* Infections in Europe exceeds 3 billion euros (€) every year (Efsa, 2014). The substantial economic cost and disease burden caused by *Salmonella* has driven the need for effective intervention methods to reduce prevalence within the food production industry.

1.1.1. Characteristics and taxonomy of *Salmonella*

Salmonella is widespread in nature, which as a result, makes it one of the most commonly isolated foodborne pathogens (Eng et al., 2015). Members of the *Salmonella* genus are Gram-negative, facultative anaerobic, non-spore forming, rod shaped bacteria. *Salmonella* bacteria can effectively and efficiently colonise and elicit disease in humans and a range of animals. *Salmonella* possesses long, peritrichous flagella which aids their motility.

The *Salmonella* genus is diverse, with thousands of antigenic variants arising as a result of within-host evolution (Tanner and Kingsley, 2018). Traditionally, *Salmonella* lineages were determined by biochemical properties which lead to the establishment of two species – *enterica* and *bongori.* Most clinically relevant species are found within *enterica* which is further sub-divided into 6 subspecies: *enterica* (I), *salmae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) *and indica* (VI) (Figure 1). Each subspecies contains multiple serovars which are determined by the combination of antigens present on their cell surface. This

classification system is referred to as the Kauffmann-White Scheme which utilizes diversity found within the somatic antigen (O Antigen) and flagella antigen (H Antigen) (Grimont and Weill, 2007). The O antigen is determined by the oligosaccharides which are associated with the lipopolysaccharides present on the outer membrane of *Salmonella.* The H antigen is determined by the flagellin variant which constitutes the flagella. Most *Salmonella are* capable of a process known as phase variation by which the flagellin subunit constituting the H antigen may be one of two variants controlled by the expression of either the *fliC* or *fljB* genes. *Salmonella* that are only capable of expressing one form a flagellin, due to a loss of one allele, are termed monophasic. Examples include *Salmonella enterica* subsp. *Enterica* sv 1,4,[5],12:1:-, a monophasic variant of *Salmonella* Typhimurium (Petrovska et al., 2016). In addition, some serotypes lost both flagellin genes resulting in a non-motile phenotype. For example, *Salmonella* Galinarum is non-flagellated and non-motile (Li et al., 1993). An overview of the current recognised *Salmonella* classification scheme is shown in Figure 1. More recently, the application of routine whole genome sequencing of *Salmonella* isolates has led to the proposed addition of new species and sub-species. The proposal promotes arizonae (IIIa) from sub-species to species level and establishes a new species, *Salmonella englandensis* which contains five new sub-species – londinensis (VII), brasiliensis (VIII), hibernicus (IX), essexiensis (X) and reptilium (XI) (Chattaway et al., 2023)

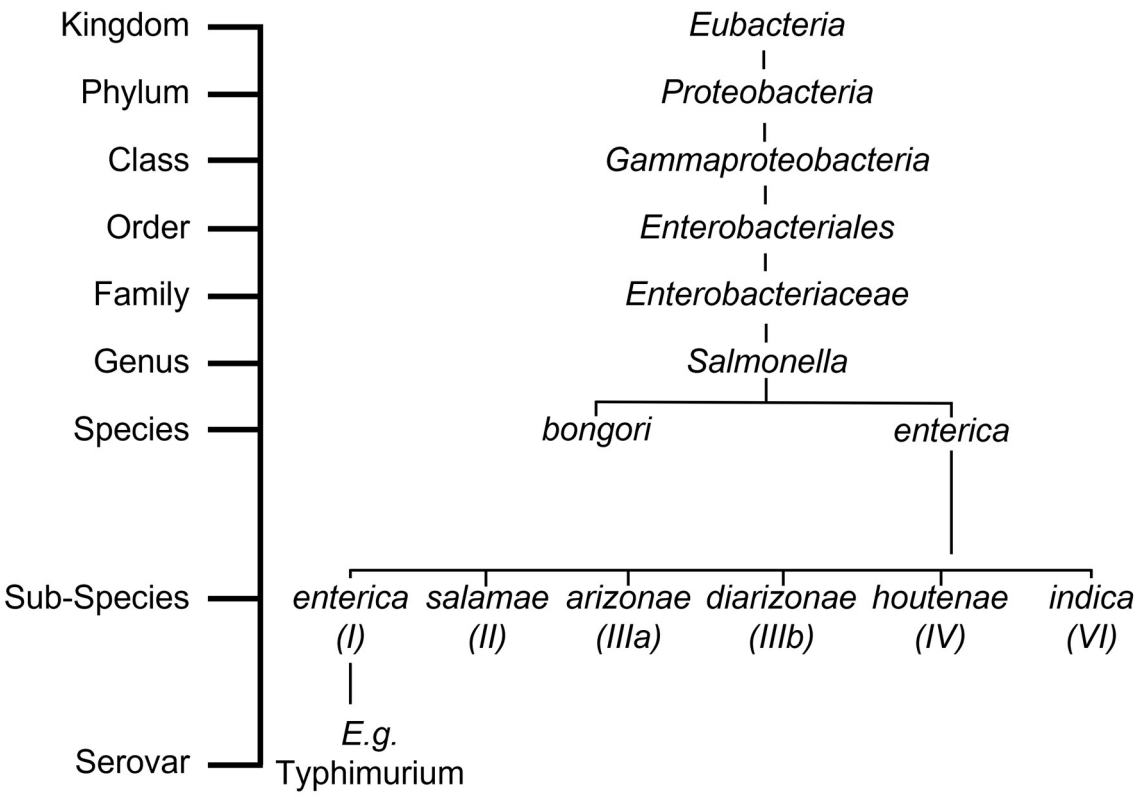


Figure 1 - Overview of the current classification of Salmonella.

1.1.2. Population Structure of *Salmonella* Typhimurium

Salmonella host-pathogen interactions have been extensively studied, although the vast majority of published research uses a small selection of lab strains, particularly *Salmonella enterica* sv. Typhimurium SL1344 (Branchu et al., 2018). While combined research has resulted in a vast understanding of these strains, such approaches fail to properly consider the extant diversity observed within both Typhimurium and the *Salmonella* genus as a whole. The advent of next generation sequencing revealed the genetic diversity underlying the observed antigenic diversity (Callow, 1959). More recently, phylogenetic analysis using a large collection of diverse *Salmonella* Typhimurium strains revealed the remarkable diversity of a serotype (Bawn et al., 2020). The population structure consists of multiple lineages present in two high order clades (α and β) (Figure 2). Clade α contained strains associated with well documented epidemics in domestic animals, whereas Clade β

contained strains generally associated with infection of wild avian species. The distinction of these clades is well reflected in the distribution of antimicrobial resistance genes, which are far more common in strains belonging to clade α. Similarly, the presence of hypothetically interrupted coding sequences (HDCS) was far more common in clade β, a signature generally associated with host adaption (Branchu et al., 2018, Wheeler et al., 2018).

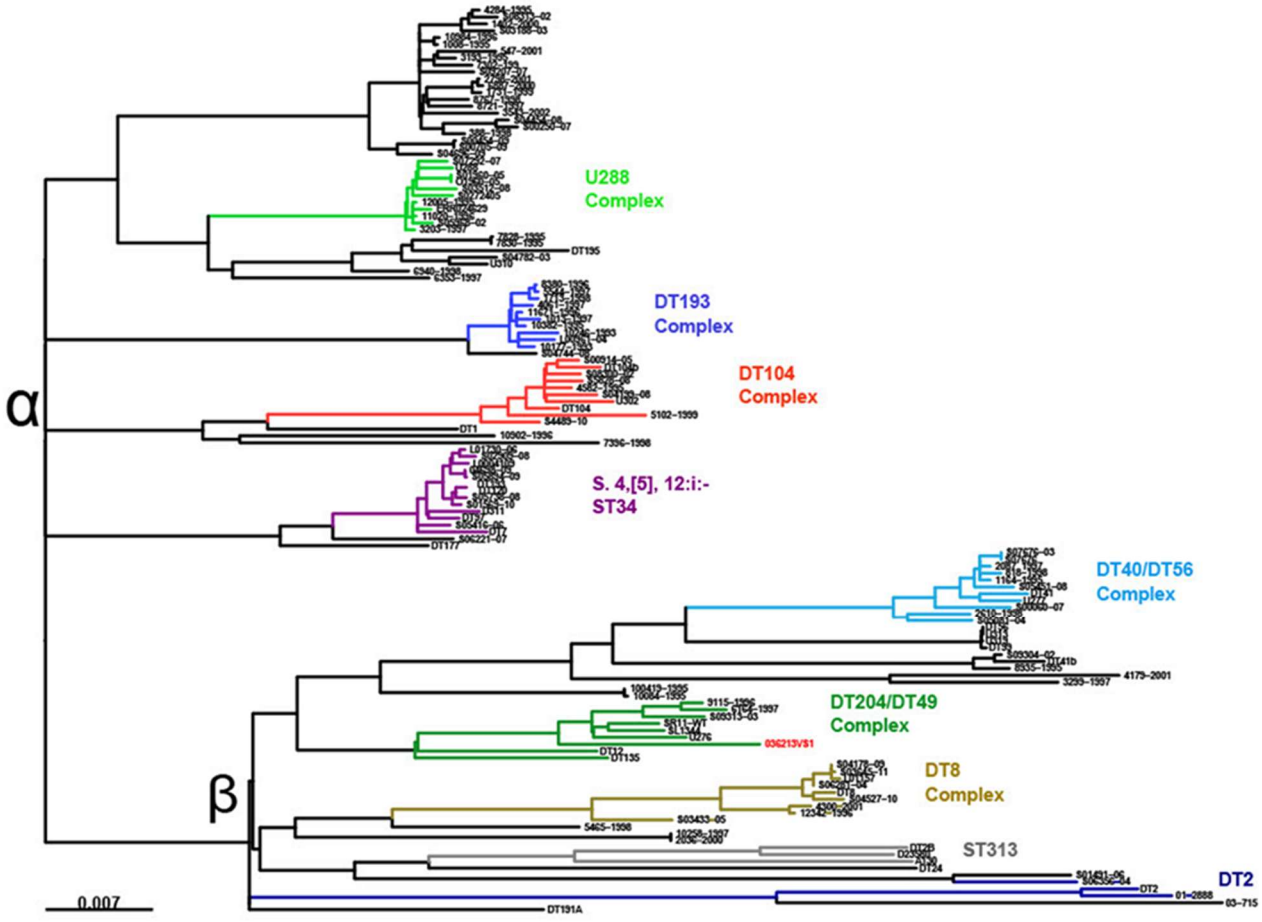


Figure 2 - Maximum likelihood phylogenetic tree based on sequence variation (SNPs) in the core genome of 131 *Salmonella* Typhimurium strains with reference to *S*. Typhimurium SL1344. Phylogenetic Tree is rooted to ST36 strains which clustered separately from the remaining 131 isolates. Phage types associated with each clade are displayed in colours. Tree is based on phylogeny previously reported in Bawn et al 2020

1.1.3. Salmonellosis

The genotypic diversity exhibited within the *Salmonella* genus is reflected in the types of diseases that they cause. Depending on the serovar, *Salmonella* is capable of causing a self-limiting gastroenteritis or an enteric fever such as typhoid or paratyphoid fever caused by *S*. Typhi and *S*. Paratyphi respectively (Coburn et al., 2006). *Salmonella* is also capable of causing bacteraemia, however this is often associated with specific serovars infecting specific hosts, for example *S*. Dublin infection of cattle. More recently, *Salmonella* bacteraemia has also been associated with *S*. Typhimurium and *S*. Enteritidis infection, associated with susceptible people and specific genotypes in sub-Saharan Africa (Feasey et al., 2012). The type of disease associated with a *Salmonella* infection is dependent on both host susceptibility and serovar. Serovars Dublin, Typhimurium and Choleraesuis can cause disease in both humans and animals. In humans, these serovars cause gastroenteritis. However, in cattle, Dublin is associated with bacteraemia and spontaneous abortion (Coburn et al., 2007). Similarly, Typhimurium causes invasive disease in mice which is similar to Typhoid in Humans (Mittrücker and Kaufmann, 2000). *Salmonella* transmission occurs via the fecal oral route, with more than 95% of cases are caused by the consumption of contaminated food or water (Acheson and Hohmann, 2001). Although far less common, other methods of transmission include contact with infected animals, nosocomial transmission, direct human contact and contaminated drugs. In the case of NTS (non-typhoidal *Salmonella*), the infectious dose required to cause infection can be as little as $<10^3$ cells (Blaser and Newman, 1982). The typical incubation period is around 6-72 hours and symptoms usually last for around 4 days without the need for antibiotics. Antibiotics may be prescribed for immunocompromised individual or if the infection develops complications such as severe dehydration, bacteraemia, osteomyelitis or reactive arthritis.

The likelihood of complications developing is low and is dependent on the host and virulence of the infective strain.

1.1.4. *Salmonella* outer envelope structure

The cell envelope is a complex and multilayered structure which serves as the interface between a bacterium and the environment (Rowley et al., 2006). Being Gram-negative, *Salmonella* are surrounded by a thin peptidoglycan layer, which itself is surrounded by an outer cell membrane. The function of the cell envelope is to protect the cell from environmental stress whilst allowing the transport of key nutrients into the cell. The outer membrane of Salmonellae consists of two key components; Lipopolysacharride (LPS) and outer membrane proteins. Exposure of both of these components on the cell surface is exploited by bacteriophages, many of which use LPS and different outer membrane proteins as receptors (Shin et al., 2012).

1.1.4.1. Lipopolysacharride and Outer Membrane Proteins

Lipopolysaccharide is the predominant cell surface carbohydrate present on *Salmonella,* with the exception of *S.* Typhi which additionally possess Vi antigen (Robbins and Robbins, 1984). LPS is composed of a lipid A tail, a phosphorylated glucosamine disaccharide associated with fatty acids, which anchors the LPS structure into the bacterial outer membrane. The lipid A is attached to a core domain consisting of oligosaccharide sugars as well as non-carbohydrate components such as phosphates. The core domain is attached to O-antigen which forms the outer most domain of LPS and consists of a repetitive glycan polymer containing multiple repeating units of one to eight sugar residues (Greenfield and Whitfield, 2012). LPS heterogeneity is mainly achieved in O-antigen structure and the chemical composition varies between serovars. The O-antigen protects the cell from actions of the innate immune system and is itself immunogenic (Bertani and Ruiz, 2018).

Modifications within the O-antigen can be achieved through a variety of mechanisms. Much of the genetic diversity in O-antigen is a consequence of sequence variation within the *rfb* gene cluster. Therefore, horizontal gene transfer of *rfb* genes contributes to O-antigen modification. Additionally, determinants outside the *rfb* region have also been identified and demonstrated to provide O-antigen modification. The O-acetyltransferase, *oafA,* causes serotype modification through acetylation and the O-antigen polymerase, *wzy*, can alter chain length (Slauch et al., 1996, Kong et al., 2011). Glucosyltransferase operons (*gtr*) further contribute to variation in LPS structure. *Gtr* operons consist of three genes *gtrA*, *gtrB* and *gtrC* which act together to add glucose molecules onto the galactose moiety of repeating O-antigen units and are commonly localised within bacteriophage associated regions (Davies et al., 2013, Makela, 1973).

Outer membrane proteins (OMPs) embedded within the outer membrane of *Salmonella* fulfil are variety of functions which are integral to cell homeostasis (Futoma-Kołoch et al., 2019). These include translocation of solutes and proteins, signal transduction and enzymatic digestion (Dirienzo et al., 1978). The structure of OMPs differs from other membrane bound proteins as they do not contain α-helical domains and instead fold into antiparallel β-barrel structures. OMP are often used as receptors by bacteriophages to infect bacteria. Many OMP were first known to be receptors for bacteriophages before their physiological function was determined (Koebnik et al., 2000). For example, the maltoporin, LamB, facilitiates the diffusion of maltodextrin across the membrane and was first identified as the receptor for bacteriophage λ (Chang et al., 2010)

1.1.5. *Salmonella* Pathogenicity

All *Salmonellae* are pathogenic to one or more host species, which is a result of the acquisition of *Salmonella* pathogenicity islands (SPI's). SPI's are genetic islands of the *Salmonella* genome containing genes involved in pathogenicity. SPI's are often located next to tRNA regions and often possess an average GC content which is different from the rest of the genome, suggesting that they were horizontally acquired (Lou et al., 2019). The number of SPI's is variable between strains, however all *Salmonella enterica* possess SPI-1 and SPI-2 which are the best studied examples.

1.1.5.1. *Salmonella* Pathogenicity Island 1 (SPI-1)

Horizontal acquisition of SPI-1 caused the divergence of *Salmonella* from an *Escherichia coli* ancestor around 100-160 million years ago (Mirold et al., 2001). Acquisition of SPI-1 allows *Salmonella* to inject effector proteins into host cell membranes via a Type 3 secretion system (T3SS-1). A T3SS is a large, supramolecular needle-like structure used to translocate effector proteins across host cell membranes. All *Salmonella* invade host epithelial cells using this mechanism. SPI-1 contains all genes necessary for production of the T3SS-1 as well as most of the effector proteins that it can translocate. Invasion is triggered by rearrangement of the host cell cytoskeleton promoting membrane ruffling (Patel and Galán, 2005). This allows *Salmonella* to avoid the harsh environment of the lumen and instead gain access to the protected intracellular environment. Invasion of host cells provokes a proinflammatory response from the host. This triggers the release of signalling molecules, including cytokines which recruit immune cells and limit the systemic spread of the infection and instead keep it contained locally and repair damaged epithelial cells (Carvajal et al., 2008). However, *Salmonella* can benefit from the inflammatory environment as it can make the lumen environment more favourable. For example,

increases in nutrient availability can favour expansion of *Salmonella* populations and promote transmission. Additionally, inflammation can lead to disruptions in other members of the gut microbiota limiting competition and further favouring the growth of *Salmonella*. Expression of the T3SS-1 is extremely energetically costly for the bacterium and therefore its regulation is tightly controlled, through specific regulators and gene silencing mediated by H-NS nucleoid-like protein (Silphaduang et al., 2007). Many environmental stimuli, such as pH, osmolarity and oxygen tension have been shown to induce the expression of SPI-1 genes (Lou et al., 2019). Such tightly regulated expression ensures the timely expression of the T3SS-1.

1.1.5.2. *Salmonella* Pathogenicity Island 2 (SPI-2)

SPI-2 is only found in *Salmonella enterica.* Although it also encodes a T3SS, It is functionally distinct from the T3SS found on SPI-1 as mutations in SPI-1 are not complemented by any genes found on SPI-2 (Marcus et al., 2000). However, cross talk between SPI-1 and SPI-2 is achieved through the transcription factor HilD (Bustamante et al., 2008). Expression of SPI-2 occurs within phagocytic cells such as macrophages. Many SPI-2 encoded genes have been found to be essential for the persistence and survival of *Salmonella* within macrophages. After invading the host cell, *Salmonella* is engulfed in a membrane bound compartment called the *Salmonella* containing vacuole (SCV). The SPI-2 T3SS is used to translocate effectors across the membrane of the *Salmonella* containing vacuole (SCV) which modifies the membrane and prevents fusion with host cell lysosomes and subsequent degradation (Kolodziejek and Miller, 2015). Additionally, SPI-2 secreted effectors help intracellular *Salmonella* to acquire nutrients leading to proliferation. The resulting progeny can infect other cells and, in some cases, may lead to systemic infection (Figueira and Holden, 2012).

1.1.6. *Salmonella* Epidemiology

Salmonella is a zoonotic pathogen, meaning it can be transmitted between animals and humans. Whilst there are many serotypes of *Salmonella*, infections in both humans and animals are predominantly causedby serovars such as *Salmonella enterica* serovar Typhimurium and Enteritidis. Host adaption means that each serovar has varying degrees of host specificity. Some serovars are host adapted meaning they are commonly associated with infection within a given host. Examples of these serovars include *Salmonella* serovar Dublin which is commonly associated with infection of cattle. Additionally, *Salmonella* serovar Pullorum is often linked to poultry infection. Alternatively, serovars can also become host restricted resulting in only being able to infect a specific host. An example of host restriction is *Salmonella* serovar Typhi which can only cause infection in humans. Host adaption and host restriction is driven by genome degradation of genes which caused the serovar to be a generalist and acquisition of specific virulence factors (Tanner and Kingsley, 2018).

Majority of human infections are the result of consumption of contaminated animal protein, however diversity of *Salmonella* causing infections in humans is distinct from serotypes observed in the food chain as well as in farmed animals (APHA, 2022). Therefore, it is likely that the food chain represents an evolutionary bottleneck for *Salmonella* diversity. The mechanisms driving diversity are poorly understood, but are likely due differences in opportunities to enter the food chain, survive in food production and food matrices and the ability to cause disease in people (Humphrey, 2004, Pye et al., 2023).

1.1.7. Food associated outbreaks of *Salmonella* and Intervention

A foodborne disease outbreak is defined as at least two infections from the same source. The risk that *Salmonella* possess to food safety is well known. At particular risk are fresh and ready-to-eat (RTE) foods as they do not have additional intervention methods, for example, heat from cooking, that prevent the pathogen from being consumed (Mukhopadhyay and Ramaswamy, 2012). In fact, foods that are intended to be consumed raw often have no intervention method to prevent pathogens entering the final product at any stage in their production. This is particularly concerning as the popularity of RTE food has increased in the past decades and the need for effective intervention methods has never been greater. The most common sources of *Salmonella* are chicken, pork, turkey, beef and eggs. However, in recent years fresh produce has been linked to an increase in foodborne disease, due to contamination from animal waste (Kilonzo-Nthenge and Mukuna, 2018). The major sources of outbreaks from fresh produce include: alfalfa sprouts, cucumbers, papayas and cantaloupes (Angelo et al., 2015, Mahon et al., 1997, Singh and Yemmireddy, 2021, Munnoch et al., 2009). Additionally, outbreaks associated with the consumption of dried and low moisture foods have also been reported, including peanut butter and baby formula, potentially due to the ability of *Salmonella* to survive in this environment (Sithole et al., 2022, Mukherjee et al., 2020).

Intervention steps included in the product manufacture can often limit the contamination of food products and increase food safety. Current intervention methods include chemical washes such as organic acids (Yang et al., 2017b). Although easy to implement, these washes have been found to be no more effective than washing with hot water (Koohmaraie et al., 2005). Perhaps a more promising method is the use of pulsed UV light which has been shown to offer as high as 2 log reductions of *Salmonella* in food (Rajkovic et al., 2017).

However, it is likely to only have an effect on bacteria on the food surface and the type of food matrix is likely to also determine the effectiveness.

1.1.8. *Salmonella* and Antimicrobial Resistance

Antimicrobial resistance (AMR) is one of the most concerning public health issues in the modern world. Antibiotic resistant infections already cause 700,000 death per year, which is projected to rise to 10 million by 2050 (Tagliabue and Rappuoli, 2018). Complications arising from *Salmonella* infections are often coupled with antibiotic resistance which makes treatments increasingly difficult (Kariuki et al., 2015). Antibiotic resistance genes in *Salmonella* are now widespread (Neuert et al., 2018), leading to the World Health Organisation classifying AMR *Salmonella* as a high priority pathogen with an imminent public health threat (Savoldi et al., 2020). An increase in AMR is a direct result of horizontal gene transfer (HGT) of resistance genes. HGT of antimicrobial resistance genes often occurs on plasmids with perhaps the most famous example being the MCR-1 gene, which confers resistance to colistin (Chiou et al., 2017). However, AMR genes can also be encoded within the chromosome and transferred to other bacteria (Liu et al., 2022). *Salmonella* Genomic Island 1 (SGI-1) is an integrative conjugative element (ICE) which confers the resistance profile ACSSuT (Ampicllin, Chloramphenicol, Streptomycin, Sulphonamides and Tetracycline) and is found in the *Salmonella* Typhimurium clone DT104 which emerged in the 1980's and disseminated worldwide (Doublet et al., 2005).

Most worryingly, AMR bacteria are increasingly common in food associated outbreaks making public exposure to dangerous and difficult to treat pathogens more common. High numbers of AMR pathogens found on farms and in meat are likely to be the result of intense antibiotic use in agriculture. Although antibiotics are now banned for use as growth

promoters in agriculture, recent studies show that 31% of *Salmonella* isolated from retail chicken showed resistance to at least 3 antibiotics (Mikanatha et al., 2010).

1.2. Introduction to Bacteriophages

Bacteriophages, also referred to as phages, are viruses which are capable of infecting and replicating within bacterial hosts. It is estimated that there are more than 10^{31} phages on the planet, playing important roles in microbial physiology, population dynamics and evolution (Bossi et al., 2003). Phages are incredibly diverse, evolving a wide range of different morphologies and DNA structures. Most commonly, phages possess a dsDNA genome, however ssRNA, dsRNA and ssDNA are also possible genome structures (Callanan et al., 2020). Phage genome size varies from 3.3kb to 735kb (Friedman et al., 2009, Al-Shayeb et al., 2020). Phages with genome sizes >200kb are termed 'Jumbo Phages', which are rarely isolated by conventional protocols and represent a small proportion of the phage genomes depositing in GenBank (Yuan and Gao, 2017).

Phages were discovered independently by Frederick Twort in 1915 and Felix d'Herelle in 1917. D'Herelle began using phages to successfully treat human infections, however, the discovery of antibiotics in 1928 led to phage therapy being largely ignored in favour of antibiotic treatment (Gordillo Altamirano and Barr, 2019). In recent years, the inappropriate use of antibiotics has led to increasingly heavily antibiotic resistant bacterial strains which has sparked new interest in phage therapy as potential alternative or adjunct to traditional antimicrobials (Altamirano and Barr, 2019, Chan et al., 2013). One potential advantage of the use of bacteriophages is that they are highly specific against their hosts. Unlike antibiotics, bacteriophages only kill their target and do not directly affect other

bacteria. This makes treatment complications which are a result of the loss of diversity in the gut microbiota far less common than traditional antibiotic treatment. The potential of bacteriophages means it is important that we fully understand all aspects of bacteriophages for their successful application to treat infections in humans, animals and also their use as biocides in the food industry.

1.2.1. Bacteriophage Taxonomy

The responsibility of creating and maintaining appropriate viral taxonomy currently belongs to the International Committee on the Taxonomy of Viruses (ICTV). The ICTV comprises of 6 subcommittees, of which, the bacterial and archaeal viruses (BAVS) subcommittee holds responsibility of bacteriophage taxonomy. The ICTV system is based on a hierarchical system devised by Linnaeus used to classify plants and animals. Historically, phages were classified using morphology alone. This lead to the establishment of 3 families of tailed dsDNA phages, *Myoviridae* (long contractile tail), *Siphoviridae* (Long non-contractile tail) and *Podoviridae* (short non-contractile tail) (Nelson, 2004). However, the advent and common application of next generation sequencing revealed diversity which was not previously known and allowed the classification of bacteriophages with higher resolution. One of the issues with the previous classification system which NGS revealed was that members of the same family sometimes had very little sequence similarity. For example, members of the genera *Myxoctovirus* and *Lederbergvirus* were classified within the *Podoviridae* based on morphology, however they share no orthologous genes (Turner et al., 2021). The desire for monophyletic phage families has encouraged phage taxonomists to move away from a purely morphology based approach and instead adopt a more sequenced based rationale to define taxa. Since 2021, the current taxonomic system groups viruses together based on shared characteristics such as genome sequence

(Walker et al., 2021). For example, phages within the same genus share >70% nucleotide sequence similarity. Beyond this, phages within the same species share >95% sequence similarity. Therefore, two phages are said to be different species if >5% of their nucleotide sequence is different to each other across the entire genome length.

All tailed dsDNA viruses belong to the realm Duplodnaviria (Figure 3), whilst non tailed dsDNA phages are classified into the realm Varidnaviria (Forterre and Gaïa, 2021). All members of the Duplodnaviria realm encode the HK97 fold within the major capsid protein (Duda and Teschke, 2019). Viruses in this realm also share other characteristics such as an icosahedral capsid, a capsid portal and DNA packaging enzymes. Duplodnaviria contains only a single kingdom, Heunggongvirae, which is subdivided into two phyla, Uroviricota and Peploviricota. The phylum Peploviricota contains animal viruses which cases infections such as, herpes, chickenpox and glandular fever (Gatherer et al., 2021). The Phylum Uroviricota contains a single class, Caudoviricetes which is subdivided into 7 orders (as of 2022 taxonomy release from ICTV) . At the time of writing, there are also many "unclassified Caudoviracetes" this is due to the removal of the order Caudovirales and the abolishment of the families*, Myoviridae, Siphoviridae* and *Podoviridae* to make way for new taxa based on genomic similarities (Turner et al., 2021).

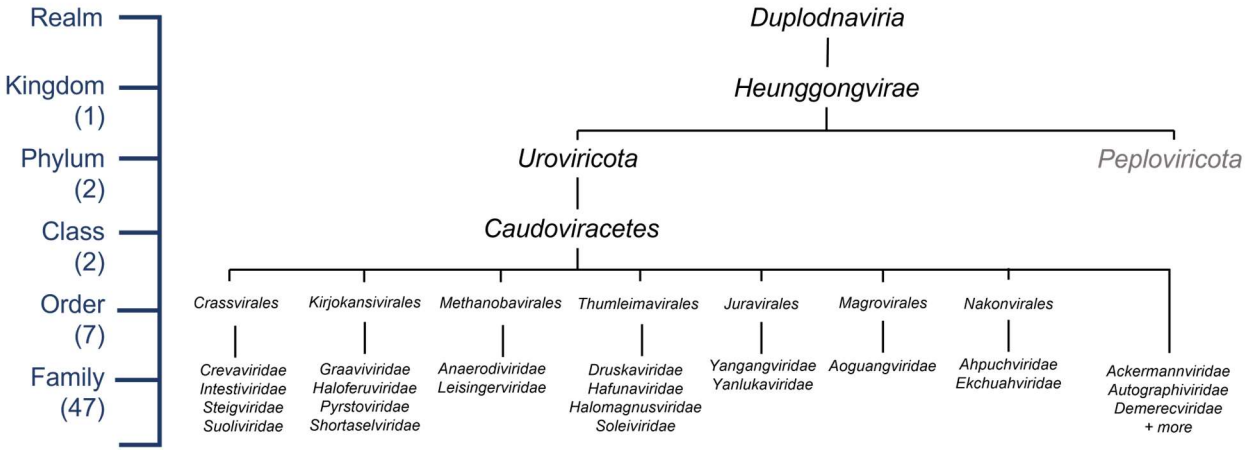


Figure 3 - Classification of members of the Realm Duplodnaviria to family level. Based on Taxonomy Release of the International Committee on Taxonomy of Viruses (ICTV) in March 2023 https://ictv.global/taxonomy (Accessed: March 2023) (ICTV, 2023)

1.2.2. Bacteriophage Lifecycles

Phages commonly exhibit two distinct lifestyles and their use of one or both have important consequences for their therapeutic use (Figure 4). As obligate parasites, phages are unable to complete either of their life cycles without the presence of their bacterial host. Infection begins via binding of the phage to a cell surface receptor located on the host (Salmond and Fineran, 2015). The bacterial cell envelope represents a unique barrier that the phage must overcome in order to inject their genome into the host. Phage tail proteins are large, complex, supramolecular structures which modulate attachment and penetration of the bacterial cell envelope (Hofer, 2016). The binding of the phage tail protein and cell surface receptor is highly specific which, amongst other factors, contributes to host range (Kutter, 2009). Common phage receptors include LPS (Lipopolysaccharide), flagella, pilli and outer membrane proteins (Stone et al., 2019). Phages initially bind to a primary receptor in a reversible manner. This initial interaction helps bring the bacteriophage in to contact with the host bacterium. Following initial contact in susceptible hosts, phages commonly bind

irreversibly to a secondary receptor where the infection cycle can progress. A well-studied model of this interaction has been elucidated using the model phage P22. Bacteriophage P22 initially binds to O-antigen and uses depolymerase activity present within its tail fibre to sequentially degrade O-antigen to bring the virion into close proximity to the bacterial cell (Knecht et al., 2019). In the case of *Salmonella,* and many gram negatives, a common receptor is LPS (Dowah and Clokie, 2018). However receptors of non-model phages remain largely uncharacterised (Stone et al., 2019). This makes their use as antimicrobials more challenging. A summary of the lifecycle of a bacteriophage is shown in Figure 4.

1.2.2.1. Lytic Cycle

Strictly Lytic phages only replicate through the lytic cycle which involves production of new

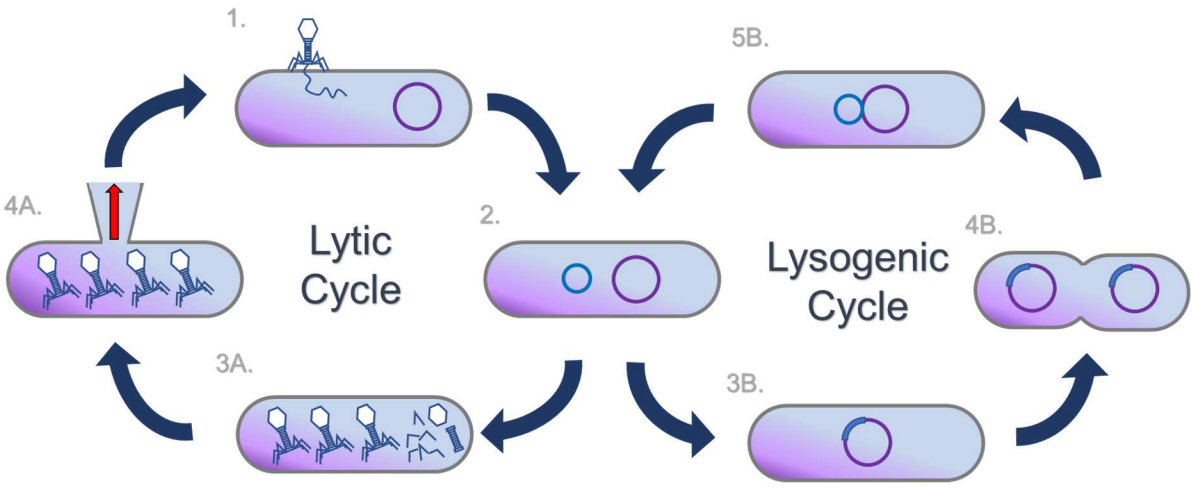


Figure 4 - Summary of bacteriophage lifecycles. Lytic phages attach to the host cell via a receptor and inject their DNA into the host (1). The Phage DNA circularises within the host (2). In the Lytic cycle (Left), the host transcribes new phage particles (Virions) which are assembled into phage progeny (3A). The host cell is lysed and the phage progeny are released. In the Lysogenic cycle, phage DNA integrates into the chromosome forming a stable intermediate termed 'Prophage' (3B). The prophage replicates along with the host genome (4B) and may excise from the genome and enter the lytic cycle (5B).

phages, termed 'virions', and their release from the host cell via cell lysis. The lytic cycle begins following adsorption and injection of the viral nucleic material into the host cell, termed infection (Lim et al., 2019). Upon infection, the host cell typically uses host encoded enzymes to replicate the viral nucleic material and produce new viral proteins. The newly

produced proteins are then assembled, typically starting with the capsid (Prevelige and Cortines, 2018). The capsid is the head portion of the phage which is packaged with a new viral genome. Upon capsid maturation, the tail proteins are assembled. Tail protein assembly is complex and is variable between phage family (North et al., 2019). The end point of phage infection is the production of a phage encoded holin or endolysin which lyses the host cell and releases the phage progeny (Ajuebor et al., 2016). The burst size of a phage is equal to the number of phage progeny that are released during lysis and varies between phage species (Krasowska et al., 2015). The lytic cycle can begin again once the newly produced phage progeny adhere to a new susceptible host.

1.2.2.2. Lysogenic Cycle

Some phages, termed temperate phages, can also replicate via the lysogenic cycle where phage DNA integrates into the bacterial chromosome forming a stable genomic region referred to as prophage. Horizontal gene transfer in this way plays an important role in bacterial evolution (Fortier and Sekulovic, 2013). Prophages are replicated along with the bacterial genome and parsed down generations during bacterial replication. The lysogenic cycle can begin following nucleic acid insertion into the host. Unlike the lytic cycle, new phage progeny are not produced. Instead, the phage nucleic acid inserts into the host genome forming a stable entity called a prophage. In order to do this, lysogenic phages harbour an integrase gene which directs site specific recombination into the host genome (Calos, 2016). Different integrases target different recognition sites within the genome and therefore phages which possess different integrases insert into different genomic loci. This allows a single bacterium to carry multiple prophages. A recent study found that *Salmonella* genomes contain between 2-8 prophages on average (Mottawea et al., 2018). Phages can also lose integrase function producing a cryptic prophage which is unable to excise from

the genome (Campbell, 1998). Integration of a lysogenic phage may lead to the host phenotype being changed; this is called lysogenic conversion. Phages can also carry virulence genes which can offer a selective advantage and drive the evolution of pathogens (Tassinari et al., 2020, Wang et al., 2019). Additionally, prophages increase the ability to tolerate environmental stress associated with infection (Wang et al., 2010). The paradigm for prophage induction is set by model phages such as phage λ and P22. In these models, induction is triggered by the proteolytic cleavage of a repressor gene which prevents phage induction. Cleavage by the antirepressor protein results in prophage induction (Lemire et al., 2011) . This mechanism is conserved in *Salmonella* prophages Gifsy-1, Gifsy-2 and Gifsy-3 (Figueroa-Bossi et al., 1997). In this case, the antirepressor is activated in response to RecA activity, an activator of the SOS response (Mayola et al., 2014). The SOS response is triggered by direct damage to DNA. This can be achieved using chemical agents such as mitomycin C, antibiotics such as ciprofloxacin or UV light (Otsuji et al., 1959, Barnhart et al., 1976, Goerke et al., 2006). Prophages are commonly induced in the gut microbiota, in particular as a result of infection (Manrique et al., 2017). Inflammation leads to the release of reactive oxygen species (ROS) which can direct DNA damage, trigger the bacterial SOS response and leads to induction of prophages (Henrot and Petit, 2022).

1.2.3. Prophages of *Salmonella enterica*

In common with other bacteria, *Salmonella enterica* genomes contain multiple prophages which can make up to 5% of the genome content. On average, *Salmonella* genomes contain 5.29 prophages accounting for up 30% of the accessory genome – suggesting a high frequency of polylysogeny (Bobay et al., 2013). Prophages commonly integrate between non-translated RNA genes, the most common being tRNA genes in *Salmonella* (Canchaya

et al., 2004). Many *Salmonella* phages, including well studied examples such as Gifsy-1, Gifsy-2 and Gifsy-3, can be induced by common induction treatments as well as spontaneously induce at low titres (Garcia-Russell et al., 2009). Once inserted, some prophages lose the ability to excise and form virions. An example in *Salmonella* is the prophage ST64B present in *Salmonella enterica* sv Typhimurium. In *S*. Typhimurium strain SL1344, ST64B cannot be induced by mitomycin C due to a mutation affecting virion assembly (Figueroa-Bossi and Bossi, 2004). In contrast some prophages spontaneously induce at high titre. *Salmonella* Typhimurium sequence type ST313, which causes bloodstream infections in sub-Saharan Africa, has a distinct prophage repertoire compared to ST313 strains from the UK (Kingsley et al., 2009). Amongst these phages, BTP-1 has the highest reported spontaneous induction rate of any prophage – with 10^9 viral particles per ml of stationary phase culture (Owen et al., 2017b).

Salmonella prophages are also known to drive the emergence and clonal expansion of epidemic strains. The lysogenic phage mTmV habors the virulence factor, *SopE*, which encodes a guanine nucleotide exchange factor (GEF). SopE is a type III secreted effector protein which contributes to actin-mediated membrane ruffling promoting microbial uptake within the lumen (Müller et al., 2009). Ancestral state reconstruction has demonstrated that the acquisition of *sopE* resulted in clonal expansion of the pandemic monophasic *S*. Typhimurium sequence type ST34 clone (Tassinari et al., 2020). This demonstrates the effect prophages can have on the population structure in *Salmonella*.

1.2.4. Bacteriophage Defence Mechanisms

Phage predation represents a strong selection pressure for bacteria (Bossi et al., 2003). Many bacteria have mechanisms to evade phage predation leading to an evolutionary arms

race (Stern and Sorek, 2011). Phage-bacteria dynamics is an example of the Red Queen Hypothesis (Valen, 1977). This evolutionary hypothesis states that in tight co-evolutionary scenarios, adaption on one side may lead to near extinction of the other. The only way the non-adapted side can survive and maintain fitness it to counter adapt. In the case of phage defence, if a bacterium evolves resistance to a phage, then the bacteria has increased fitness and the phage lacks a host. The only way for the phage to survive is to evolve and overcome the resistance. For example, bacteriophage P1 habours an antirestriction system, DarAB, which prevents restriction by the bacterial encoded type I restriction system (Iida et al., 1987). There are many well studied examples of phage defence mechanisms in bacteria using model phages (Ofir and Sorek, 2018, Benson and Roth, 1997, Labrie et al., 2010) (Figure 5). Characterisation of defence mechanisms of non-model phages remains limited.

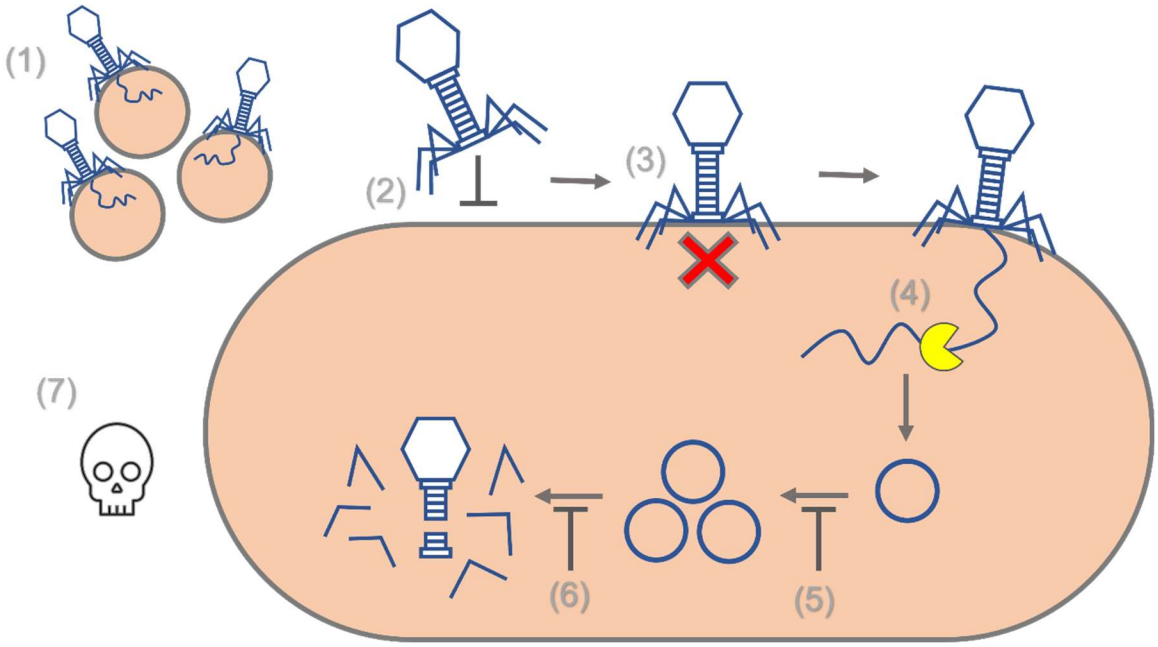


Figure 5 - ***Summary of common bacteriophage defence mechanisms in bacteria.*** 1) OMV as phage decoys. 2) Prevention of adsorption to cell surface receptor. 3) Blocking DNA injection. 4) CRISPR system. 5) Prevention of Viral DNA Replication. 6) Prevention of Viral Assembly. 7) Abortive Infection

1.2.4.1. Outer Membrane Vesicles as Bacteriophage Decoys

Outer Membrane Vesicles (OMVs) are spherical, non-replicating nanostructures of endolytic origin produced by Gram negative bacteria (Jan, 2017). They are naturally produced and play important roles in cell signalling and biofilms (Schwechheimer and Kuehn, 2015). More recently, they have been proposed as potential drug delivery system (Gnopo et al., 2017). Since OMV's are made of bacterial membrane, they often contain phage receptors. This allows phages to bind to OMVs and inject their nucleic acid. However, since OMVs lack cellular machinery, such as ribosomes, no phage progeny are produced and the infected phages are effectively titrated out (Manning and Kuehn, 2011). For this reason, OMVs have been described as the first line of defence against phage infection. Co-incubation of the model phage T4 with OMVs showed fast, irreversibly binding and a reduction in phage activity (Manning and Kuehn, 2011). Similarly, secreted OMVs provided *Vibrio cholera* immunity against infecting phage when the OMV expressed the appropriate phage receptor (Reyes-Robles et al., 2018). More recent work has used Transmission Electron Microscopy (TEM) and identified the presence of OMVs in T4 and T7 phage lysates (Mandal et al., 2020), likely left behind from their propagation. This further highlights their implications with phage infection.

1.2.4.2. Prevention of Bacteriophage Adsorption

Since the initial step in phage infection is the appropriate binding to a receptor on the bacterial cell surface, prevention of this binding results in no phage infection. There are a number of ways bacteria can block phage binding to their receptor resulting in resistance. Mutations or deletions of the phage receptor have been shown to grant immunity to phage. For example, the outer membrane porin, OmpC, is commonly found in *E.coli* and is

used by some phages as a phage receptor (Hejair et al., 2017). This protein is commonly deleted in order to evade phage predation (Morita et al., 2002). However, as the Red Queen Hypothesis states, phages can acquire mutations to overcome this and target alternative receptors. In the case of *Salmonella*, some strains are capable of phase variation for expression of phage receptors (as discussed in 1.1.1). This allows them to vary the receptor expressed on their cell surface in a phase dependant manner. If the phage receptor is located in a genomic region that is switched off in the current phase, then no phage infection can occur. However, since the phage receptors can play an important roles in survival and virulence, this often comes at a cost of reduced fitness (Cota et al., 2015). Phase variable gene expression affecting genes involved in phage receptor synthesis can impact phage sensitivity. For example, glucosylation of O-antigen by phase variable *gtr* operons effects LPS composition and has previously be shown to impact infection by the model phage P22 ((Davies et al., 2013, Broadbent et al., 2010)

1.2.4.3. Blocking DNA Injection

Prophages often encode Superinfection Exclusion Systems (SIEs). These systems prevent secondary infection by preventing the injection of DNA from the same or closely related phage. Many phages encode SIEs including the P22 phage which possesses the *sieA* and *sieB* genes. Both genes act independently and confer immunity to *Salmonella* phages MG40 and MG178 (Susskind et al., 1971). The gene product of SieA has been shown to localise to the bacterial membrane, but exactly how it moderates superinfection exclusion is unknown (Hofer et al., 1995). More well studied examples of SIEs include that of the T4 phage which encodes Imm proteins (Lu and Henning, 1994) . Imm proteins have previously been shown to alter the conformation of the DNA injection site in order to prevent DNA entry to the cell (Lu and Henning, 1989).

1.2.4.4. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Perhaps the most well-known phage defence mechanism is CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). CRISPR remains the only adaptive bacterial immune response currently discovered (Kim et al., 2012). All systems work by the enzymatic modification of injected viral nucleic acid in order to prevent infection. In the case of CRISPR, viral nucleic material is degraded by the Cas enzyme. Bacteria which possess the CRISPR loci are able to gain adaptive immunity to bacteriophages by integrating short 21-48bp fragments of viral DNA into their genomes (Dupuis et al., 2013). These spacers, once transcribed into the CRISPR RNA, guide the Cas enzyme to cleave complementary nucleic acids upon injection into the host cell. However, phages are able to overcome this system in many different ways. The most simple mechanism, is by acquiring point mutations within the regions which are complementary to the spacer sequence, which prevents Cas binding. In addition, anti CRISPR genes have been identified in *Pseudomonas* phages which encode proteins which interact with the CRISPR-Cas complex and prevent their degradation of phage nucleic material (van Gent and Gack, 2018). More recently, phages which possess their own CRISPR system have also been discovered which inactive a host encoded phage inhibitory island and results in phage susceptibility (Seed et al., 2013).

1.2.4.5. Interference in Phage Replication and Assembly

Inhibition of viral nucleic acid replication can be achieved through the BREX (Bacteriophage Exclusion) system. This system was initially discovered in *Bacillus*, and has been found to be very common amongst bacteria (Goldfarb et al., 2015). The BREX system works by methylation of the viral nucleic acid at the fifth position of a 5-TAGGAC-3 hexamer

sequence (Bhushan, 2017). However the methylated DNA is not degraded, instead the methylation is believed to aid self/non-self discrimination which prevents the viral nucleic acid from being transcribed (Goldfarb et al., 2015). In addition to inhibition of replication, the host can also prevent proper phage infection by interfering with correct phage assembly. The best studied example of this involves a phage inducible chromosomal island (PICI) found within *Staphylococcus aureus.* Upon phage infection, the PICI is excised from the genome, circularised, and packaged. During virion assembly, phages are packaged with the PICI instead of the phage genome. Subsequent phage progeny are not able to produce downstream infections (Martínez-Rubio et al., 2017).

1.2.4.6. Abortive Infection

Bacterial abortive infection (Abi) systems are one of the only altruistic behaviours exhibited by living organisms (Dy et al., 2014). Abi systems co-ordinate cell death in response to phage infection to limit the levels of viral replication. Most Abi systems are found on mobile genetic elements such as prophages or plasmids (Shabbir et al., 2016). Phage infection begins in a typical fashion, with adsorption and injection of nucleic acid. However, phage progeny development is interrupted, often leading to very few or no viral particles released. As a result, the infected cell dies, however the general bacterial population survives. Many Abi systems have been well characterised in *Lactococcus lactis* with the interest of better understanding the bacterium's dynamics during cheesemaking (Chopin et al., 2005). However, Abi systems have also been found in pathogens such as *Salmonella* (Barker, 1988). The RexAB system, encoded by phage lambda, protects the host bacterium from other coli phages by inducing a loss in membrane potential which prevents ATP

production (Snyder, 1995). Abortive infection mechanisms usually work by toxin anti-toxin systems. Recently, it has been shown that phages can spontaneously mutate to avoid triggering the toxin-antitoxin system and therefore evade abortive infection (Chen et al., 2017).

1.2.5. Phage Resistance and Antibiotic Resistance

Bacteriophage infection represents a strong selection pressure for bacteria. As previously discussed, phages exploit a variety of different structures produced by bacterial cells in order to initiate infection. Bacteria often modify or lose these structures when faced with a selective phage pressure. However, these structures have functions within the bacterial cell lifestyle. For example, TolC is an outer membrane component of the multidrug efflux pump AcrAB-TolC which has been shown to play a crucial role in efflux of a wide range of molecules (Nolivos et al., 2019). Inactivation of TolC has been shown to result in susceptibility to antibiotics, detergents, dyes and organic solvents (Zgurskaya et al., 2011). In *E.coli*, resistance to phage U136B by mutations in TolC also led to increased antibiotic susceptibility, particularly to tetracycline (Burmeister et al., 2020). This can be described as a collateral sensitivity dynamic, where resistance to one antimicrobial leads to increased sensitivity to another. Interestingly, mutations were also detected in LPS synthesis genes, resulting in a phage resistant phenotype and decreased resistance to Colistin. The evolutionary trade-offs resulting in phage resistance at the cost of antibiotic resistance is particularly relevant to phage-based therapies. Bacteriophage treatments could be used in conjunction with antibiotic treatments which would likely result in resistance to both treatments occurring at a much lower frequency (Liu et al., 2020). Additionally, the phenomena of Phage Antibiotic Synergy (PAS) whereby treatment with sub-lethal doses of antibiotic promotes the production of virulent phages within the host bacterium can also

increase treatment effectiveness (Ryan et al., 2012). The application of phage-antibiotic cotreatment is therefore being explored for clinical use. Initial evidence suggests that some phage-antibiotic combinations can have an antagonistic effects, resulting in decreased effectivity of treatment (Abedon, 2019). Therefore, it is likely that specific phage-antibiotic combinations would need to be extensively assessed before becoming effective therapies.

1.2.6. Applications of Bacteriophages

As previously discussed, (In 1.2), Bacteriophages are not a new discovery and despite the lack of use in phage therapy, they remain a very important tool for molecular biology. The ability of bacteriophages to bind to bacteria, transfer genetic material and kill bacteria have all been exploited in recent years and has contributed to our knowledge of phage infection.

1.2.6.1. Phage Typing

Phage typing remains an important epidemiological tool used to detect strains of bacteria and trace outbreaks (Mohammed, 2017). Although following the widespread availability of NGS (Next Generation Sequencing), other methods such as MLST (Multiple Locus Sequence Typing) have become more popular (Pavón and Maiden, 2009). However, phage typing is still used due to its low cost, ease to perform and easily reproducible nature (Ferrari et al., 2017). Phage typing tests susceptibility of strains to a panel of bacteriophages to identify epidemiologically related isolates. Phage typing has been extensively used to trace *Salmonella* outbreaks (Demczuk et al., 2003). The system can distinguish more than 300 definitive phage types (DT) of *Salmonella* Typhimurium alone (Callow, 1959). Use of this method has showed that some strains have a broad host range and are widely distributed, such as DT204 and DT104 epidemics (Rabsch, 2007). However, phage typing has limitations. Some isolates are resistant to all phages in the typing scheme making typing very difficult. Additionally, some isolates react to some of the phages, but do not conform to a known

phage type making information gained from this very limited. Phage typing also requires maintenance of phage stocks by the reference laboratory and often results can be interpreted differently leading to unreliability (Callow, 1959).

1.2.6.2. Phage Detection of Pathogens

Rapid detection of pathogens is integral to the treatment, control or containment of infections. Traditional, culture-based methods are time consuming and labour intensive. Phages display exquisite host specificity and their ability to infect only a specified range of bacteria has led to their application in pathogen detection. Phage mediated lysis results in the release of many cellular components which can be easily detected. Most commonly, the ATP released during lysis is used to activate a luciferase enzyme producing measurable light emittance (Hinkley et al., 2018). Phages offer many distinct advantages to pathogen detection. Lytic phages are able to replicate very quickly, making detection very rapid. In addition, they are only able to replicate using specific and viable hosts making them very accurate and less prone to false positives (when compared to PCR based methods) (Petty et al., 2007). Phages are also relatively easy and cheap to produce on large scales compared to antibodies which are also commonly used in pathogen detection (Schmelcher and Loessner, 2014). The detection of *Salmonella* in foods such as spinach, tomatoes and milk has been successfully carried out using phages (Lakshmanan et al., 2007, Li et al., 2010, Wang et al., 2017).

1.2.6.3. Intervention in Food Processing Environments

Bacteriophages have long been considered for application within food processing to reduce the presence of microbial pathogens. Many studies have demonstrated their efficacy against pathogens commonly found in food (Ajuebor et al., 2016, Bai et al., 2016, Grygorcewicz et al., Islam et al., 2019). This has led to the commercialisation of phage

products for direct use in food (Schwarz et al., 2022). Bacteriophages have many advantages making them suitable for applications withinin food processing environments. Phages are self-replicating and self-limiting. This makes them able to be applied in small doses, and multiply and decline dependant on microbial host availability (Bai et al., 2016). Phages are also highly specific, only affecting the host bacterium, leaving majority of the background food microbiome unaffected (Kutter, 2009). Phage production can also be scaled up on a commercial level in order to produce enough phages to meet industrial demand (Mutti and Corsini, 2019). Finally, and most crucial, many studies have demonstrated their safety in humans (Bruttin and Brüssow, 2005).

The successful use of phages against *Salmonella* in different food matrices has been demonstrated. For example, bacteriophage Felix O1 has been shown to achieve over 2 log reductions in counts of *Salmonella* Typhimurium strain DT104 in frankfurters (Whichard et al., 2003). However, the potential use of phages exceeds just their application in food products. Studies have also demonstrated their efficacy pre-harvest such as the use of the commercially available phage preparation SalmoFREE® in chicken farming. Application of phages to drinking water of challenged broiler chickens reduced *Salmonella* to an undetectable level in cloacae samples after 33 days (Clavijo et al., 2019). This study highlights the range of applications of bacteriophages in food production.

1.3. Summary of Aims of the Study

Food-bourne diseases, such as *Salmonella,* pose a large problem to maintaining a safe food supply. The use of bacteriophages is a promising, but underutilised method to excludepathogens from food products. Despite their promise successful application of phages remains challenging. The high degree of host specificity means that extensive

knowledge of host range and mechanism of infection is required to optimise formulation combinations of phages, needed to limit emergence of resistance. Additionally, it is clear that more is required to better understand the interaction bacteriophages have with their targeted hosts as well as other members of the food microbiome in order to properly determine the effect bacteriophages have on composition of food products.

This study aims to explore the extent of pathogen contamination within food production through a case study of a producer of raw pet food (referred to as 'Company P). The application of functional genomics will identify combinations of newly isolated and characterised bacteriophages which are effective when used as a phage cocktail to both increase the host range and decrease the occurrence of phage resistance. The efficacy of phage cocktail combination for the reduction of *Salmonella* in raw pet food will be assessed using metagenomic sequencing. This will contribute to safe and effective use of bacteriophages within many settings including the food industry.

Main hypothesis

- *Salmonella* bacteriophages will reduce the levels of *Salmonella* present in raw pet food and the factory environment applying an informed selection process that considers cellular targets and host range, generating combinations of phages which work more effectively.

Aims

1. Explore the extent of pathogen contamination within a raw pet food production environment.

2. Isolate and characterise bacteriophages which infect variants of *Salmonella* which are particularly prevalent in raw pet food.
3. Use functional genomics to determine how each bacteriophage interact with a host including characterisation of host receptors and resistance genes.
4. Use metagenomics to study the effect of adding bacteriophages to raw pet food products, specifically identifying changes within the microbial communities and reductions in *Salmonella* abundance

2. Materials and Methods

2.1. Bacterial and Bacteriophage Culture and Maintenance

Unless otherwise stated, bacteria were cultured in Lysogeny Broth (1% tryptone (w/v), 0.5% yeast extract (w/v) and 1% NaCl (w/v) (LB-Miller, Formedium, UK) and incubated at 37^{o}C with 200rpm shaking. Bacteria were stored as LB glycerol stocks and maintained at -80^{o}C in 25% glycerol. Where appropriate, cultures were grown in Kanamycin (50 µg/ml), Chloramphenicol (25 µg/ml), Ampicillin (100 µg/ml) or Hygromycin (75 µg/ml) by supplementation of LB broth or agar. Bacteriophages were stored in LB broth at 4 ^{o}C. Bacteriophages were routinely cultured using original isolation strains (detailed in chapter 4). For bacteriophage culture, 200 µl of bacteriophage lysate was added to 200 µl of exponential phase *Salmonella* culture (OD = 0.3). The *Salmonella*-phage mixture was used to inoculate 10 ml of LB broth and incubated overnight at 37 ^{o}C with shaking. The overnight culture was centrifuged at 3220 x G for 10 minutes, and supernatant was passed through a 0.45 µm PES (polyethersulfone) filter.

2.2. Sampling and Swabbing at Company P

The sampling procedure followed APHA (Animal and Plant Health Protection Agency) guidelines for sample collection and testing. Briefly, equipment used to collect raw pet food samples was cleaned with bleach before and after sample collection. Equipment included hand axes, hand saws, hammers and plastic scoops. Where possible, dry samples were mixed prior to collection. The minimum weight of each sample was 500g and two samples per collection were collection, with one being retained as a backup. Samples were stored at -20^{o}C until use. Samples were sent to an UKAS (United Kingdom Accreditation) accredited laboratory and tested using the ISO standard testing procedure. For each swabbing location a 10cm^{2} area was identified and swabbed each week. The same location was swabbed each successive collection unless not physically possible. *Enterobacteriaceae*

swabs were collected using Hygiene Surface Swab Kits (Technical Service Consultants) and immediately placed in 10ml neutralising buffer following collection. Swabs for detection of *Listeria* and *Salmonella* were collected using EnviroScience Surface Swabs (Technical Service Consultants). Swabs were stored at 4 ^{o}C until sent for testing.

2.3. Sampling of Raw Pet Food

Samples of raw meat pet food manufactured by Company P using materials from different suppliers were collected. Samples were stored at -20^{o}C until used. Samples consisted of tripe, which were from either bovine or ovine origin, and chicken. Each sample was made as a different production batch from three different production locations.

2.4. Isolation of *Salmonella* from Raw Pet Food

The following method is an adaption of the International Organization for Standardisation (ISO 6579-1:2017) (ISO, 2022) protocol for enrichment of Salmonella from food samples (Figure 1). This method was used for the isolation of *Salmonella* in Chapter 3 and Chapter 6. Briefly, 25g of each food sample was transferred into 225ml of buffered peptone water (BPW 1% Triton x100, Formedium, UK) and incubated for 18 hours at 37^{o}C with shaking. Selective enrichment was performed by adding 1ml of BPW culture to 9ml Muller-Kaufmann Tetrathionate-Novobiocin (MKTTn) broth or 0.1ml BPW culture to 9.9ml Rappaport-Vassilidis (RV) broth and incubated at 37^{o}C and 42^{o}C respectively. Selective enrichments were each plated onto xylose-leucine deoxcholate (XLD) and brilliant *Salmonella* agar (BSA) agar and incubated at 37^{o}C overnight. At least one suspected *Salmonella* colony with a typical morphology was selected from each plate where present, a maximum of four colonies were picked from a single plate. For XLD plates, suspected *Salmonella* colonies were defined as black colonies with a red halo. On BSA agar, suspected *Salmonella* colonies were defined as purple colonies. Detection of *Salmonella* was carried

out using polymerase chain reaction (PCR) using primers which amplified regions of the 16s (16S_FW/16s_RV) and *invA* genes (InvA_FW/InvA_RV). Primer sequences are available in supplementary table 2. The 16s region was used as a positive control, whereas the *invA* gene region was selected to confirm the isolate was *Salmonella*. Briefly, colonies were

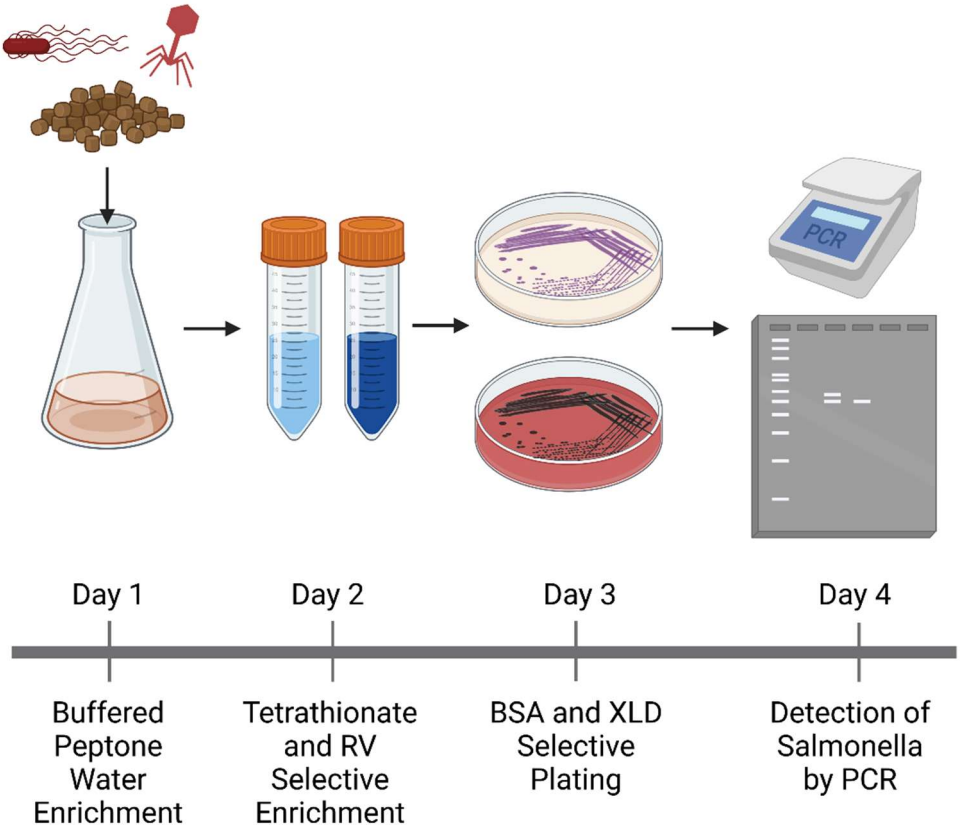


Figure 1 – Method used for the isolation of *Salmonella* from food products. Method is adapted from ISO 6579:1:2017. Detection is performed by PCR. *Salmonella* and or Bacteriophages were only added when used in Chapter 6.

suspended in 15 µl lysis buffer (2ml 10mM Tris-HCL pH 8.5, 2ul Triton x100) and boiled for 5 minutes using a thermocycler. A 1µl aliquot of boiled colony suspension was used as the template for the PCR reaction. The sequence for each primer used can be found in Supplementary table 2. Primer Design was done by Dr Gaëtan Thilliez. PCR products were visualised using agarose gel electrophoresis at 110 volts for 30 minutes.

2.5. Bacterial Whole Genome Sequencing

Chromosomal DNA from bacteria was isolated using either Wizard Chromosomal DNA isolation kit or Maxwell RSC Cultured Cells DNA kit (Promega, USA). Isolation was performed following the manufacturers protocol for both kits. Assessment of quality and determination of the quantity of DNA was carried out using Nandrop and Qubit 3.0, respectively. Library preparation for sequencing using Illumina Next-Seq 500 was carried out by myself or QIB Core Sequencing team. Library preparation was carried out using Nextera XT library preparation kits. Genomic DNA was normalised to 5ng/ml in sterile ultra-pure water. A master mix of 0.9 µl tagment DNA buffer (Illumina), 0.09 µl tagment DNA enzyme and 4.01 µl PCR grade sterile water were mixed with 2 µl of each normalised genomic DNA sample. Samples were heated to 55 ^{o}C for 10 minutes. The PCR master mix was made using 10 µl KAPA 2G Fast Hot Start Ready Mix (Merck) and 2 µl PCR grade water per sample. A 12 µl aliquot of PCR master mix was combined with 1 µl (10 µM) Illumina barcodes and 7 µl of tagmentation genomic DNA mix. The PCR programme was run following the Illumina specifications. Library preparations were pooled together and final pool concentration was determined using Tapestation (Agilent). The pool was sequenced at a final concentration of 1.5 pM on an Illumina NextSeq500 using a Mid Output Flowcell following the recommended loading and denaturation procedures. Quality control of raw sequencing reads was carried out using fastP with default parameters (Chen et al., 2018). Reads that passed quality control were assembled *de novo* using SPAdes based assembler, Shovill (Seeman, 2018, Bankevich et al., 2012). Where appropriate, *Salmonella* serovar was predicted using Seqsero2 (Zhang et al., 2019) and prophage identification was performed using PHASTER (Arndt et al., 2016).

2.6. Phylogenetic Reconstruction of Bacterial Strains using variation in genome sequence.

For Phylogenetic reconstruction of bacterial strains of multiple genera of the order Enterobacterales, the 16s rRNA gene was identified using barrnp (https://github.com/tseemann/barrnap). A multiple sequence alignment of each 16s rRNA gene was generated using MAFFT (Katoh et al., 2002) and trimmed using TrimAL (Capella-Gutiérrez et al., 2009), resulting in a 1536 bp multiple sequence alignment. Phylogenetic reconstruction was performed using IQ-tree with a bootstrap value of 1000 and model selection using ModelFinder. (Nguyen et al., 2015, Kalyaanamoorthy et al., 2017). The selected substitution model was HKY+F+R2. For Phylogenetic reconstruction of *Salmonella,* paired end sequences were aligned to the SL1344 reference genome (FQ312003) using the rapid haploid variant calling and core SNP phylogeny pipeline SNIPPY (https://github.com/tseemann/snippy). Maximum likelihood phylogenetic trees were contructed using multiple sequence alignment RaxML, using the GTRCAT model and a bootstrap value of 100 (Stamatakis, 2014). Trees of *Salmonella enterica* subspecies I were rooted to *Salmonella Bongori* whole genome sequence assembly (GCA_000252995.1).

2.7. Identification of Bacterial Encoded genes

To identify antimicrobial resistance genes within the whole genome sequence of *Salmonella* isolates, quality controlled, short read sequences were mapped and locally assembled to a database of candidate genes using ARIBA (Hunt et al., 2017). The candidate genes database was prepared using sequences present in the Resfinder database (Bortolaia et al., 2020). To identify heavy metal and biocide resistance genes, a database of experimentally confirmed genes from BacMet 2.0 was used (Pal et al., 2014). Genes were identified by aligning against amino acid sequences of bacterial isolates using blastP. The

threshold for discovery was set to 80% coverage and 80% sequence identity. Initial Bacmet analysis was carried out by Rafal Kolenda.

2.8. Isolation of Bacteriophages

Environmental samples for phage isolation were collected between November 2019 and February 2020. Samples included eight wastewater treatment samples, eight retail meat samples, nine lake/river samples and six samples of drain water from a pet food production factory, Company P. Wastewater treatment samples were collected from Norwich, United Kingdom. Retail meat samples were purchased from supermarkets within Norfolk, United Kingdom. Lake and River samples were collected from the University of East Anglia, Norwich, United Kingdom. Samples of food were enriched, as previously described, in buffered peptone water (BPW). For bacteriophage isolation, 25ml of each sample was centrifuged at 3220 xG for 10 minutes. The supernatant was filtered through a 0.45nm pore size PES filter and 5ml was added to 40ml of 2x LB broth with a 200 µl mixture of each bacterial strain cultured to mid exponential phase (OD 600nm of 0.6). Samples were enriched by incubation at 37 ^{o}C for 18 hours with shaking. The enrichment culture was centrifuged and filtered through a 0.45 µm pore size filter. Samples were checked for phages using a double agar overlay method. A 200 µl aliquot of exponential phase culture was mixed with 4 ml of 0.75% LB agar and poured over a 1.5% agar plate. After drying, 10ul of filtered and serial diluted phage enrichment was spotted on the surface of each plate. Plates were incubated at 37 ^{o}C for 18 hours. Presence of an enriched bacteriophage was determined by identifying areas of lysis of the host bacterium. If multiple plaque morphologies were identified, lysates were deemed to contain two different phages. Individual plaques were picked from each plate and further enrichment was carried out until single plaque morphologies were identified.

2.9. Bacteriophage Sequencing, Assembly and Annotation

Genomic DNA from bacteriophages was prepared using phage lysates at a concentration exceeding 10^9 PFU/ml, generated using the original enrichment strain. A 1ml aliquot of each phage was treated with 1 µl DNase at a concentration of 2,000 units/ml (New England Biolabs, USA) at 37 ^{o}C for 40 minutes. Phage virions were concentrated by precipitation using 500 µl a solution containing 24% polyethylene glycol and 1M NaCl at 4 ^{o}C overnight. Samples were pelleted by centrifugation, supernatant discarded and resuspended in 200 µl nuclease free water. Nucleic Acid purification with proteinase K digestion was performed using Maxwell® RSC Total Viral Nucleic Acid Purification Kit following the specification of the manufacturer (Promega, USA). Whole Genome Sequencing libraries were prepared using NexteraXT and sequenced using Illumina NextSeq500 as previously described. Preparation of whole genome sequencing libraries and sequencing was performed by QIB core sequencing team. Quality control of raw sequencing reads was carried out using fastP with default parameters (Chen et al., 2018). Reads that passed quality control were assembled *de novo* using SPAdes based assembler, Shovill (Seeman, 2018, Bankevich et al., 2012). Reads were mapped back to assembled contigs using BWA-mem (Li and Durbin, 2010). Assembled phage genomes were annotated using Prokka with the Prokaryotic virus Remote Homologous Groups (PHROGS) database (Seemann, 2014, Terzian et al., 2021). Sequencing reads from enrichment hosts were mapped to phage contigs using BWA-mem to check from induction of prophages (Li and Durbin, 2010).

2.10. Bioinformatic Characterisation of Bacteriophage Genomes

Phages were termed SPLA (*Salmonella* Phage Luke Acton) and allocated a number to distinguish between them. The lifestyle of each bacteriophage was predicted using Bacphlip (Hockenberry and Wilke, 2021). Intergenomic similarity of each SPLA phage was

calculated and plotted using VIRIDIC (Moraru et al., 2020). SPLA phages were placed in a phylogenetic context by constructing a phylogenetic tree based on proteome sequence using VIPtree (Nishimura et al., 2017). Trees were annotated using updated taxonomic information from Inphared (Cook et al., 2021).

2.11. Host Range and Growth Curve analysis

Bacterial strains were cultured in LB (Lysogeny broth) overnight at 37 ^{o}C at 200 rpm, adjusted to 10^7 cfu/ml and 180 µl was added to each well of a 96-well microtiter plate. Bacteriophages were inoculated into each well at a multiplicity of infection (MOI) of approximately 1. Plates were placed into either a Flurostar Omega Microplate reader (BMG LABtech, Germany), for host range testing (Chapter 4) or LogPhase600 (Aglient, USA) for mutant growth curve phenotyping (Chapter 5). Plates were incubated at 37 ^{o}C with shaking and readings were recorded at 15 minute intervals for 18 hours. Optical density readings were corrected to baseline readings and plotted against time using a mean of 3 technical replicates. The difference in area under the curve compared to a non-phage treated control was calculated using a method previously described (Xie et al., 2018). This generated a score which corresponded to the virulence of the phage in culture, termed liquid assay score (LAS).

2.12. Cocktail selection using Phage Host Range

With 12 bacteriophages, there are 4096 different possible phage cocktail combination each with a different number of phages (n). All combinations were assessed using two methods. Max50 is the number of strains that the cocktail scores a LAS above 50. To calculate the Max_{50} score, the number of strains each cocktail combination scored a LAS of above 50 was determined. Additionally, the $Max_{average}$ was calculated as the highest average LASfor each

cocktail combination. Using both methods, the optimal phage cocktail for each n (number of phages) was selected. Statistical analysis was performed by George Savva.

2.13. Temperature Stability Assays

Five phages (SPLA1a, SPLA4, SPLA5b, SPLA9 and SPLA11) were selected for stability testing as they were determined to represent the optimal penta-phage cocktail combination using the $Max_{average}$ method. To test phage stability under heat stress, a 100 µl aliquot of each phage lysate was placed in a heat block for 60 minutes. Phages were tested in parallel at 25^{o}C, 50^{o}C and 75^{o}C. Survival was assessed using a double agar overlay method. The host bacterium for each phage was their initial isolation host. Lysates were plated in triplicate and a mean was calculated. Representative data from two independent biological replicates is presented. Plaque counts were enumerated to calculate phage titre and statistical analysis was carried out using a t-test comparing heat treated titres to controls. Experiments were designed and analysed by Luke Acton, experiments were performed by Owen Mullen.

2.14. Simulated Intestinal Fluid, Simulated Gastric Fluid and pH Stability Assays

For pH stability assays, LB broth was adjusted to pH 3 (1.138M) and pH 5 (14.25µM) using Acetic Acid. Using the Penta-Phage cocktail candidates, 50µl samples of each lysate were added to 4.96ml of pH adjusted LB, and a non-adjusted control (pH 7). Samples were exposed for 5 minutes before stability was assessed using the same method previously described for temperature stability. Simulated intestinal fluid (SIF) was prepared using 1mg/ml pancreatin in 50ml 1M KH_2PO_4 adjusted to pH 6.8. SIF was prewarmed to 37 ^{o}C prior to use. Simulated Gastric Fluid (SGF) was prepared 3.2mg/ml pepsin in 50ml 1M NaCl adjusted to pH 2 and pH 3 using HCl. Stability in SIF and SGF was tested using that same method used for pH stability testing, however stability was tested by recovering

bacteriophages after 1 hour treatment. All stability assays were plated in triplicate, statistical significance was assessed using a t-test and representative data from 2 biological repeats is presented. Experiments were designed and analysed by Luke Acton; experiments were performed by Owen Mullen.

2.15. Construction of Tn5 saturating transposon mutant library

To prepare a Tn5 transposon mutant library in *Salmonella* Typhimurium ST4/74, transposon DNA was amplified by polymerase chain reaction (PCR) using P-Tn5Km-01 and P-Tn5Km-03 oligonucleotides (sequences in supplementary table 2) and pHPTTn5Km plasmid DNA as template (Sequence in supplementary) PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany), and 200ng of purified transposon DNA was mixed with 2µl 100% glycerol and 4µl EZ-Tn5 transposase (Lucigen, USA) to form transposomes. Electrocompetent *S*. Typhimurium ST 4/74 was prepared by incubating colonies in 5ml LB overnight at 37 ^{o}C with 200 rpm shaking. A 500µl aliquot of overnight culture was sub-cultured in 50ml 2xYT broth broth (1.6% tryptone, 1% yeast extract, 85.6 mM NaCl) and cultured to mid log phase (OD = 0.3). Cells were harvested by centrifugation at 3500g and subsequently washed three times with 10% glycerol to remove excess salts. The final washed cell pellet was resuspended in 600 µl of 10% glycerol and 60 µl samples were used for electroporation with 2µL sterile nuclease free water, 2µL TypeOne Restriction Inhibitor (Lucigen, USA) and 0.4µL transposome, on ice. Following electroporation, cells were immediately recovered with 1ml SOC (2% typtone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl, 10mM MgCS04 and 20mM glucose) prewarmed to 37 ^{o}C. Cells were incubated at 37 ^{o}C for 90 minutes. To calculate the number of transformants, 10 µl and 100 µl aliquots were plated onto LB agar containing 50 µg/ml

kanamycin. In total, 35 electroporations were performed to generate the transposon mutant library. The mutant library was generated by Hannah Pye.

2.16. Selection of Tn5 Mutant Library using Bacteriophages

Six bacteriophages (SPLA1a, SPLA1b, SPLA2, SPLA5b, SPLA5c and SPLA11) were selected to challenge the transposon mutant library. The Tn5 Insertion library was cultured in LB broth at 37 ^{o}C with 200 rpm shaking for 18 hours. The culture was adjusted to approximately 10^7 CFU/ml in 10ml LB broth containing each bacteriophage at an MOI of roughly 10. SPLA1a was used at an MOI of 1. A negative control containing no phage was included. Transposon library cultures were incubated at 37 ^{o}C with 200 rpm shaking and viable counts were performed each hour for 5 hours by plating serial dilutions on LB agar and enumerating colony counts. After 3 hours, a 2ml sample of each culture was harvested by centrifugation and genomic DNA was extracted using Maxwell Cultured Cells DNA extraction kit (Promega, USA). Two independent biological replicates were carried out.

2.17. Preparation of Transposon Library DNA for TraDIS

Extracted genomic DNA from bacterial cultures following phage selection and bacterial cultures of non-selected libraries was diluted to 11.1 ng/µl and tagmented using MuSeek DNA fragment library preparation kit (ThermoFisher, USA) Fragmented DNA was purified using AMPure XP beads (Beckman Coulter, USA) DNA was amplified by PCR using biotinylated primers specific to the transposon and primers for the tagmented ends of DNA (Primers in supplementary table 2). PCR products were purified using AMPure XP beads and incubated overnight with streptavidin beads (Dynabeads) to allow for capture of DNA fragments with the transposon. A subsequent PCR step using barcoded sequencing primers allowed for the pooling of samples. Streptavidin beads were magnetically removed, and samples were further purified, and size selected using AMPure XP beads. Quantification of

DNA was performed using Qubit 3.0 (Invitrogen, USA) and Tapestation (Aligent, USA). Sequencing was carried out as previously described. Reads were mapped to the reference genome (*Salmonella enterica* sv Typhimurium strain ST4/74) and differences in insertion frequency was calculated using BioTraDIS (Barquist et al., 2016). Significant changes ($P < 0.05$) were tested using the BioTraDIS pipeline.

2.18. Construction of mutant strains of *Salmonella* by allelic exchange using lambda-red Recombineering

Construction of mutant strains was performed using one-step inactivation using an adapted version of a method previously described (Datsenko and Wanner, 2000). The recombineering plasmid used was pSIM18 (Datta et al., 2006) and selection for this plasmid used hygromycin. Electrocompetent cells containing the pSIM18 plasmid were made as previously described. Competent cells were transformed by electroporation using PCR products (Supplementary table 2). All primers amplified the *aphII* gene from the plasmid pKD4 and tagged it with 50bp regions which were homologous to the target for mutagenesis. Following electroporation, cells were recovered for 2.5 hours and selected using LB agar supplemented with 50 µg/ml kanamycin.

2.19. Bacteriophage Inactivation of *Salmonella* in Company P Products

Tripe was selected as the product for bacteriophage testing as *Salmonella* contamination is commonly found during Company P testing, as well as being one of the highest selling products. A single product of Tripe was purchased in Norwich, Norfolk. Asceptically, 25g was sampled and inoculated with either 10^3 CFU or 10^5 CFU *Salmonella* Typhimurium ST 4/74. For method optimisation, Samples were incubated at room temperature for 30 minutes and subsequently inoculated with 100 µl of penta-phage cocktail at four different concentrations (10^2 PFU/ml, 10^4 PFU/ml, 10^6 PFU/ml, 10^8 PFU/ml). Innoculation was

performed by using a pipette, spreading liquid across different parts of the sample and incubating at room temperature for 30 minutes. A non-phage inoculated control treated with phage buffer was also included. Detection of *Salmonella* was performed using the International Organization for Standardisation (ISO 6579-1:2017) protocol with PCR detection as previously described. For the main experiment, samples were inoculated with 10^3 CFU *Salmonella* and treated with 10^7 PFU phage cocktail. For cocktail preparation, each component was used in equal parts and total concentration equalled that of single phage preparations. Phage were typically diluted from titres that exceeded 10^{10} PFU/ml. Twenty replicates of non-phage and phage treated samples were carried out.

2.20. Extraction of metagenomic DNA from Company P products

Metagenomic DNA extractions were carried out on total mesophilic growth of enrichment cultures of Company P tripe samples. Enrichment cultures were setup as previously described. Cultures were allowed to settle for 30minutes and 1ml of culture was transferred to a Lysing E Matrix Tube (MP Biomedicals, USA) . Cells were lysed by bead beating following the FastDNA Spin Kit using the FastPREP bead beater (MP Biomedicals, USA). Samples were placed in the bead beater for three 60 second cycles. Samples were placed on ice in between cycles. DNA purification was carried out using Maxwell RSC as used previously (Promega, USA) . DNA quantification was performed by Qubit 3.0. Samples were normalised, pooled and library Preparation was carried out by QIB Core Sequencing and Sequencing was carried out by Source BioScience using Illumina NovaSeq (Illumina, USA).

2.21. Metagenome Analysis

Raw sequencing reads were quality controlled and filtered using fastP with default parameters (Chen et al., 2018). For bacterial metagenomic analysis, metagenome profiling

was performed using MetaPhlan 4.0 (Blanco-Míguez et al., 2023). Alpha and Beta diversity was calculated using Phyloseq (McMurdie and Holmes, 2013). For primary analysis, statistically significant changes in *Salmonella* relative abundance were tested using a t-test. For exploratory analysis, detecting significant changes in other species was performed using a t-test with Benjamini-Hochberg correction. For Viromics analysis, quality-controlled reads were assembled using MEGAHIT (Li et al., 2015). Assembled contigs were mined for virus sequence using GnomAD (Karczewski et al., 2020). Quality and completeness of assembled viral contigs was assessed using CheckV. Viral Contigs were manually filtered by removing contigs containing greater than 20% host genes or no coding sequences. Contigs were annotated using Prokka with the PHROGS database (Seemann, 2014, Terzian et al., 2021). Viral contigs were classified using VConTACT2 with annotation from Inphared as a reference database (Bolduc et al., 2017, Cook et al., 2021). Alpha and beta diversity analysis was performed using MetaPop (Gregory et al., 2022) and Phyloseq (McMurdie and Holmes, 2013). Reads were mapped to genomes of the spiked phage cocktail to check for abundance of phages in each sample using BBmap using 100% sequence identity threshold (Bushnell, 2014).

2.22. Data availability

Genomes of SPLA phages are deposited in NCBI under the following accessions listed in Table 1 in Chapter 4. Sequencing reads of bacteria are deposited in SRA/NCBI and are listed in supplementary table 1. TraDIS sequencing reads are deposited in SRA and available under the project PRJNA1004457. Bacteriophage sequencing reads are available under the project code PRJNA1031836

3. Isolation and Characterisation of *Salmonella* Bacteriophages

3.1. Introduction to Chapter Four

Due to high bacterial load and taxonomic diversity, wastewater is a commonly used source for the isolation of bacteriophages, especially those which infect bacteria of the human gut microbiome (Adriaenssens et al., 2021). In order to use isolated bacteriophages for specific applications, its crucial to carry out further characterisation and better understand their biology. In this study, *Salmonella* serovars which commonly contaminate raw pet food were used as enrichment strains for phage isolation. The whole genome sequence was determined for phage isolated to pure lysates and taxonomy, phylogenetic relationship and prediction of lifestyle were investigated using computational methods. In addition, their survival in a range of food chain related stresses as well as their host range against a collection of bacteria isolated from food was determined. The aim of this chapter is to isolate bacteriophages which infect *Salmonella,* characterise their host range against a diverse collection of *Salmonella* and assay their survival in different stress conditions.

3.1.1. Beneficial Characteristics of Bacteriophages

Bacteriophages possess diverse characteristics which are important to determine so that suitable phage are selected for particular applications such as phage therapy or interventions in food processing environments. For bacteriophages used as interventions in food processing, one desirable characteristic is a broad host range. The host range of a bacteriophage is defined by the number of different species, serovars or strains a phage can infect. Broad host range phages are more useful for this application as they are able to kill many different strains of *Salmonella* which commonly contaminate food. Additionally, phages for use as antimicrobials should be highly lytic, with no potential lysogeny genes present within their genomes. Lysogenic phages are not as useful for this purpose as whilst they can kill their host in certain conditions, they can also cause lysogenic conversion and

provide their host with genes which enhance their survival or virulence (Tassinari et al., 2020). Phages used in the food chain will encounter a range of environmental stresses and therefore should be able to tolerate them so that they remain active when they encounter their targeted host.

3.2. Results

3.2.1. Selection of Strains for Phage Enrichment

Twelve *Salmonella enterica* strains were used for the enrichment of environmental samples for the isolation of bacteriophages (Supplementary table 1). Strains selected were of serotype Montevideo, Panama, Mbandaka, Kedougou, Infantis, Derby, Newport, Enteritidis and Typhimurium. Eleven of the strains were previously isolated from food products within the UK, including Company P products (Chapter 1). The remaining strain was an isogenic mutant of *Salmonella enterica* sv Typhimurium (*S.* Typhimurium) strain with the prophages Gifsy-1, Gifsy-2, ST46B, SopEΦ and P4-like phage removed. (From Jay Hinton Lab, Liverpool, UK). Strains used for phage enrichment were selected due to their ability to cause disease in humans and food production animals and because they were isolated from food or a food production environment. A range of serovars across the phylogeny were selected in order to incorporate as much diversity as possible. In order to investigate the relatedness of each enrichment strain, we constructed a maximum likelihood phylogenetic tree (Figure 1)

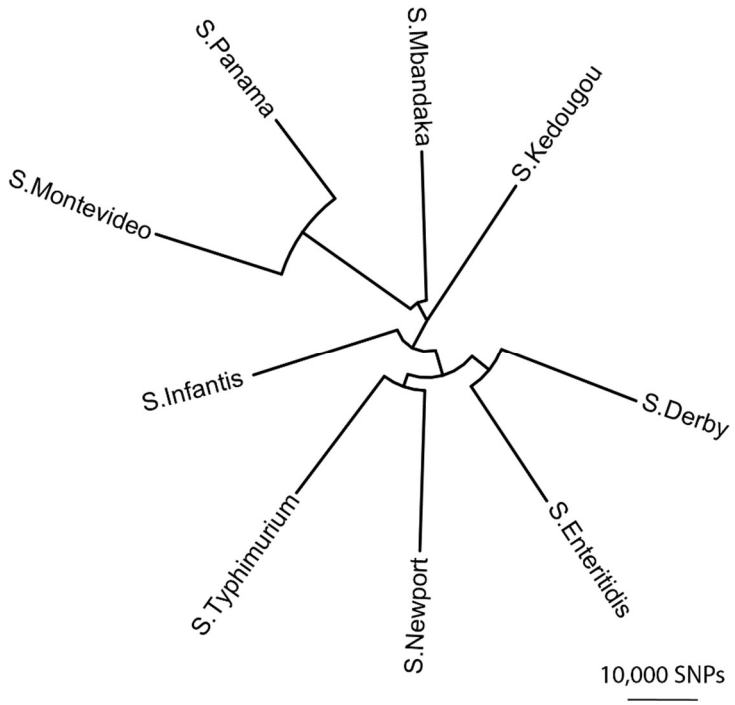


Figure 1 – Maximum likelihood phylogeny of *Salmonella* enterica strains based on non-repetitive, non-recombinant SNPs using RaxML with 1000 bootstrap replicates.

3.2.2. Isolation of SPLA Phages from the Environment

Salmonella bacteriophages were isolated from wastewater, river and food samples. Samples were collected from sites detailed in the materials and methods section. Phages which exhibited distinct plaque morphologies were further enriched and purified by subsequent enrichment cultures. In total, twelve phages were isolated, by propagation of plaques on seven different enrichment strains (Table 1 and Figure 2). The phages were termed SPLA1a, SPLA1b, SPLA2, SPLA3, SPLA4, SPLA5a, SPLA5b, SPLA5c, SPLA9, SPLA10, SPLA11 and SPLA12. The whole genome sequence of each phage was determined and 150bp Illumina reads assembled into a single bacteriophage contiguous sequence (contig). Contigs ranged from 39,459 bp to 240,593bp. Bacteriophage lifestyle prediction using Bacphlip suggested that all phages were more likely lytic phages than lysogenic phages with

the exception of SPLA1B that was predicted to be lysogenic with a score of 0.00 / 1.00 (Lytic to Lysogenic probability).

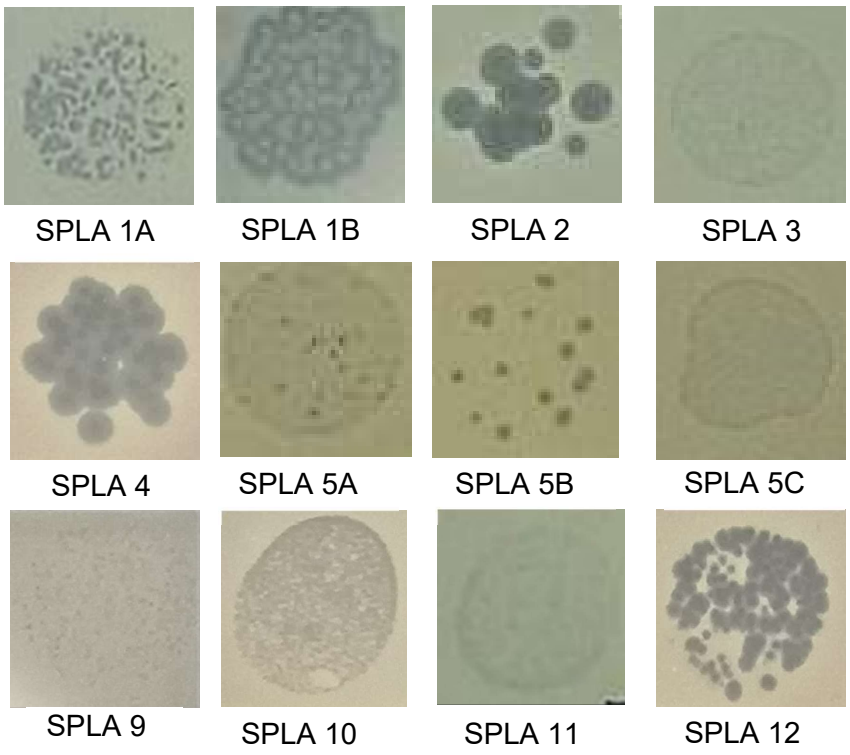


Figure 2 – Plaque Morphologies of 12 SPLA Phages on in 0.75% Agar infecting Isolation Host

Table 1 – Summary of SPLA bacteriophages used in this study

	Isolation Host Serovar	Source	Genome Size (bp)	Predicted Family	Predicted Genus	Lifestyle probability (Lytic/Lysogenic)	Accession
SPLA1a	*S.* Typhimurium	Food	240348	*Unclassified*	*Seoulvirus*	0.76 / 0.24	OR413578
SPLA1b	*S.* Typhimurium	Food	39459	*Unclassified*	*Lederbergvirus*	0.00 / 1.00	OR413579
SPLA2	*S.* Newport	Food	53075	*Unclassified*	*Rosemountvirus*	0.94 / 0.06	OR413580
SPLA3	*S.* Mbandaka	River	240593	*Unclassified*	*Seoulvirus*	0.77 / 0.23	OR413581
SPLA4	S. Mbandaka	Factory drain	39540	*Autographiviridae*	*Berlinvirus*	1.00 / 0.00	OR413582
SPLA5a	*S.* Montevideo	Wastewater influent	151208	*Unclassified*	*Seunavirus*	0.81 / 0.19	OR413583
SPLA5b	*S.* Kedougou	Wastewater influent	107599	*Demerecviridae*	*Tequintavirus*	0.89 / 0.11	OR413584
SPLA5c	*S.* Infantis	Wastewater influent	239099	*Unclassified*	*Seoulvirus*	0.76 / 0.23	OR413585
SPLA9	*S.* Montevideo	Wastewater influent	51694	*Unclassified*	*Rosemountvirus*	0.89 / 0.11	OR413586
SPLA10	*S.* Montevideo	Wastewater influent	210197	*Unclassified*	*Phikzvirus*	0.88 / 0.12	OR413575
SPLA11	*S.* Panama	Wastewater influent	52451	*Unclassified*	*Rosemountvirus*	0.81 / 0.19	OR413576
SPLA12	*S.* Infantis	Wastewater influent	51974	*Unclassified*	*Rosemountvirus*	0.83 / 0.17	OR413577

3.2.3. SPLA1b is an Induced Prophage from *Salmonella* Typhimurium

Since SPLA1b was predicted to be a prophage based on genome sequence analysis, the possibility that this phage was an activate prophage from one of the enrichment strains was investigated further. Illumina sequence reads of each enrichment strain (Supplementary Table 1) were mapped to each assembled viral contig (Figure 3). Log10 transformed coverage was plotted against genomic position and a phage was judged to be a prophage if coverage was high across the whole length of the genome. SPLA1b was the only phage which appeared to be an induced prophage. Coverage was moderately high for many of the enrichment strains but only consistently high across the whole length of the genome a single Typhimurium strain, suggesting that it was induced from this strain and similar regions are present in the genomes of other enrichment strains.

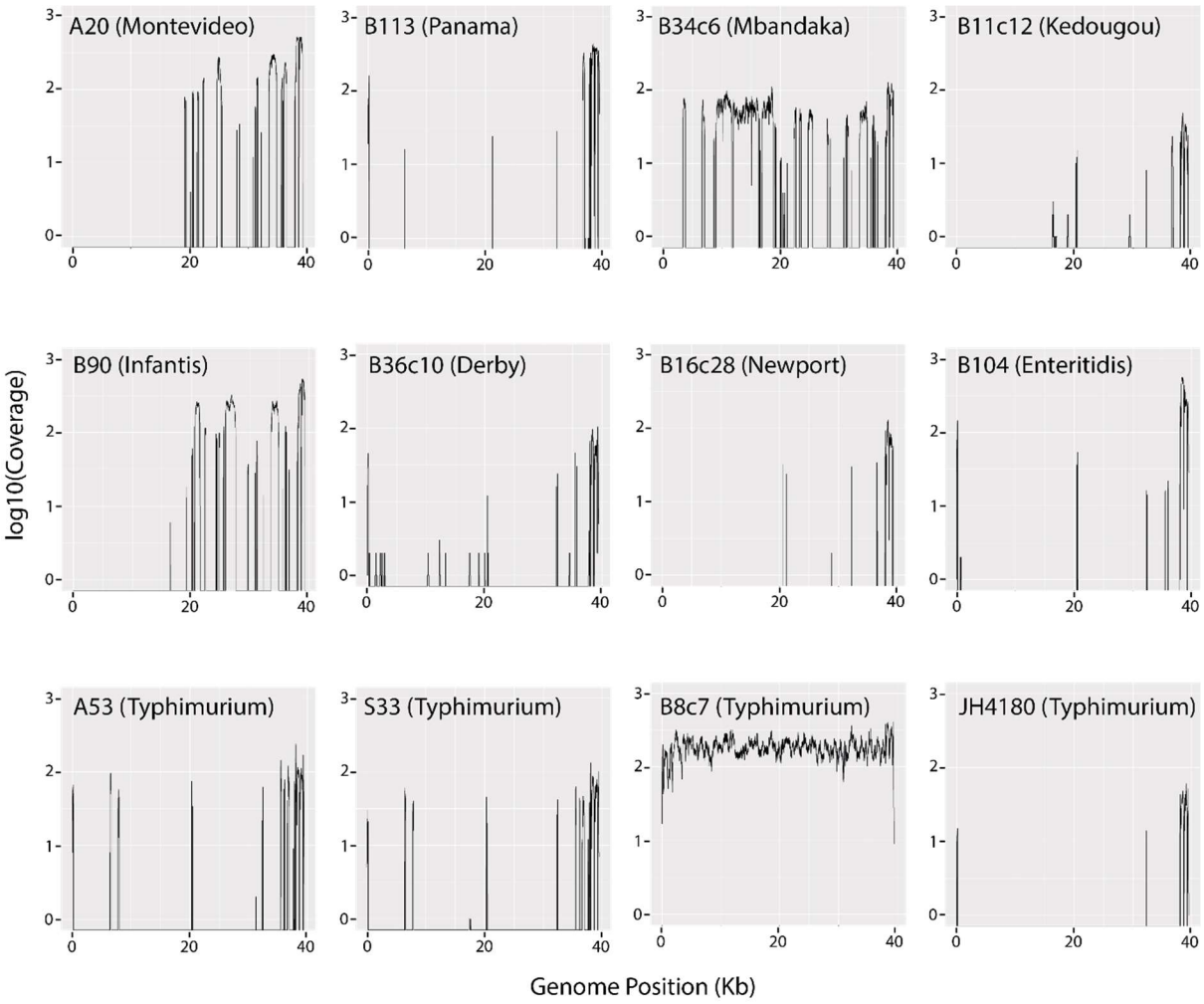


Figure 3 – Log10 Coverage of *Salmonella* enrichment strain sequence reads aligned to the SPLA1b genome sequence assembly.

3.2.4. Genomic Comparison of SPLA phages using VIRIDIC

In order to initially investigate the diversity of isolated bacteriophages, genomes were compared using Virus Intergenomic Distance Calculator (VIRIDIC). This software compares and assigns an intergenomic similarity score to between viral genomes. A score of 100 suggests identical genomes and a score of 0 indicates completely unrelated genomes. Members of the same viral genus had high intergenomic similarity scores. For example, SPLA1a, SPLA3 and SPLA5c, all seoulviruses, shared greater than 96 intergenomic similarity at the nucleotide level. Additionally, SPLA2, SPLA9, SPLA11 and SPLA12, belonging to the *Rosemountvirus* genus all shared more than 94 intergenomic similarity across the whole

length of their genome. Genomes of members of different viral genera did not align and had poor intergenomic sequence similarity < 1 between each other highlighting diversity amongst SPLA phages.

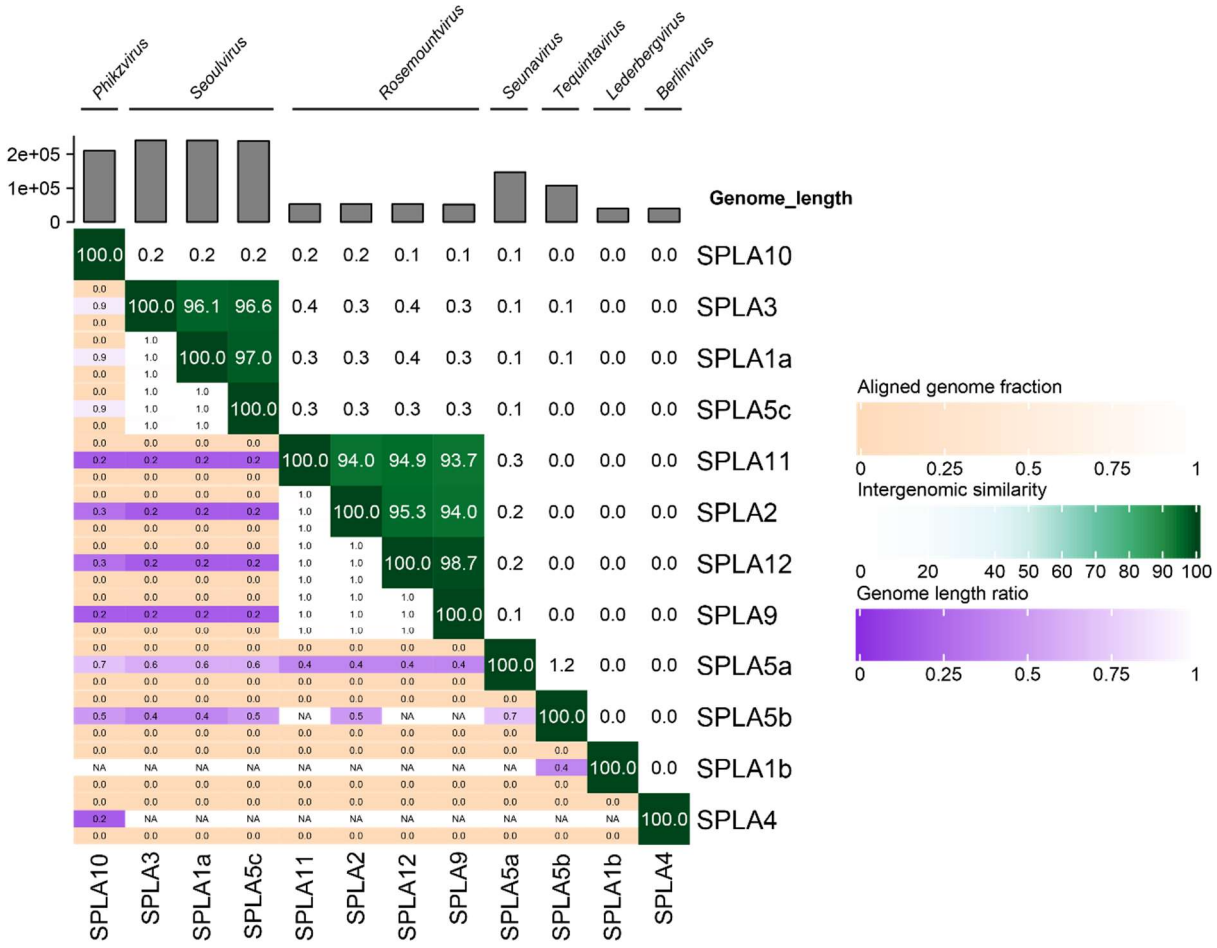


Figure 4 – Nucleotide base intergenomic similarities of SPLA bacteriophages calculated using VIRIDIC. A heatmap of clustering based off intergenomic similarity values as percentages is shown in top-half (White-Green heatmap). The bottom heat map indicates the aligned fraction (beige heatmap) and genome length ratio (purple heatmap) for each phage pair where a darker colour indicates less similarity between each phage.

3.2.5. SPLA Phages are distributed among 7 different viral lineages

To determine the evolutionary relationship of each phage in available databases, phylogenetic context based on protein sequence similarity of SPLA phages and phages infecting Gammaproteobacteria was determined by phylogenetic reconstruction (Figure 5). SPLA phages clustered in 7 deeply rooted lineages which were well distributed among gammaproteobacteria phages which is consistent with the diversity observed using

VIRIDIC. Additionally, members of each genera clustered with other known members of the same predicted genera, further supporting the clusters of related phage genomes. Each phage genome within the tree was assigned a taxonomic classification based on the Inphared database consisting of complete bacteriophage genomes deposited into Genbank. Only two phages, SPLA4 and SPLA5b, have a currently assigned viral family of *Autographiviridae* and *Demerecviridae*, respectively.

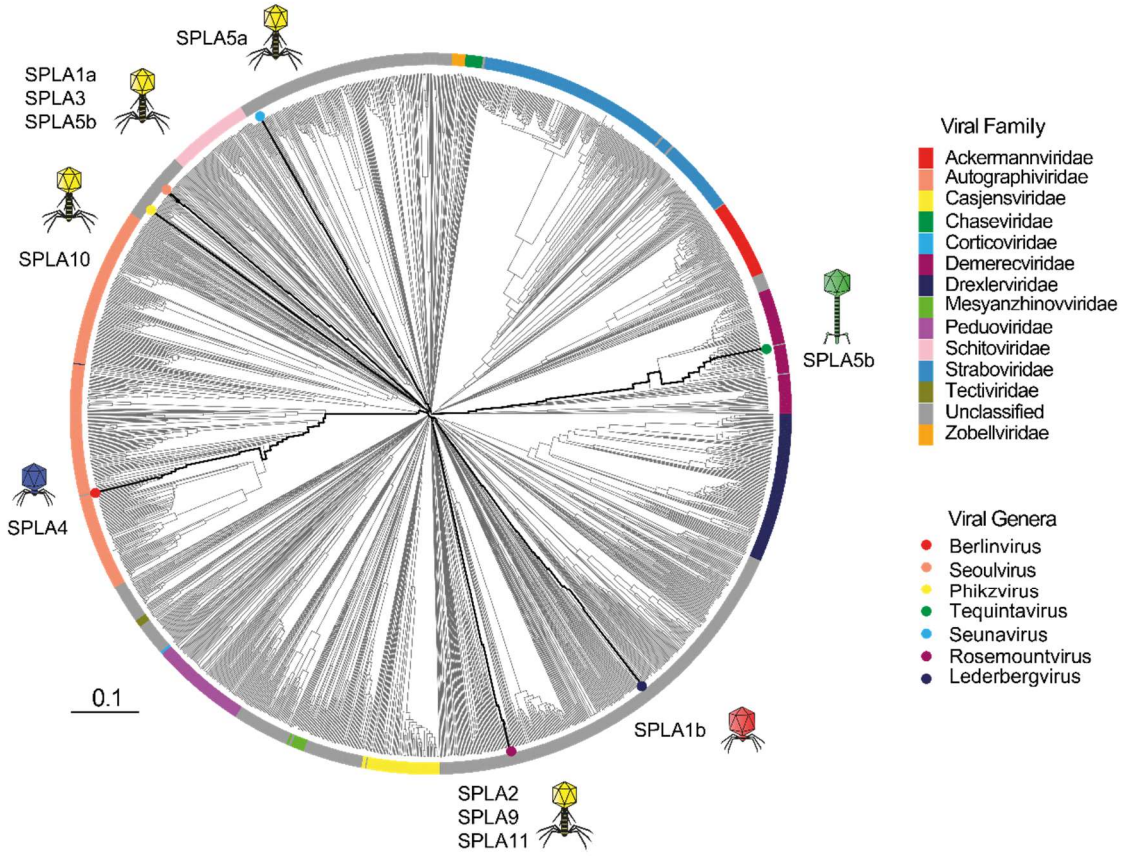


Figure 5 – Phylogenetic relationship of SPLA phages proteome in the context of known diversity of viral proteomes from viral families known to infect Gammaproteobacteria. The dendogram was generated using VIPtree and viral family annotation was assigned using Inphared database. SPLA phages are indicated using circles and diagrams of predicted morphology.

3.2.6. SPLA Phages have limited virulence against non-*Salmonella* strains

To investigate the ability of SPLA phages to infect gammaproteobacterial species, eleven strains from ten different genera were tested including ten *Enterobacteriales* and one *Aeromonaceae*. A broth culture assay that indicates virulence based on the difference in growth in the presence and absence of phage resulting in a Liquid Assay Virulence Score (LAS) was used (Figure 6). All non-*Salmonella* gammaproteobacterial strains exhibited very little susceptibility to SPLA phages with the exception of *Hafnia alvei* which had low susceptible to several SPLA phages, notably the podophages SPLA2 and SPLA4, both of which had LAS greater than 35.

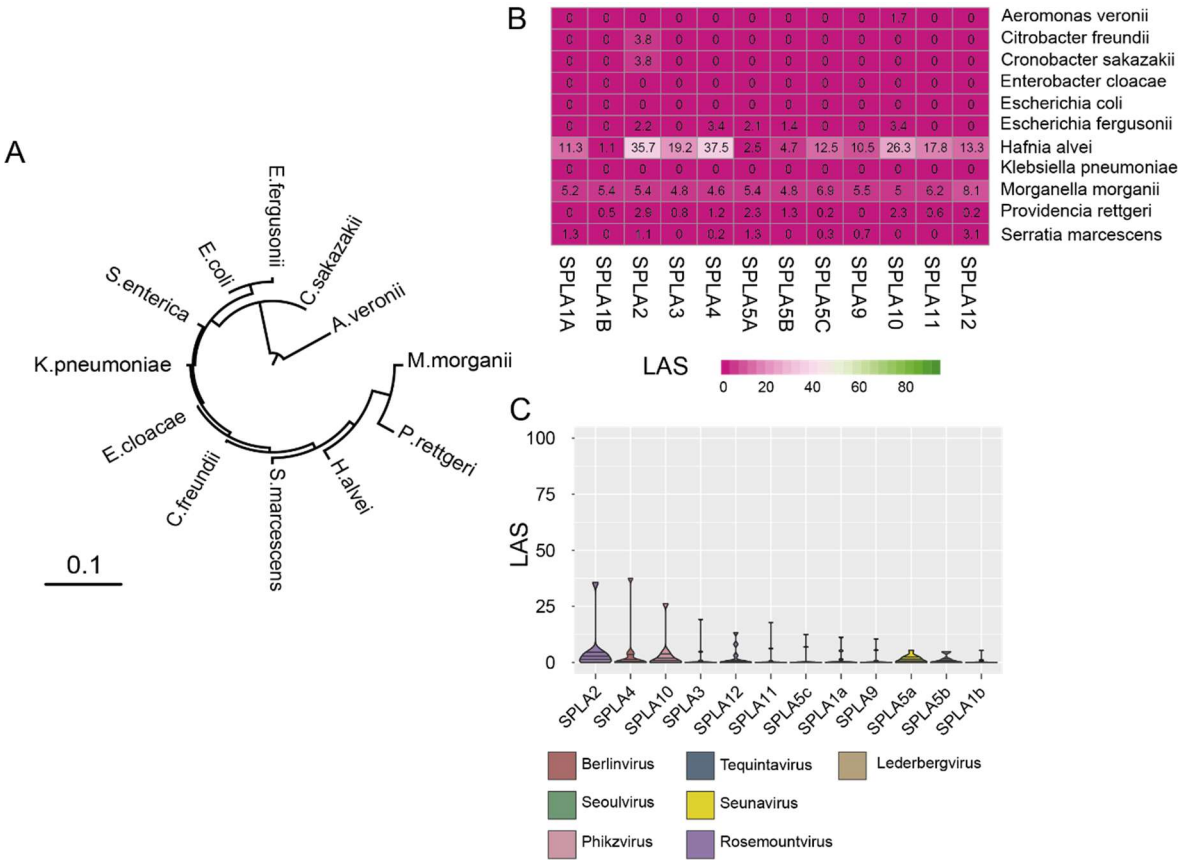


Figure 6 – Host Range testing of SPLA phages against diverse Gammaproteobacteria. A) Maximum likelihood phylogenetic reconstruction of host range strains, including *Salmonella*, based on sequence variation in the 16s rRNA region. Tree is rooted to *Aeromonas veronii* as out group. B) Heatmap of Liquid Assay Virulence Scores (LAS) ranging from 0 (no change in growth) and 100 (no growth) of each strain the presence of each SPLA phage. C) Violin plots of LAS for each SPLA phage against gammaproteobacterial strains.

3.2.7. SPLA Phages have variable virulence against *Salmonella enterica* serovars

To determine the virulence of SPLA phages for isolates of diverse *Salmonella* serovars, thirteen strains of 12 different serovars of *Salmonella enterica* were investigated (Figure 7). Two phylogenetically distinct strains of *S*. Newport were investigated. Susceptibility of S.*enterica* to SPLA phage was highly variable. No SPLA phage exhibited greater than moderate virulence (33-66 LAS) against all strains tested. The broadest host range was observed in SPLA9, SPLA11 and SPLA1a which exhibited at least moderate LAS in 10 of the 13 serovars tested. Nonetheless, all SPLA phages, with the exception of SPLA5a, SPLA5b and SPLA1b, exhibited at least moderate virulence against more than half of the strains tested suggesting relatively broad host range.

Amongst the most phage resistant serotypes were *S*. Kentucky and *S*. Heidelberg with the highest LAS of 12.4 and 23.9 by SPLA9 and SPLA1a respectively, suggesting that these strains are not well infected by SPLA phages. In contrast, the most susceptible strains were *S*. Enteritidis where all SPLA phages achieved at least moderate virulence, and both *S*. Newport strains where all except SPLA1B and SPLA5B scored moderate virulence.

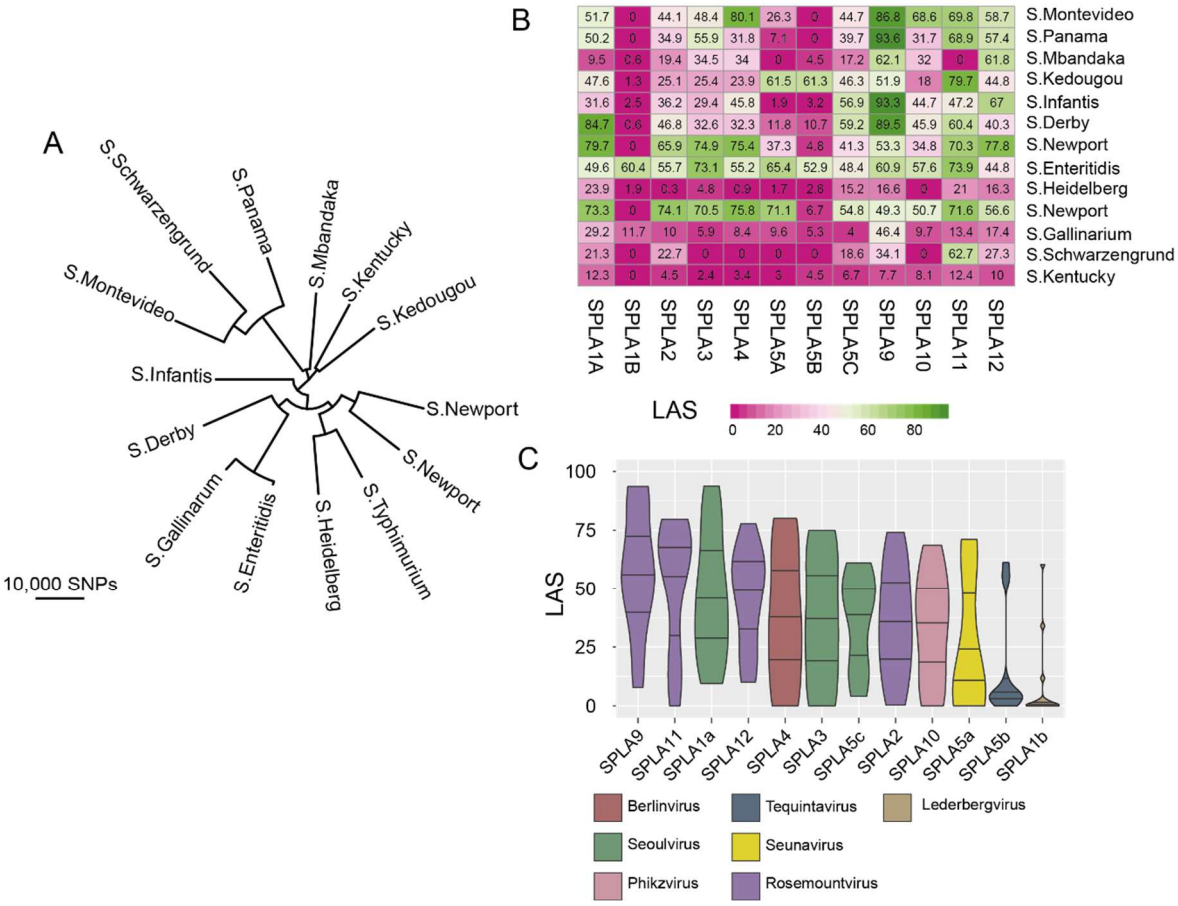


Figure 7 – Host Range testing of SPLA phages against diverse *Salmonella Typhimurium*. A) Maximum likelihood phylogenetic reconstruction of host range strains, including *Salmonella*, based on sequence variation in the core genome (SNPs). Tree is rooted to *Salmonella bongori* as out group and removed from the analysis. B) Heatmap of Liquid Assay Virulence Scores (LAS) ranging from 0 (no change in growth) and 100 (no growth) of each strain the presence of each SPLA phage. C) Violin plots of LAS for each SPLA phage against Salmonella enterica strains.

3.2.8. SPLA phages have variable virulence against *Salmonella* Typhimurium

Twelve *Salmonella* Typhimurium strains, which were well distributed amongst the population structure were chosen to further investigate the virulence of SPLA phages in more detail (Figure 8a). Typhimurium was selected for further investigation as this serotype has a broad host range and commonly causes disease in humans and many farmed animals (Bawn et al., 2020). Despite the much closer relationship between Typhimurium strains relative to *Salmonella* enterica, considerable variation in virulence was still observed

(Figure 8b). SPLA1a was particularly virulent, reflected in high LAS (>66) for many of the strains tested. Additionally, SPLA9 and SPLA11, both Rosemountviruses, were moderately virulent against a broad range of Typhimurium strains. Even among very closely related strains, variation in susceptibility was observed. For examples, S04698 and A53 are both part of the monophasic *S*. Typhimurium ST34 epidemic which has emerged in the last three decades, and differ by 51 core SNPs, yet these strains exhibited distinct differences in susceptibility to at least five SPLA phages (Tassinari et al., 2020). Most strikingly, *S*. Typhimurium with prophages cured from its genome resulted in a decrease in susceptibility to SPLA phages compared to wild-type.

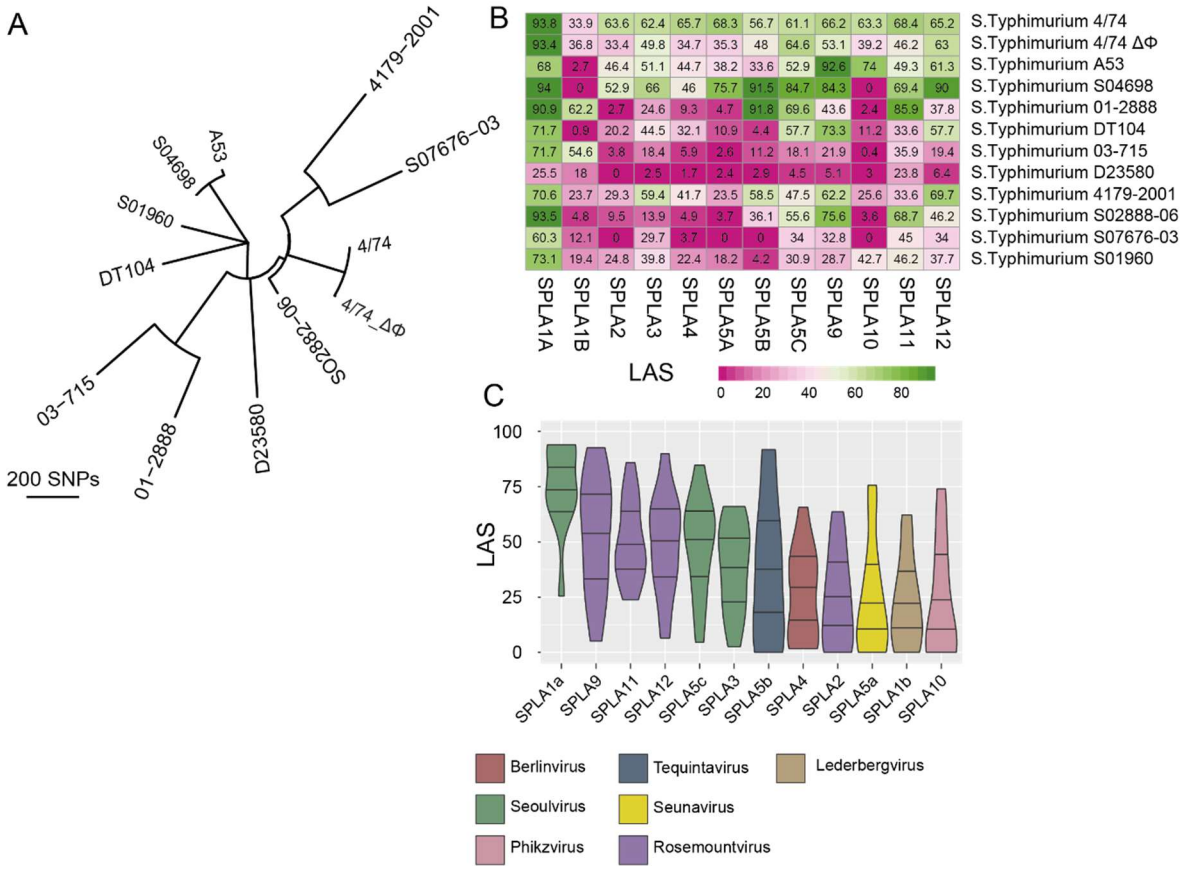


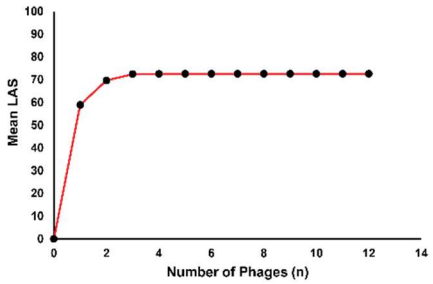
Figure 8 – Host Range testing of SPLA phages against diverse *Salmonella enterica*. A) Maximum liklehood phylogenetic reconstruction of host range strains, including *Salmonella*, based on sequence variation in the core genome (SNPs). Tree is rooted to *Salmonella* bongori as out group and removed from the analysis. B) Heatmap of Liquid Assay Virulence Scores (LAS) ranging from 0 (no change in growth) and 100 (no growth) of each strain the presence of each SPLA phage. C) Violin plots of LAS for each SPLA phage against *Salmonella* enterica strains.

3.2.9. A penta-phage cocktail is sufficient for maximum coverage of host range.

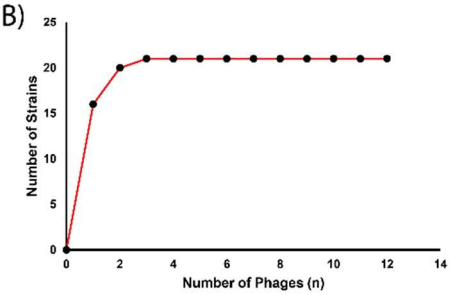
Phage cocktails are combination of phages mixed together and used simultaneously. This approach is used as multiple phages can infect a broader range of strains than single phage preparations. The LAS for each phage - host combination was used to predict the best possible phage combination containing each possible number of phages (n) (Figure 9). Two metrics were used for calculation: Max average where selection was based on the highest mean LAS for each combination and Max_{50} where the best possible combination was

selected based on the number of strains it scored LAS >50. The Max_{50} metric suggested that a tri-phage cocktail containing SPLA1A, SPLA9 and SPLA11 was optimal. This cocktail achieved LAS greater than 50 for 21 of the *Salmonella* strains tested (Figure 7 and 8). Both of these metrics selected for a phage cocktail with a broad host range and high LAS scores indicating high virulence. Conversely, the $Max_{Average}$ metric suggested that the addition of SPLA4 and SPLA5B would broaden the host range and result in a more effective cocktail. This cocktail had a mean LAS of 72.536. In both metrics, adding additional phages beyond this point did not improve respective scores.

A)



B)



Number of Phages (n)	Optimal Phage Combination	Mean LAS
0		0
1	SPLA1A	58.844
2	SPLA1A, SPLA9	69.62
3	SPLA1A, SPLA9, SPLA11	72.4
4	SPLA1A, SPLA4, SPLA9, SPLA11	72.5
5	SPLA1A, SPLA4, SPLA5B, SPLA9, SPLA11	72.536
6	SPLA1A, SPLA1B, SPLA4, SPLA5B, SPLA9, SPLA11	72.536
7	SPLA1A, SPLA1B, SPLA2, SPLA4, SPLA5B, SPLA9, SPLA11	72.536
8	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5B, SPLA9, SPLA11	72.536
9	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA9, SPLA11	72.536
10	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA11	72.536
11	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA10, SPLA11	72.536
12	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA10, SPLA11, SPLA12	72.536

Number of Phages (n)	Optimal Phage Combination	Strains with >50 LAS
0		0
1	SPLA1A	16
2	SPLA1A, SPLA9	20
3	SPLA1A, SPLA9, SPLA11	21
4	SPLA1A, SPLA1B, SPLA9, SPLA11	21
5	SPLA1A, SPLA1B, SPLA2, SPLA9, SPLA11	21
6	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA9, SPLA11	21
7	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA9, SPLA11	21
8	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA9, SPLA11	21
9	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA9, SPLA11	21
10	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA11	21
11	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA10, SPLA11	21
12	SPLA1A, SPLA1B, SPLA2, SPLA3, SPLA4, SPLA5A, SPLA5B, SPLA5C, SPLA9, SPLA10, SPLA11, SPLA12	21

Figure 9 – Phage cocktail selection based on statistical analysis of LAS. A) $Max_{average}$ scores for each optimal phage cocktail for containing each number of phage (n). B) Max_{50} scores for each optimal phage cocktail for containing each number of phage (n).

3.2.10. SPLA1a is resistant to heat stress

Penta phage cocktail candidates (SPLA1a, SPLA4, SPLA5B, SPLA9 and SPLA11) were tested for resistance to heat (Figure 10). Heat resistance is a useful characteristic as it allows phages to be spray dried which improves shelf life and aid phage delivery (Chang et al., 2017). Heat resistance was measured by recovery of phages after 1 hour of treatment. All phages were stable at both 25°C and 50°C with the exception of SPLA1a where a significant reduction of around 1 log was observed (t=8.2609, df=2.0233, p-value=0.01385) compared to no treatment. After treatment at 75°C, SPLA1a was the only phage which could be recovered, although there was a significant 2 log reduction (t=4.4505, df=2.0171, p-value=0.04623), nonetheless this phage remained recoverable, at high titre, where all other SPLA phages were completely inactivated.

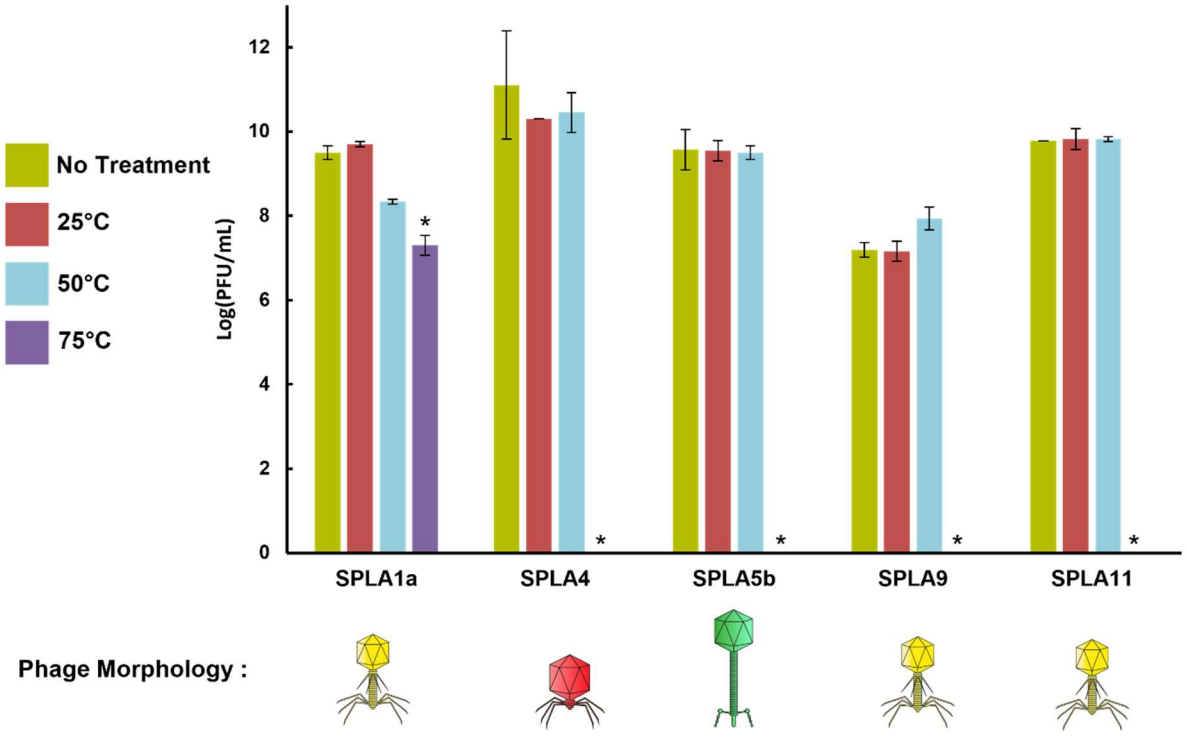


Figure 10 – Survival of SPLA Penta-Phage Cocktail candidates (SPLA1a, SPLA4, SPLA5b, SPLA9, SPLA11) under heat stress measured by log PFU/ml recovered after 1 hour incubation at 4 different temperatures (No treatment, 25°C, 50°C and 75°C). Statistical significance is show using asterisk (*). Phage morphology is represented by icons.

3.2.11. SPLA1a is resistant to Acetic Acid, SGF and SIF treatments.

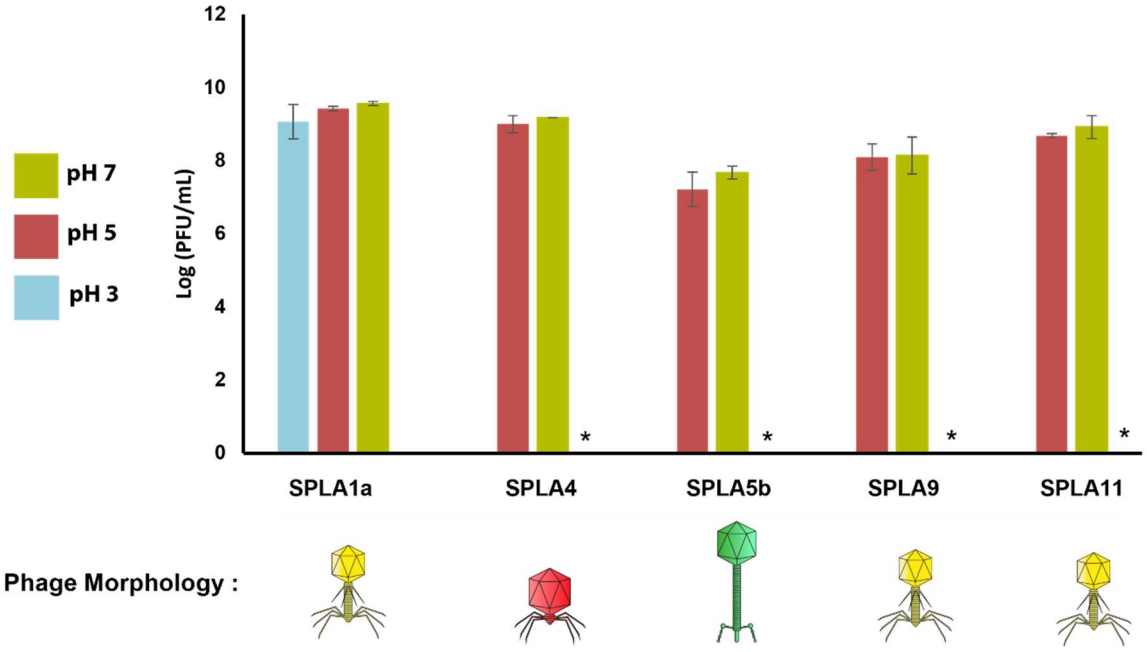


Figure 11 – Survival of SPLA Penta-Phage Cocktail candidates (SPLA1a, SPLA4, SPLA5b, SPLA9, SPLA11) under acetic acid stress measured by log PFU/ml recovered after 5 minutes incubation at 3 different pH (pH7, pH 5 and pH 3). Statistical significance is show using asterisk (*). Phage morphology is represented by icons.

Stability in acid stress was determined by subjecting phages to acetic acid for 5 minutes at pH 5 and 3 (Figure 11). Acid Stability allows phages to remain active in low pH conditions such as acid washes used in food processing or within the human stomach. No significant reduction in phage titre was observed after 5 minutes of exposure to acetic acid at pH 5 compared to pH 7. At pH 3, SPLA4, SPLA5b, SPLA9 and SPLA11 were all completely inactivated however SPLA1a remained stable and no significant reduction in titre compared to pH 7. To further investigate the pH stability of SPLA Phages in the penta-phage cocktail, stability in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was tested following 1 hour of exposure (Figure 12). In SIF, SPLA4 was the only phage with a significant reduction in titre after 60 minutes and was completely inactivated with no recoverable phages ($t=4.4376$, $df = 2$, p-value = 0.04721). In simulated gastric fluid adjusted to pH 3,

once again all phages with the exception of SPLA1a were non-viable, however there was a significant reduction in SPLA1a titre with a roughly 2.5 log reduction observed (t=8, df=2, p-value=0.01527). When SGF was adjusted to pH 2, no viable phages were recovered amongst all SPLA phages.

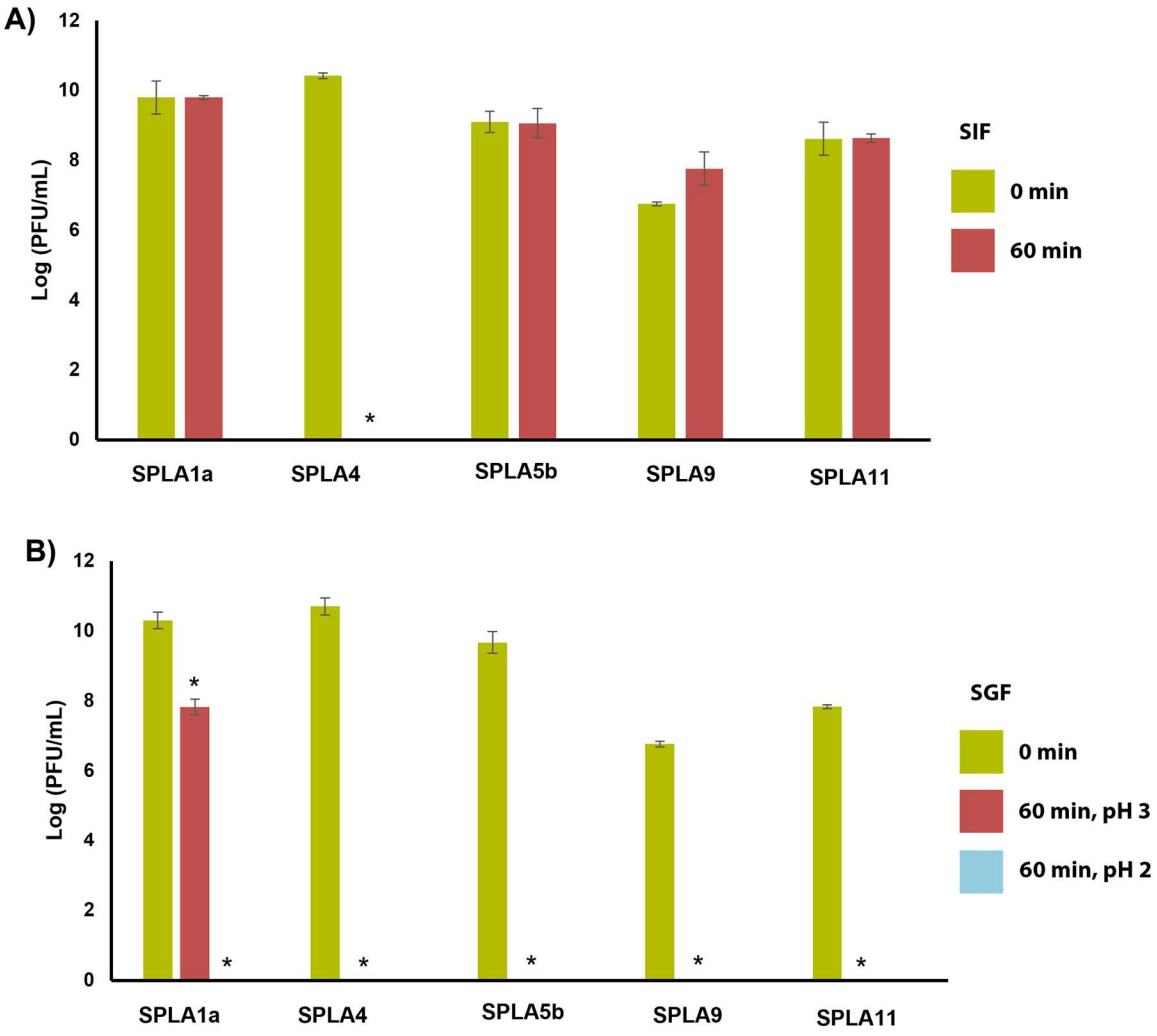


Figure 12a-b – Survival of SPLA Penta-Phage Cocktail candidates (SPLA1a, SPLA4, SPLA5b, SPLA9, SPLA11) exposed to A) Simulated Intestinal Fluid (SIF) and B) Simulated Gastric Fluid (SGF) measured by log PFU/ml recovered after 1 hour incubation. Statistical significance is show using asterisk (*). Phage morphology is represented by icons.

3.3. Discussion

Isolation of bacteriophages is often most successful using samples which are high in bacterial diversity and therefore able to support a more diverse virome. It is therefore unsurprising that wastewater was a successful source of *Salmonella* phages in this study, as it has previously be shown to contain high viral diversity, and in particular phages (Adriaenssens et al., 2021). *Salmonella* were also found in the Lakes/River samples, the locations of which were not in the immediate vicinity of a waste water treatment plant. Despite different sampling locations, closely related phages were often isolated, with 4 *Rosemountviruses* and 3 *Seoulviruses* purified from food, river and wastewater samples. The newly isolated *Seoulviruses* are closely related to previously isolated phages and share greater than 96 % ANI to SPFM phages (Thanki et al., 2019) All *Seoulviruses* are jumbo phages which are classified as phages with genomes larger than 200kb. SPFM phages were mostly isolated from sources of pig origin, including a wild boar reserve and multiple pig farms. Whilst SPLA1a was isolated from food samples which included pork products, SPLA3 and SPLA5c were isolated from river and wastewater samples suggesting that *Seoulviruses* are not exclusively of pig origin.

SPLA phages were not shown to affect the growth of non-*Salmonella* strains with the exception of *Hafnia alvei* where SPLA phages show low virulence. Bacteriophages which are able to infect multiple different genera are rarely reported and are usually restricted to genera within the same family , for example vB_HalM_SPARTY is able to infect both *Salmonella* and *Hafnia* (Bullen et al., 2022). However, *Hafnia* phage Ca is able to infect *Hafnia* and *Aeromonas sobria*, a member of the family *Aeromonadaceae* (Pan et al., 2021*).* It is unclear whether SPLA phages are able to fully complete their infection cycle within the

Hafnia strain tested. The low LAS scores could instead indicate lysis from without, a process where phage adsorption results in destruction of the cell without production of phage progeny. This is common at high multiplicity of infection (MOI), such as the MOI used in this study, and *Hafnia* could have been more susceptible to lysis from without which may explain why virulence was achieved against all phages tested.

SPLA phages were much more virulent against *Salmonella* strains. Amongst the most phage resistant serovars were *S.* Heidelberg, *S*. Gallinarium and *S*. Kentucky, suggesting that a cocktail of SPLA phages would have limited virulence against these serovars, and further phages would need to be isolated to generate a cocktail effective against these strains. In contrast, the SPLA phages were effective against *S*. Typhimurium, *S*. Newport and *S*. Derby. These serotypes represent almost 60% of Salmonella isolations from Pigs by APHA in 2021 which highlights their potential application within the pork production chain as feed additives or processing aid. Seoulviruses, similar to SPLA1a, have previously been used as feed additives which led to significantly reduced *Salmonella* colonisation in *Salmonella* challenged piglets (Thanki et al., 2022). Additionally, the phages were effective against Montevideo, Mbandaka, Kedougou and Infantis which are contribute to around 56% of serovars isolated from chickens, highlighting another potential application within agriculture.

Amongst the most phage resistant strains was *Salmonella* Typhimurium D23580, an invasive, multidrug resistant isolate associated with blood stream infection in sub-Saharan Africa (Kingsley et al., 2009). Despite close genomic similarities to other Typhimurium tested, this strain harbours a unique prophage repertoire, carrying 5 complete prophages, BTP1, BTP5, Gifsy-1, Gifsy-2 and ST64B, with BTP prophages being unique within this

lineage (Owen et al., 2017a). Crucically, BTP-1 is known to harbour a phage defence gene, *bstA*, which results in phage resistance to diverse bacteriophages via abortive infection (Owen et al., 2021). This could explain why this isolate was resistant to many SPLA phages.

Combining bacteriphages into cocktails is a useful approach to increase host range and limit phage resistance (Lood et al., 2022). Selecting phages for cocktails should consider the host range of the individual phages. Using two approaches, SPLA1a, SPLA9 and SPLA11 were picked as cocktail candidates. These phages were effective against multiple serovars of *Salmonella*. The addition of SPLA4 and SPLA5B resulted in a small increase in mean LAS and a slightly more effective cocktail. However, this approach does not consider any antagonistic (lower efficacy than the most effective bacteriophage acting alone) or synergistic (greater efficacy than the most effective bacteriophage acting alone) effects of bacteriophages within the cocktail which could result in changes in efficacy. For example, cocktail selection based on virulence of single phage preparations against *Escherichia coli* resulted in synergistic, neutral and antagonistic effects (Niu et al., 2021). This highlights the requirement to test for phage-phage interactions when formulating cocktails to ensure most successful applications.

Another important consideration for bacteriophage cocktail candidates is their stability within environmental stress conditions. The type of stress should consider the potential application. For example, phages used within food production should be stable to changes in temperature consistent with the food manufacturing process. Conversely, phages with applications for oral therapies should be able to tolerate changes in pH such as those present in the human stomach and gastrointestinal tract (Dąbrowska, 2019). We

demonstrate that SPLA1a is tolerant to heat and acid stress, in addition to its broad host range and high virulence scores. This is an unusual phenotype as phages are typically non-viable following exposure to high temperatures, specifically as high as 75^{o}C (Jończyk et al., 2011). A similar stability phenotype has been reported in SPFM10, a Seoulvirus with 98% sequence similarity to SPLA1a. Single nucleotide polymorphism analysis has linked the heat and pH tolerance phenotype to a non-synonymous mutation in a hypothetical protein. The adenine to thymine mutation led to a threonine to serine substitution and was the only SNP consistently not present in four other closely related phages which did not display the phenotype. Increases in tolerance to stresses expands the potential downstream application of bacteriophages. Many applications require stability at ambient temperatures, which is best achieved by spray drying (Vinner et al., 2019). The process of spray drying requires prolonged exposure to high temperatures, making SPLA1a a good candidate for spray drying. Additionally, spray dried bacteriophages are suitable for oral administration. Oral administration is the most convenient delivery method for bacteriophages to treat many infections, the stability of SPLA1a in simulated gastric fluid and simulated intestinal fluid will likely contribute to increased efficacy for oral administration.

Host range, infection dynamics and stability to external stress are important factors for selecting bacteriophages for downstream applications. Both host range and stability should consider the intended downstream application to ensure that bacteriophages are effective against selected targets as well as remain viable within the niche. However, it is not possible to fully predict the effectiveness of a bacteriophage cocktail using these factors alone as it does not consider phage synergy or antagonism. In order to determine this, more

information relating to the phage host interaction is required. This should include host receptor and resistance mechanisms to ensure there is limited overlap between cocktail candidates.

4. Elucidating Phage-Host Interactions using Functional Genomics

4.1. Introduction to Chapter Five

The interaction of a bacteriophage with a susceptible host is complex. Phage infection is initiated by attachment to a receptor located on the cell surface. Attachment is a signal for the genetic material of the phage housed in the capsid to be injected into the cytoplasm. Subsequently, the phage genome is replicated and new viral progeny are produced within the host bacterium. Specificity is mediated by the receptor binding proteins (RBP) located on the phage tail fibres and the host receptor. Different RBPs bind to different host receptors, giving phages the potential to utilize a variety of different cell surface receptors including lipopolysaccharide, outer membrane proteins and flagella proteins (Broeker and Barbirz, 2017). However, bacteria may encode a variety of different mechanisms to prevent phage infection. Phage resistance mechanisms can target different stages of the phage infection process including receptor binding and nucleic acid injection (Barker, 1988, Bondy-Denomy et al., 2013, Goldfarb et al., 2015). In order to use phages for downstream applications, its important the target receptor and phage defence mechanisms are properly characterised as this information can help develop more effective treatments. Using phages which target alternative receptors makes application more effective as phage resistance can occur at lower frequency (Chan et al., 2013).

Genome wide transposon insertion mutant libraries combined with high throughput sequencing of transposon insertion sites has been widely used to assay essentiality or conditional essentiality of every gene within a bacterial genome. This approach has been applied to identify genes involved in antibiotic tolerance, biofilm formation, antibiotic production and bacteriophage infection (Holden et al., 2021, Yasir et al., 2020, Xu et al., 2017, Cowley et al., 2018). The approach uses a saturating library of transposon mutants each with a transposon inserted into a different genomic location. The insertion of a

transposon into a coding sequence is assumed to knock out the function of a gene, although since multiple insertions are normally present in each gene within the library it is possible to identify insertion sites with no effect on gene function. The library of mutants is placed under selection in a defined test condition and targeted sequencing of transposon insertion sites facilitates identification of mutants which have increased or decreased in frequency corresponding to increased or decreased fitness. To date, there have been four variations of transposon insertion sequencing; transposon sequencing (Tn-Seq), insertion sequencing (INseq), high-throughput insertion tracking by deep sequencing (HITS) and transposon directed insertion site sequencing (TraDIS). TraDIS was initially developed for investigation of essential genes and bile tolerance genes in *Salmonella* Typhi and has since been applied to other organisms including *E.coli* (Langridge et al., 2009, Yasir et al., 2020)

In this study, a functional genomics approach, employing TraDIS, was used to determine host encoded genes which contribute to susceptibility or resistance to SPLA bacteriophages. This information, along with details of host range and virulence discussed in chapter four, is used to select a combination of bacteriophages which work synergistically together leading to improved bacteriophage cocktail design.

4.2. Results

4.2.1. Distribution of Insertions within *Salmonella* Typhimurium ST4/74 TraDIS Library

A *Salmonella* Typhimurium strain ST4/74 Tn5 insertion mutant library, constructed by Dr. Hannah Pye, was used to determine the genes involved in infection by SPLA phages. This library was susceptible to SPLA phages with at least moderate LAS by all phages with the exception of SPLA1B. The mutant library was constructed by electroporating a transposome that is a mixture containing a Tn5 transposon harbouring a kanamycin

resistance gene and transposase enzyme. Sequencing of transposon insertion sites in the library cultured on Luria broth agar using TraDIS and mapping to the S. Typhimurium ST4/74 genome sequence indicated approximately 609,000 unique transposon insertions corresponding to approximately one insertion every 8 base pairs across the genome. Gene essentially was determined using BioTraDIS based on frequency distribution of insertions across replicates (Barquist et al., 2016). Sequencing reads were mapped to the *Salmonella* Typhimurium ST 4/74 reference genome and were placed alongside the location of essential genes (Figure 1). This figure was adapted from Dr Hannah Pye.

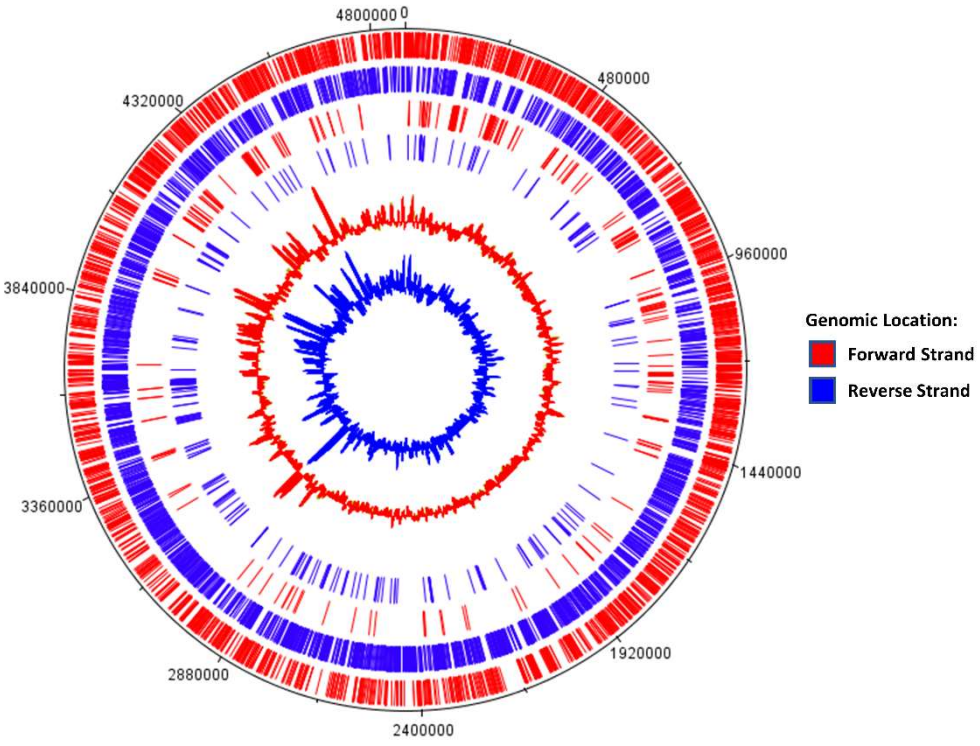


Figure 1. Insertion site map and essential genes of transposon mutant library in *S*. Typhimurium Strain ST4/74. Insertion sites of Transposon mutant library grown mapped to reference genome (inner two rings), essential genes required for growth in laboratory conditions (LB Broth, 37^{o}C) (middle two rings) and coding sequences (CDS) of ST4/74 (outer two rings). Genomic location is denoted by red (forward strand) and blue (reverse strand). Genome size is presented in base pairs. Adapted from Dr Hannah Pye, thesis.

4.2.2. Selection of SPLA phages for host interaction investigation

In order to select the library of transposon mutants, the selection pressure must be appropriate so that changes in insertion frequency can be observed. We reasoned that too much phage infection would potentially lead to complete death of the population (in some cases) and may not detect genes with a small effect on phage resistance. Too little selection would lead to no observable change in insertion frequency. Selection pressure can be controlled by varying the multiplicity of infection (MOI). Viable counts of the TraDIS library were measured in 1-hour intervals to ensure that an appropriate level of selection was achieved (Figure 2a). An appropriate level of selection was deemed to be a 2 log reduction as this has previously been used for TraDIS experiments using Antibiotic selection (Holden et al., 2021, Yasir et al., 2020). SPLA phages were used at an MOI of 10, except for SPLA1a which was used at an MOI of 1 due to high virulence observed earlier in the host range screen. Despite the lower MOI, SPLA1a still produced the largest reduction in viable counts after 5 hours, with a log reduction of roughly 3. Interestingly, SPLA2 reduced viable counts in liquid culture after 5 hours but did not produce visible single plaques on solid medium after 18 hours of incubation (Figure 2b). SPLA3 was not able to reduce the viable counts, despite previously being able to infect the parent strain in the host range screen (Chapter 4). Due to their reduction in viable counts SPLA1a, SPLA1b, SPLA2, SPLA5b, SPLA5c and SPLA11 were selected for use in the TraDIS experiments. Three hours was selected as the selection time as this was enough time for reduction in viable counts and therefore every phage could complete its infection cycle and did not allow excess time for phage resistance mutations to emerge within the population and confound the data.

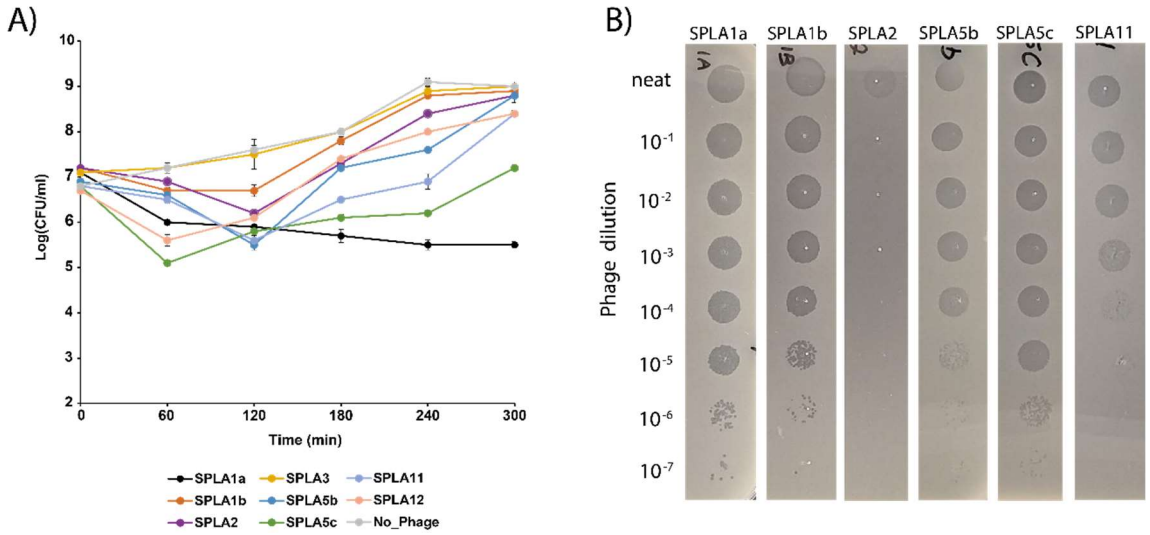


Figure 2. Viable counts of *Salmonella* Typhimurium ST 4/74 TraDIS library with infection by SPLA phages. A) Change in colony forming units (CFU) taken at 1 hour intervals for 5 hours. Error bars represent standard error (SE) B) Plaques of selected SPLA phages against ST4/74 wild type after 18 hours.

4.2.3. Distribution of Insertions following selection by SPLA phages

In order to identify host genes involved in infection by selected SPLA phages, the *Salmonella* Typhimurium transposon mutant library was exposed to SPLA1a, SPLA1b, SPLA2, SPLA5b, SPLA5c and SPLA11 for 3 hours of infection in liquid culture. Immediately following selection period, genomic DNA was extracted and sequenced using TraDIS. The transposon sequences were mapped back to the reference genome and changes in insertion frequency within genes were detected using BioTraDIS (Barquist et al., 2016)(Figure 3). Mapping of the insertions showed a large spike in a specific gene region, corresponding to LPS biosynthesis loci, which was also present in the non-phage treated control. However, this spike was dramatically reduced following treatment with SPLA5b.

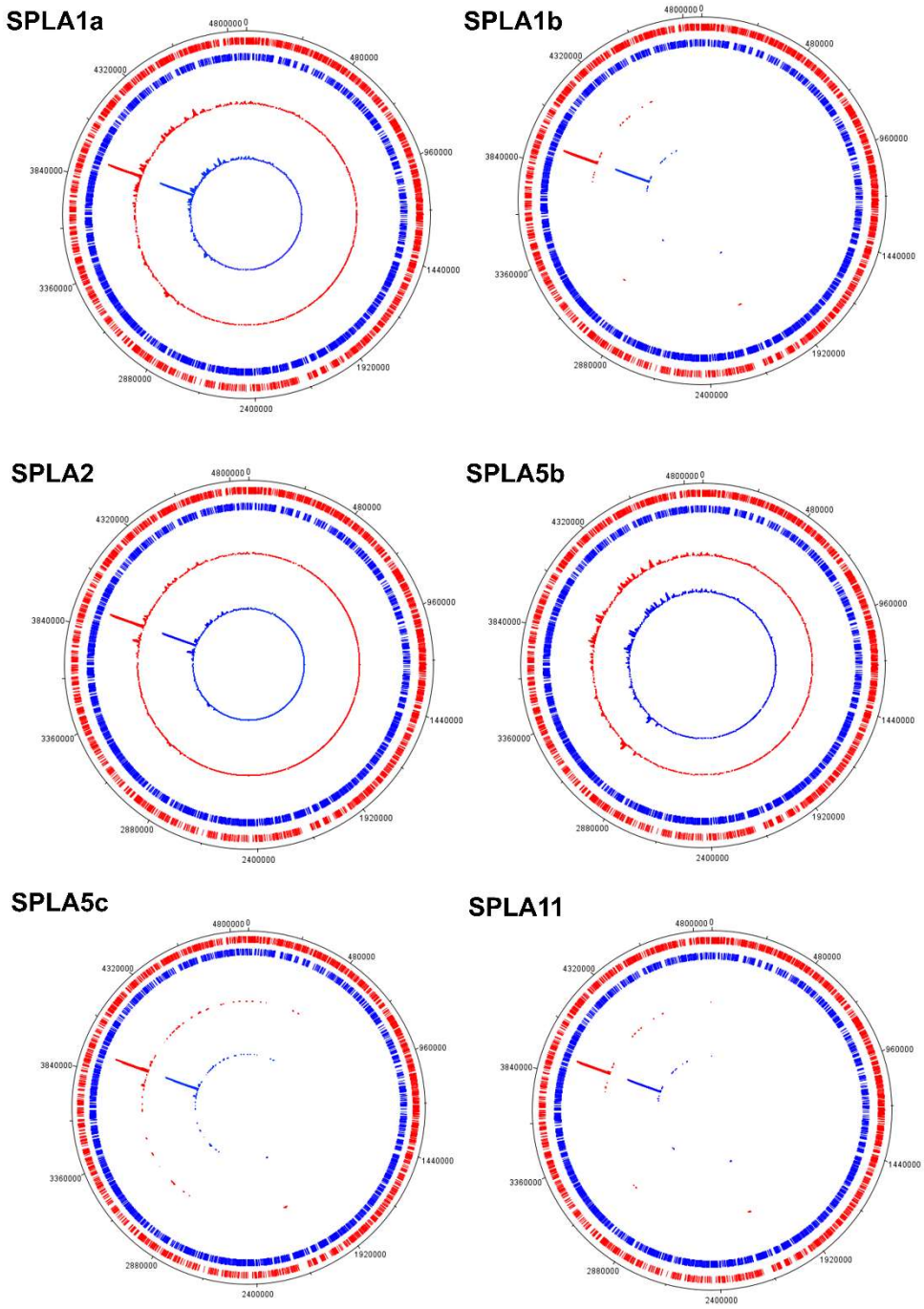


Figure 3. Insertion site maps of transposon mutant library in *S*. Typhimurium Strain ST4/74 following bacteriophage selection. Insertion sites of Transposon mutant library grown mapped to reference genome (inner two rings), and coding sequences (CDS) of ST4/74 (outer two rings). Genomic location is denoted by red (forward strand) and blue (reverse strand). Genome size is presented in base pairs.

In order to determine which genes had significant changes in insertion frequencies, a significance threshold of 0.05 (Q-value) was used. Genes containing insertions in

significantly greater relative abundance (Q <0.05) following phage treatment compared to control were termed susceptibility genes, since their interruption resulted in reduced phage infection. Conversely, genes containing transposon insertions in lower relative abundance (Q <0.05) in the transposon mutant library were termed phage resistance genes since their inactivation resulted in increased phage infection.

Most of the susceptibility genes identified were genes which encoded proteins involved in the biosynthesis of macromolecules present on the bacterial cell surface which were candidate phage receptors. For example, *btuB* that encodes the outer membrane vitamin B12 transporter appeared to be a susceptibility gene for SPLA5b infection (Figure 4D). In addition, genes involved in the synthesis of lipopolysaccharide (LPS), such as those in the *rfa* and *rfb* loci, were detected as susceptibility genes for infection by SPLA1a, SPLA1b and SPLA5c (Figures 4A, 4B and 4E). Genes involved in the synthesis of cellulose, including *yhjU*, *yhjL*, *yhjN*, *yhjQ*, *yhjR* and *yhjS* were highlighted as susceptibility genes for SPLA2 infection. Putative resistance genes were detected which when interrupted reduced bacterial survival. These included two regulatory proteins BarA (SPLA2) and OxyR (SPLA5c). In addition, RumA, involved in the methylation of uracil in 23s ribosomal RNA, and *nfi*, encoding an exonuclease, were both highlighted to provide resistance to SPLA5c infection. The glycosyltransferase gene, *gtrB*, was identified as a resistance gene in both SPLA5c and SPLA11 infection. Most notable, while insertional inactivation of genes involved in LPS biosynthesis increased survival upon infection by SPLA1a, SPLA1b and SPLA5c, inactivation of these genes decreased survival in the presence SPLA5b.

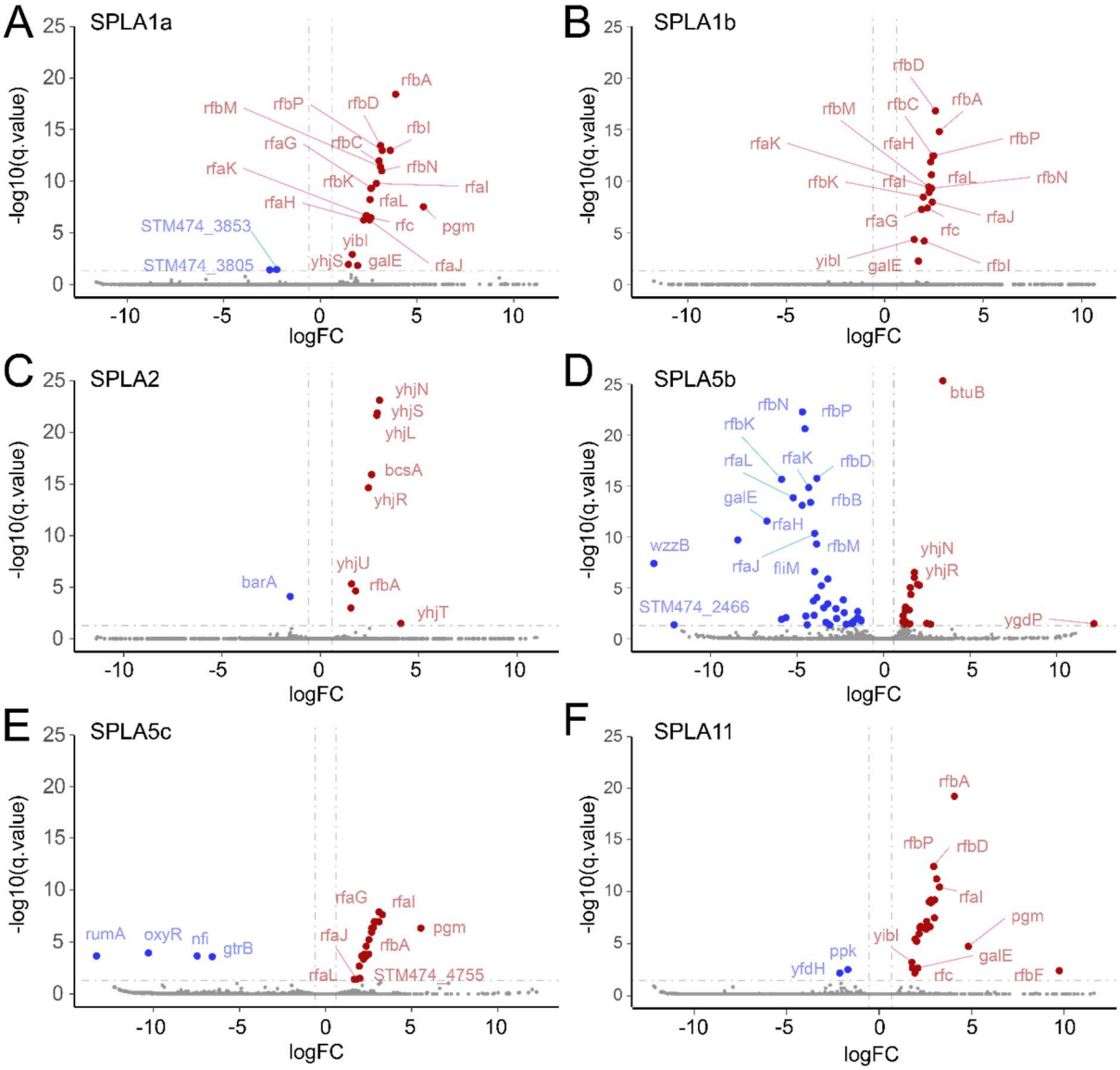


Figure 4. Volcano plots showing changes in the insertions within genes in phage treated conditions relative to non-phage treated control. Log fold change in insertions (x-axis) is plotted against significance (y-axis) using -$\log_{10}$(q-value). Each graph shows gene hits for treatment with a different phage: A = SPLA1a, B = SPLA1b, C = SPLA2, D = SPLA5b, E = SPLA5c and F = SPLA11. Blue points show a significant decrease in insertions (resistance genes) and red points show a significant increase in insertions (susceptibility genes).

4.2.4. Confirmation of TraDIS genes using isogenic mutant phenotyping

Genes where highly significant log fold changes had been identified were selected for further analysis. Ten selected genes were deleted and replaced with an *aphII* cassette, conferring resistance to kanamycin. Genes selected were; *btuB*, *galE*, *wzzB*, *oxyR*, *rfaL*, *bcsA*, *rumA*, *yhjS*, *rfbA* and *ST4/74_0756*. The resulting single isogenic mutants were

phenotyped for increases or decreases in phage sensitivity using a double agar overlay method (method described in 2.8). This method tested the ability of each phage to form plaques when infecting each mutant strain. Notably, this method assayed for phage infection after 18 hours which was in contrast 3 hours used in the TraDIS experiment. Phage infection was scored as either increased or decreased lysis compared to wild-type which included both changes in efficiency of plaquing (EOP) and confluence of plaques (Table 1). All genes resulted in a phenotypic difference to at least one phage. In general, deletion of a gene involved in the synthesis of the predicted phage receptor resulted in decreased lysis and in most cases the phage displayed no observable lysis. For example, replacement of *btuB* lead to complete resistance to SPLA5b which confirmed the large increase in insertions observed in the TraDIS data. In addition, replacement of *rfaL or rfbA ,* lead to complete resistance to SPLA1a and SPLA1b. Interestingly, replacement of the UDP-glucose 4-epimerase gene, *galE* resulted in complete resistance to SPLA1b but only partial resistance to SPLA1a. The replacement of cellulose synthase gene, *bcsA*, lead to complete resistance to four phages; SPLA2, SPLA9, SPLA12, all of which are Rosemountviruses, as well as resistance to SPLA10. Disruption of some genes also lead to increased susceptibility to SPLA phages. Replacement of *galE, wzzB, rfaL* and *rfbA,* all involved in synthesis of LPS resulted in increased sensitivity to SPLA5b. Interestingly, despite being highlighted as genes involved in resistance to SPLA5c in the initial TraDIS screen, neither *oxyR* or *rumA* provided resistance to SPLA5c in this assay.

Table 1. Plaque assay results for SPLA phages against ten *Salmonella* Typhimurium isogenic mutant strains

SPLA	WT	*Δ0756*	*ΔbtuB*	*ΔgalE*	*ΔwzzB*	*ΔoxyR*	*ΔrfaL*	*ΔbcsA*	*ΔrumA*	*ΔyhjS*	*ΔrfbA*
1A							*				*
1B				*			*				*
2								*			
3											
4											
5a											
5b			*								
5c											
9								*			*
10								*			*
11											
12								*			

	Phage activity
	Clear lysis
	Hazy lysis
	Small amount of visable lysis
	No lysis

	Knockout effect
	No change
	Increased lysis
	Decreased lysis
*	No observed lysis

4.2.5. Comparison of phage resistance and susceptibility genes

In order to identify genes which contributed to resistance or susceptibility to multiple bacteriophages, conserved resistance and susceptibility genes were compared using UpsetR (Figure 5). SPLA5b infection had the most unique genes among other SPLA phages with 34 unique significant genes identified. These genes included its receptor, BtuB as well as lower significance hits including genes involved in flagella synthesis and structure such as *flgCEFGI* and *fliFMNR.* The rest of the SPLA phages tested only had 2 unique genes each, with the exception of SPLA1B, which did not have any significant unique genes. This could be due to utilising the same receptor of SPLA1a and SPLA5c.

Many genes involved in LPS synthesis were found to have significant log fold changes for multiple SPLA genes. However, only a single gene was shared amongst all SPLA phages, *rfbA*, encoding a glucose-1-phosphate tymidylyltransferase involved in o-antigen synthesis, however this gene had contrasting roles and significance scores for each SPLA phage and was not confirmed phenotypically for SPLA2 infection (Table 1). Not including LPS genes, very few genes were conserved across multiple phages, particularly those classified in different phage genera.

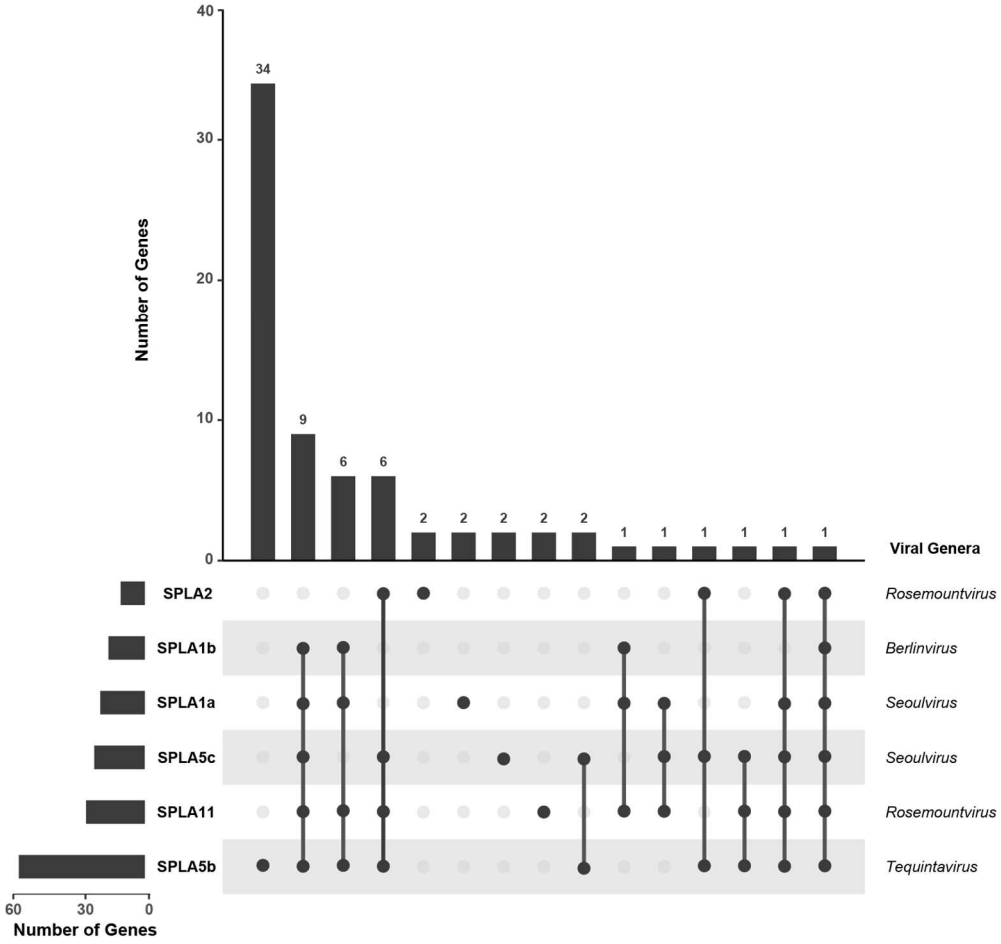


Figure 5. Distribution and Abundance of Phage susceptibility and resistance genes between SPLA phages in *Salmonella* Typhimurium strain ST4/74. UpsetR plot showing the number of genes in S.Typhimurium strain ST4/74 with significant (q-value < 0.05) log fold changes compare to non phage treated control that are shared between each SPLA phage combination.

4.2.6. Comparison of Genes involved in infection by SPLA1a and SPLA5c

SPLA1a and SPLA5c are both *Seoulviruses* and share >98% sequence identity across their genomes. The transposon mutant library screen identified 12 susceptibility genes which were required for infection by both phages (Figure 6a). These genes were mainly involved in synthesis of the LPS which is likely the receptor for both phages. LPS is a complex structure and synthesis involves a wide array of different genes. Different bacteriophages often target different parts of the LPS and therefore genes involved in LPS synthesis often have different contributions to bacteriophage infection. For example, O-antigen chain length, controlled by *wzz*, *wzy* and epigenetically by *opvAB,* affects susceptibility to bacteriophages P22, 9NA and Det7 (Cota et al., 2015). We therefore aimed to determine the contribution of each LPS synthesis gene for infection by SPLA1a and SPLA5c. Despite their high genomic similarity, unique genes were identified which were not significant for infection by the other phage. Nine genes were identified to be involved in infection by SPLA1a – seven susceptibility genes and two resistance genes. Conversely, ten unique genes were identified for SPLA5c, six susceptibility genes and four resistance genes. Significant genes shared between both phages were generally involved in the synthesis of the core LPS structure (Figure 6b), including *rfaI, rfaJ, rfaL* and *rfaG*. Genes unique to SPLA1a infection included *rfaK* and *galE,* both of which are used to add side chains onto the inner core suggesting that both phages utilize LPS as their receptor, but the exact structures required for each may differ. Most interestingly, *gtrB* (STM0558) was identified as a resistance gene for SPLA5c infection but not for SPLA1a infection further highlighting alternative targets within the LPS structure for these phages.

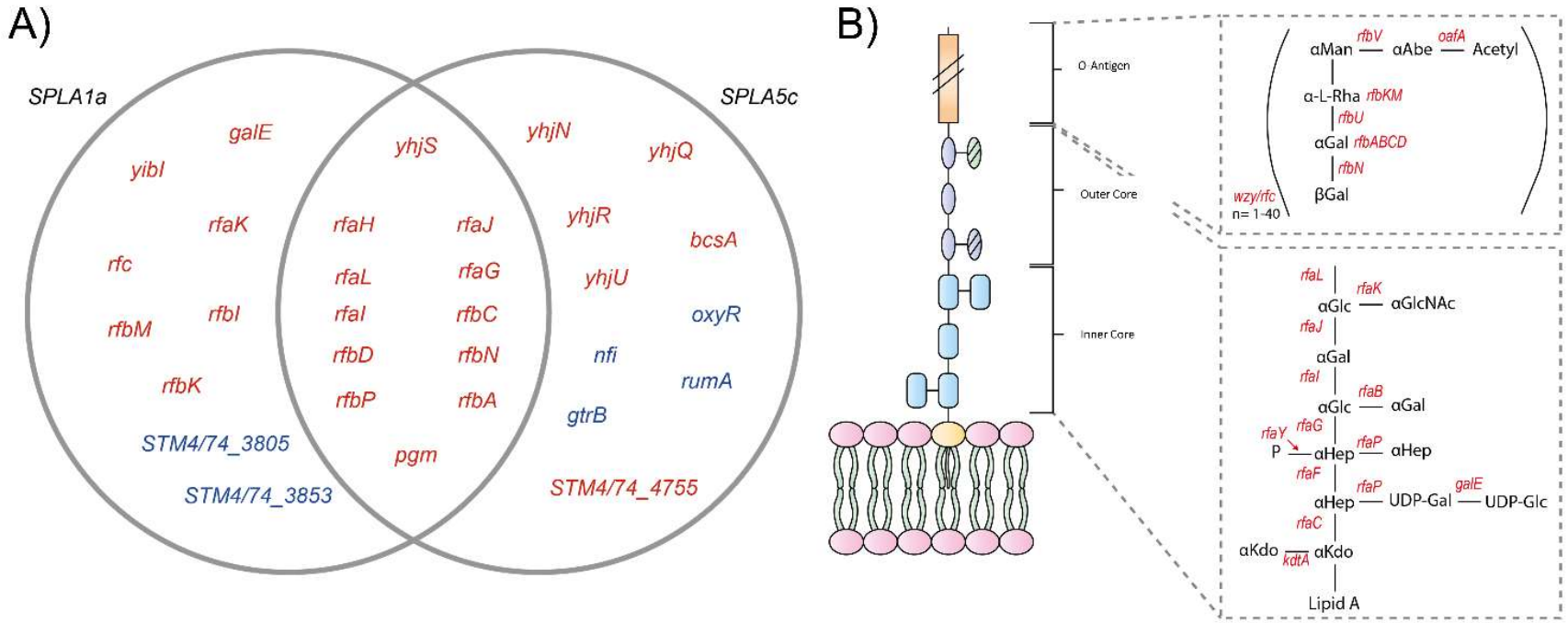


Figure 6. Comparison of Genes involved in Infection by SPLA1a and SPLA5c. A) Venn Diagram of susceptibility genes (red) and resistance genes (blue) in *Salmonella* Typhimurium ST4/74 to infection by SPLA1a and SPLA5c. B) Schematic of LPS structure and synthesis produced using information from (Schnaitman and Klena, 1993).

4.2.7. Closely related *Seoulviruses* SPLA1a and SPLA5c differ in virulence due to sensitivity to O-Antigen glycosylation by gtrB

SPLA1a and SPLA5c exhibited distinct virulence to a range of strains of *S.enterica* serotypes as well as a range of *S.*Typhimurium, despite sharing high sequence identity across their whole genome (Chapter 4). For example, SPLA1a was highly virulent against *S.*Typhimurium 4/74 (LAS = 100) while SPLA5c was only moderately virulent (LAS=61). A key difference, highlighted by TraDIS, was the sensitivity to *gtrB*, encoding a glycosyltransferase. A strain of *Salmonella* Typhimurium ST4/74 in which the *gtrB* gene was replaced by *aphII*, encoding resistance to kanamycin, resulted in an increase in LAS to 100 for SPLA5c infection and resulted in virulence and infection dynamics which were similar to SPLA1a (Figure 7). To investigate genetic determinates that could contribute to differential sensitivity to the presence of *gtrB*, the genomes of SPLA1a and SPLA5c were aligned and variation in sequence was identified (Figure 8). Average nucleotide identity was above 98% across the whole genome with the exception of a region of around 10kb affecting 17 genes which were mostly of unknown function but included virion structural proteins and a putative tail fibre protein. The ANI in this region dropped to 96.0%. Additionally, SPLA5c had three insertions relative to SPLA1a affecting a gene encoding a putative RNA polymerase subunit,

a terminase large subunit and a protein of unknown function. SPLA1a had two insertions affecting a gene of unknown function and a virion structural protein.

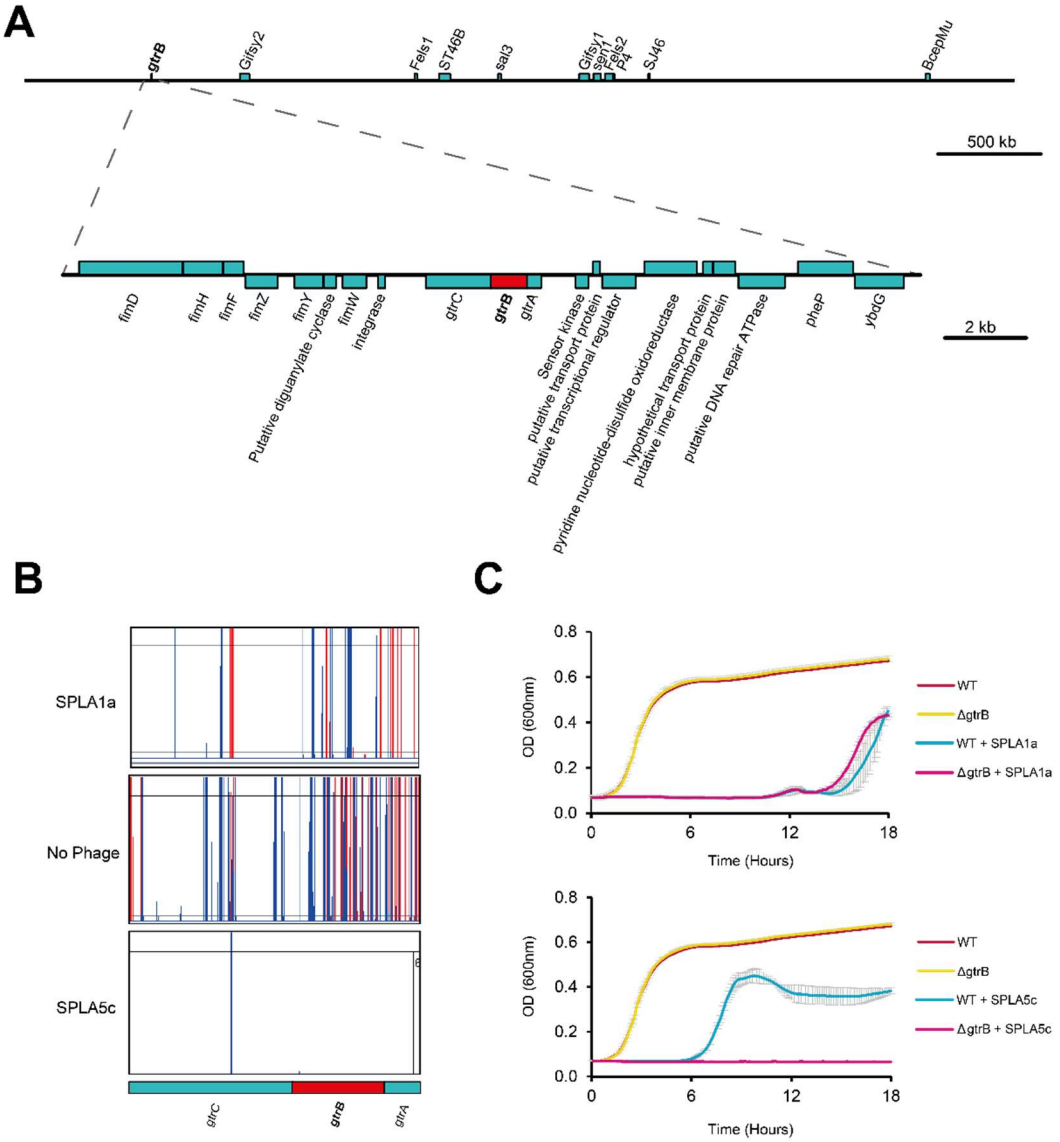


Figure 7. The role of *gtrB* in phage resistance to SPLA5c. A) Genomic context of *gtrABC* in *Salmonella* Typhimurium strain ST4/74. B) Transposon insertion sites mapped to *Salmonella* Typhimurium 4/74 in the *gtrABC* operon in the presence of SPLA1a, SPLA5c and a non-phage treated control. C) Growth of WT and ΔgtrB strains in the presence and absence of SPLA1a (top) and SPLA5c (bottom).

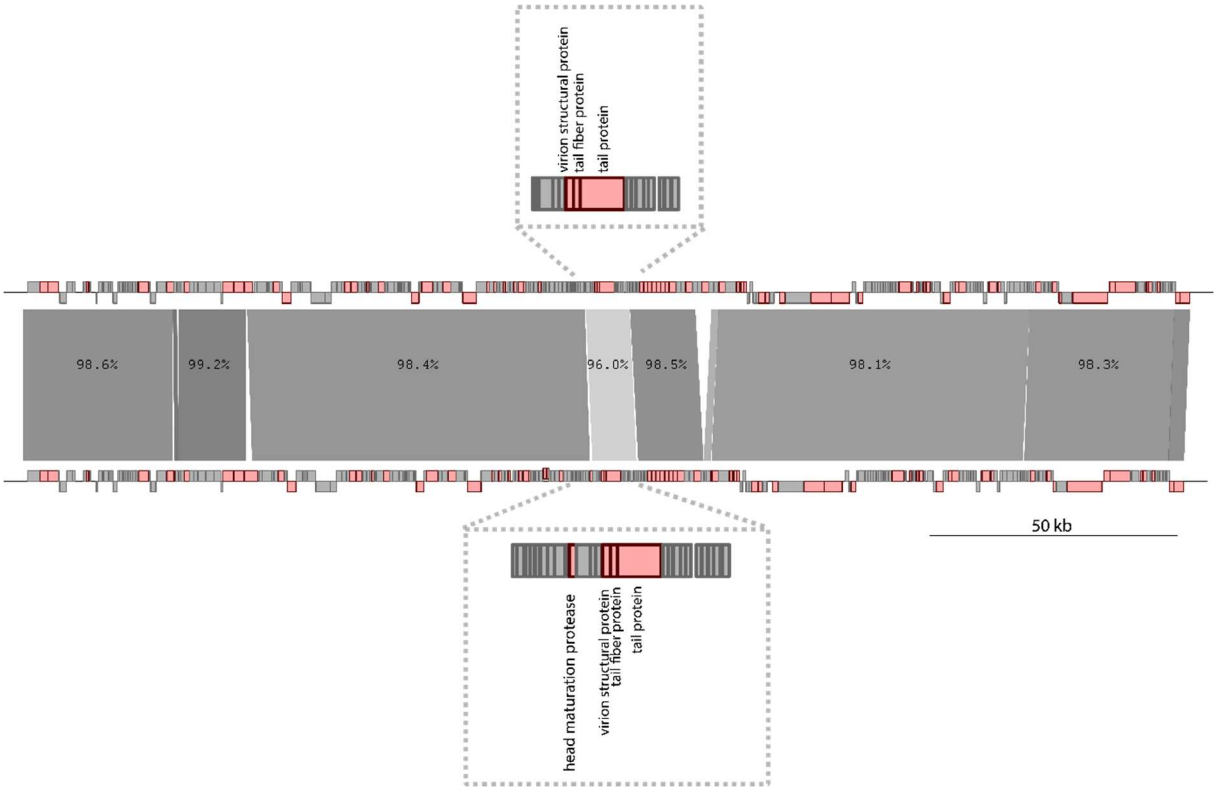


Figure 8. Whole genome alignment of *Seoulviruses* SPLA1a and SPLA5c. Hypothetical proteins are shown in grey. Annotated proteins shown in red. Average nucleotide identity (ANI) displayed as percentages.

4.2.8. TraDIS predicts collateral sensititvity of SPLA phage combinations

Collateral sensitivity is an evolutionary trade off where resistance to one antimicrobal agent results in increased sensitivity to another. We observed that many of the same LPS biosynthesis genes played contrasting roles during infection by SPLA1a or SPLA5b, that is, LPS biosynthesis genes were required for infection by SPLA1a, but acted as resistance genes for infection by SPLA5b (Figure 4). To investigate the effect of loss of LPS biosynthesis genes on the virulence of SPLA1a and SPLA5b, *rfaL, rfaK* or *btuB* were deleted in otherwise isogenic strains of *Salmonella* Typhimurium strain ST4/74 (Figure 9). Both bacteriophages formed plaques on the wildtype strain however SPLA1a plaques were far clearer compared to SPLA5b which was consistent with the much higher LAS score by SPLA1a. Deletion of the

O-antigen ligase, *rfaL*, resulted in complete resistance to SPLA1a (as observed previously) consistent with O-antigen being the receptor for this phage. In contrast, SPLA5b was still able to infect the *rfaL* mutant with plaques which were far less turbid than wild-type indicating increased virulence (Figure 10b). Loss of the gene encoding the vitamin B12 transporter, *btuB*, resulted in resistance to SPLA5b, indicated a loss of the ability to form plaques. But had no effect on SPLA1a infection consistent with BtuB being the receptor for SPLA5b. A summary of the different LPS requirements of SPLA1a and SPLA5b is shown in Figure 9

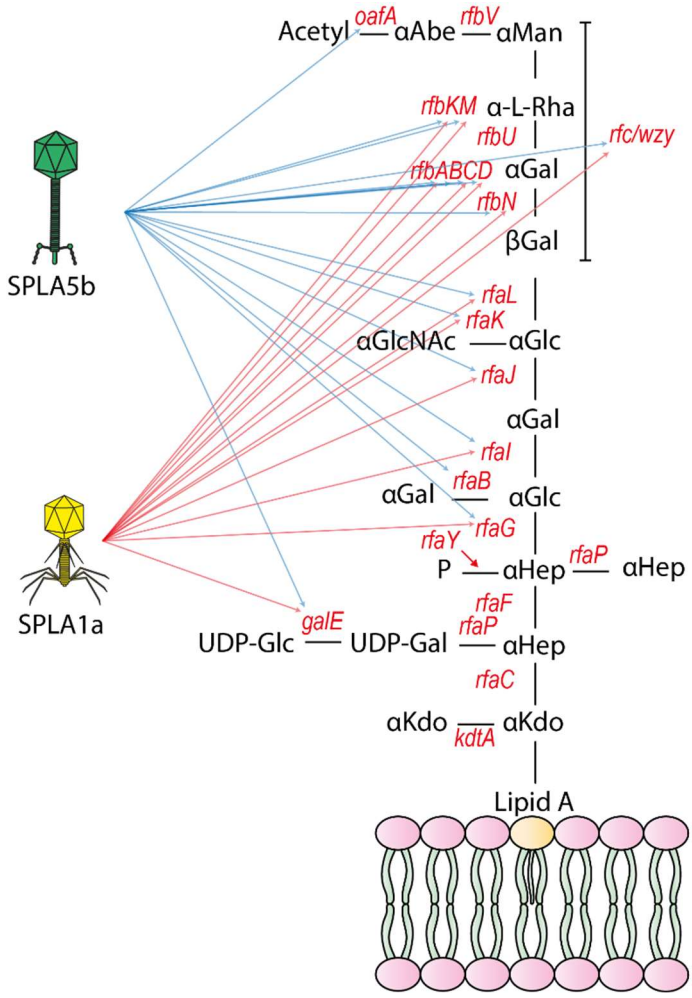


Figure 9. LPS synthesis gene requirements of SPLA1a and SPLa5b contributing to a collateral sensitivity mechanism. Genes which enhance sensitivity (red arrows) and genes which contribute to resistance (blue arrows) to SPLA1a (Yellow phage) and SPLA5b (Green phage).

To further investigate the impact of loss of LPS on the increased susceptibility to SPLA5b, ST4/74 in which the *rfaK gene,* encoding a hexose transferase involved in the synthesis of LPS, had been deleted was grown in broth culture in the presence of either SPLA1a or SPLA5b. In the presence of SPLA1a, no growth of wild-type ST4/74 was obsererved. However deletion of *rfaK* resulted in the ability to grow in the presence of SPLA1a. In contrast, under SPLA5b selection an extended lag phase was observed agaisnt wild-type ST4/74, however deletion of *rfaK* led to increased phage infection compare to wild-type which was also comparable to the infection observed for SPLA1a against wild-type ST4/74.

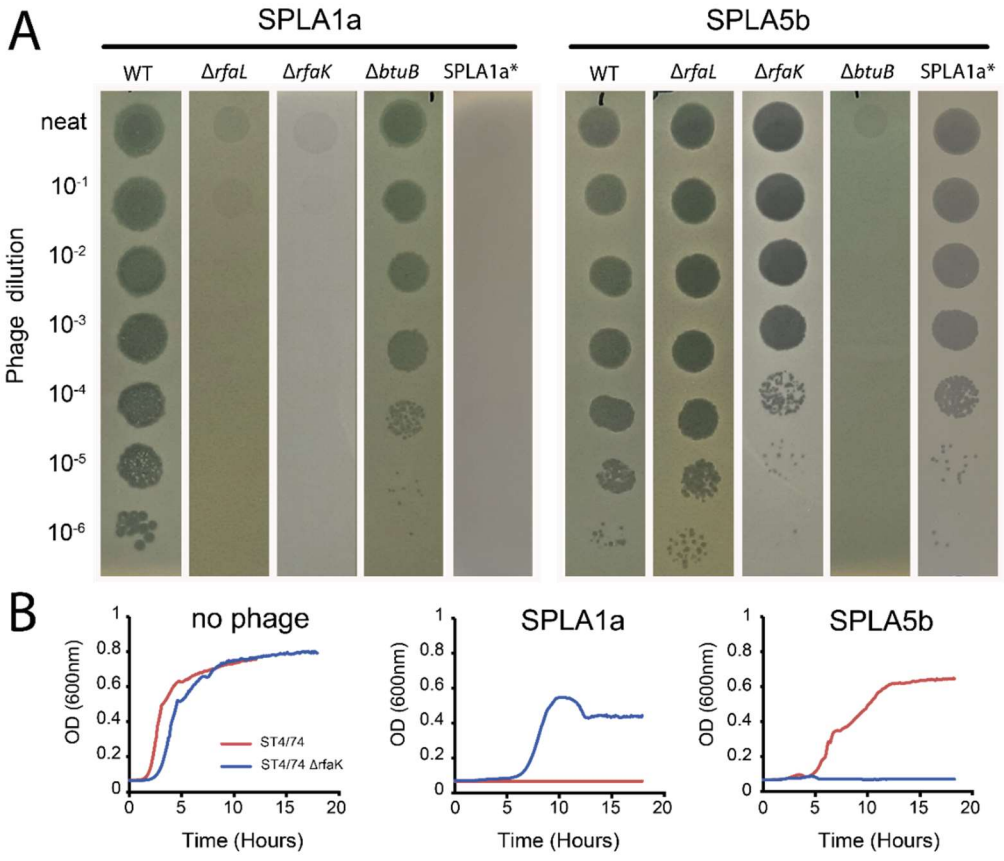


Figure 10. Collateral sensitivity dynamic of bacteriophages SPLA1a and SPLA5b. A) Plaque assays using SPLA1a (left) and SPLA5b (Right) against LPS and btuB deletion mutants in *Salmonella* Typhimurium ST4/74. B) Growth Curve analysis of wild-type (red) and *ΔrfaK* (blue) in the presence of no phages (left) SPLA1a (middle) and SPLA5b (right)

4.2.9. Collateral sensitivity leads to more effective bacteriophage cocktail design

To investigate whether a collateral sensitivity may occur when the phages were used together, a naturally occurring resistance mutant was generated by infecting *Salmonella* typhimurium ST4/74 with SPLA1a and recovering a resistant mutant that occurred at low frequency, in approximately 10% of cultures (10/96). This strain was termed *Salmonella* Typhimurium ST4/74 SPLA1a*. Sequencing and single nucleotide polymorphism (SNP) analysis revealed a single base insertion in the *pgm* which resulted in a frame shift and a predicted inactivation of this gene. (Figure 11). The *pgm gene encodes for a phosphoglucomutase,* which is involved in glycolysis as well as synthesis of precursors for LPS synthesis (West et al., 2000). This gene was also identified as a host susceptibility gene

in the TraDIS screen (Figure 4) This mutant showed a similar phenotype to the isogenic *rfaL* and *rfaK* mutant (Figure 10)

In contrast to SPLA1a, infection of ST4/74 with SPLA5b resulted in bacterial growth in 100% of cultures (96/96), although growth was associated with an extend lag phase and lower

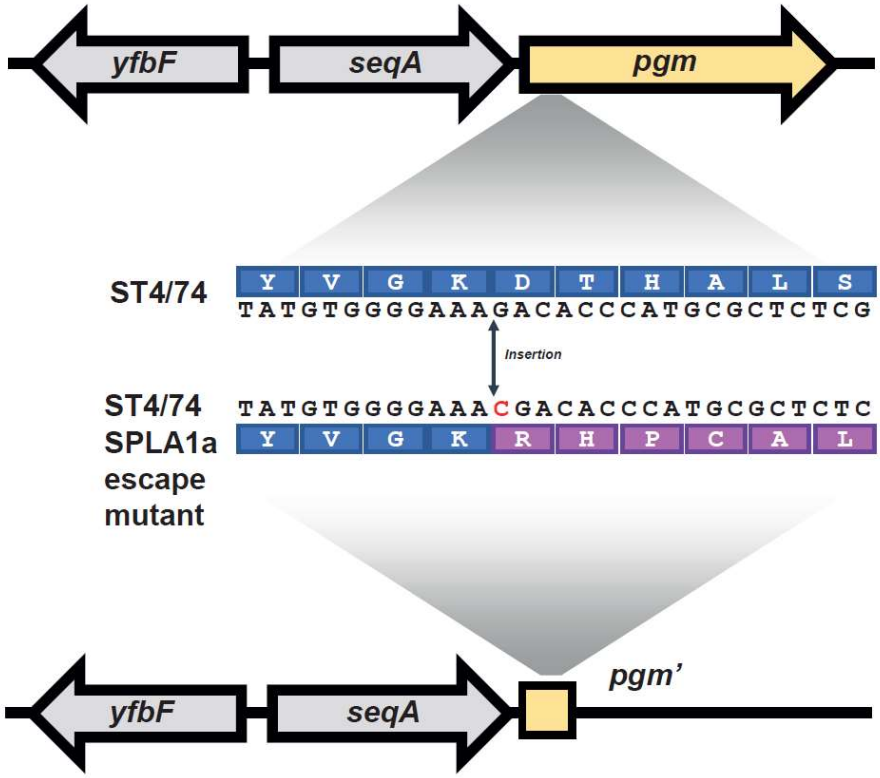


Figure 11. Schematic with sequence alignment of *Salmonella* Typhimurium ST4/74 wild-type and naturally occurring SPLA1a mutant. Insertion of C base resulted in a frame shift mutation.

end point optical density compared with non-phage treated controls (Figure 12). To investigate whether collateral sensitivity may improve phage infection and result in reduced bacterial growth, SPLA1a and SPLA5b were administered to cultures together. This limited bacterial resistance to around 1% (1/96) and resulted in a 10-fold increase compared to treatment with SPLA1a alone.

It is predicted that resistance to SPLA1a, caused by alterations or deletions in the LPS phage receptor would lead to increased resistance to SPLA5b. Therefore, these phages exhibit a collateral sensitivity dynamic, where resistance to once phage leads to increased sensitivity to another. Wild-type strains are infected well by SPLA1a, but decreased infectivity by SPLA5b is caused by masking of the btuB receptor by LPS. Mutations in LPS, such as Δ*rfaL*, lead to resistance to SPLA1a due to deletion of the phage receptor and increased sensitivity to SPLA5b as the BtuB receptor is no longer masked by LPS.

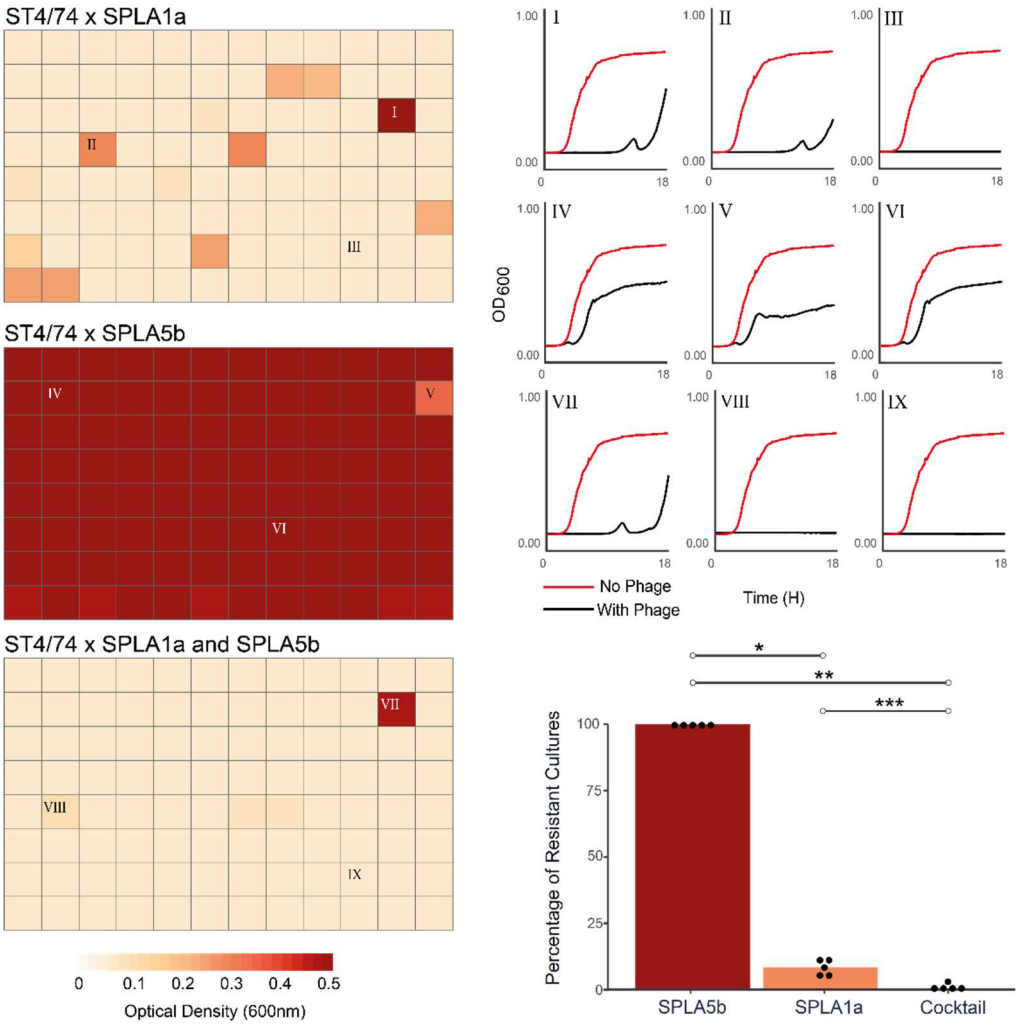


Figure 12. Growth of *Salmonella* Typhimurium strain ST4/74 in the presence of SPLA1a and SPLA5b in monotherapy and combination therapy. A) Heatmap of endpoint optical density (OD) at 600nm after 18 hours of treatment with SPLA1a (top) SPLA5b (middle) and both phages (bottom). B) Example growth curves of *Salmonella* Typhimurium ST4/74 infected by SPLA1a (I-III), SPLA5b (IV-VI) and both phages (VII-IX). C) Percentage of resistance observed across five independent biological replicates

4.3. Discussion

Two of the main challenges for the effective deployment of phage-based therapeutics is the rapid emergence of resistance within bacterial populations and their host specificity. Although highly host specific phages are useful as they avoid off target effects, it makes the development of broadly applicable phage-based products difficult. An effective solution to both challenges is the use of phage cocktails – combinations of phages used together. This approach often reduces the emergence of resistance as multiple mutations are required to occur simultaneously in order to resist the cocktail components if there is no overlap in their mechanisms of action. However, identifying host genes involved in phage infection is difficult and it is not commonplace in traditional phage cocktail design. To this end, we employ the use of functional genomics to better understand the interactions between selected SPLA phages and a bacterial host. Using this information, we differentiate closely related phages for their use in phage cocktails and formulate a combination of phages which is more effective and limits bacterial resistance better than single phage preparations.

Transposon mutagenesis combined with high throughput sequencing is an effective method to assay they function of all genes within bacteria simultaneously. Each transposon mutagenesis approach has successfully been applied to identify essential genes within different bacteria. In addition to the identification of essential genes, the same approaches have also been applied to determine genes involved under selection such as antibiotic treatment, stress response and bile tolerance (Yasir et al., 2020, Holden et al., 2021). TraDIS has previously been used to identify host genes involved in infection by model phages such as T4, λ, T2, T6 and T7 phages (Kortright et al., 2020, Cowley et al., 2018). Additionally, it has been used to identify Vi capsular antigens and genetic regulators involved in *Salmonella*

Typhi infection (Pickard et al., 2013). Crucial to experiments is the optimisation of the selection pressure. Too much selection leads to excess loss of mutants within the population and too little selection does not produce an observable effect on insertion frequency. This is easily achieved when selecting with traditional antibiotics as the concentration can be varied around the MIC to achieve the desired power of selection. When using bacteriophages, this is more challenging as varying the concentration is only effective initially as phages multiply upon successful infection. Therefore, it is likely factors such as latent period and burst size have effects on selection pressure. Highly lytic phages such as SPLA1a, are likely to require a different multiplicity of infection (MOI) compared to less virulent phages.

Interestingly, SPLA2 did not produce individual plaques when infecting the parent TraDIS library strain, *Salmonella enterica* sv. Typhimurium strain ST4/74 (Figure 2). Instead, this phage only appeared to inhibit growth at the highest concentration tested, consistent with lack of infectivity. However, this phage was able to successfully inhibit the growth when used in liquid culture. The functional genomic screen revealed genes involved in cellulose synthesis, such as *yhjU, yhjL, yhjN, yhjQ, yhjR* and *yhjS*, as susceptibility genes for SPLA2 infection. Cellulose is a cellular matrix component of bacterial biofilms (Serra and Hengge, 2019). Interestingly, when grown at 37 ^{o}C, in conditions consistent with the plaque assay utilised here, cellulose is poorly expressed and instead optimal cellulose production is achieved at temperatures between 15^{o}C and 25^{o}C (Kim et al., 2022). This could mean that this phage would become more virulent at lower temperatures, due to additional expression of the receptor. Therefore, SPLA2 could be a useful phage for applications to target biofilms in food production environments.

The transposon mutant screen combined with TraDIS was successfully applied to identify host genes involved in infection by six SPLA bacteriophages (Figure 4). Amongst the highest hits were candidate receptor genes. This is likely because loss of function leads to a dramatic reduction in phage infection and therefore a higher log fold change in insertions. Three different receptors were identified which were LPS for SPLA1a, SPLA1b and SPLA5c infection, BtuB for SPLA5b infection and cellulose for SPLA2 and SPLA11. To confirm the impact of inactivation of receptor genes on phage infection, candidate genes were replaced with the *aph* gene in an otherwise isogenic background. Mutants of candidate susceptibility genes should lead to increased phage resistance and mutants of candidate resistance genes should result in increased phage susceptibility. Despite SPLA1a, SPLA1b and SPLA5c being predicted to target LPS, its clear that these phages have alternative targets within the LPS structure. Inactivation of *rfaL*, the O-antigen ligase, lead to complete resistance to SPLA1a and SPLA1b suggesting that intact O-antigen is a requirement for infection and could be a primary receptor for this phage. Conversely, despite their high genomic similarity, inactivation of *rfaL* only resulted in partial resistance to SPLA5c, suggesting that O-antigen is beneficial to infection by this phage, but the phage is able to retain a lower level of virulence without it (Table 1). Whilst LPS is the likely primary receptor for each of these phages, it is not clear which genes may be acting as a secondary receptor as the TraDIS screen did not highlight a potential candidate. Unlike P22, which also targets LPS, but degrades the O-antigen in a sequential manner to bring itself into closer proximity to the bacterial cell, neither phage encodes enzymatic domains within its tail fibre. Interestingly, inactivation of *rfbA,* encoding a glucose-1-phosphate thmidylyltransferase involved in the biosynthesis of the O-antigen monomers resulted in complete resistance to SPLA1a, SPLA1b, and SPLA5c despite a predicted phenotype similar to *rfaL* inactivation. In addition,

inactivation of *galE*, a UDP-glucose 4-epimerase, lead to complete resistance to SPLA1b but only partial resistance to SPLA1a, further highlighting alternative targets of LPS targeting SPLA phages.

BcsA is a cellulose synthase and is a key player in the formation of biofilms (Harrell et al., 2021). Inactivation of *bcsA* resulted in complete resistance to Rosemountviruses, SPLA2, SPLA9, SPLA11 and SPLA12, suggesting that cellulose is required for infection. Interestingly, previous studies have shown that bcsA mutants are not attenuated and instead exhibit increased intracellular proliferation within macrophages and increased colonisation of the chicken spleen (El Hag et al., 2017). Additionally, loss of *bcsA* has been shown to increase association and uptake by *Acanthamoeba* which could contribute to environmental persistence (Gill et al., 2018). These considerations should be taken when considering these phages for therapeutic or veterinary interventions as treatment with these phages could select for strains with increased virulence and persistence. LPS targeting phages would make better candidates as mutations in LPS biosynthesis, such as those that could arise from treatment with LPS targeting SPLA phages, have been shown to lead to decreased virulence (Kong et al., 2011). Therefore, resistant mutants to this phage would not contribute to the emergence of more virulent strains.

The application of TraDIS to the phage selected mutant libraries also identified candidate resistance genes, although these were much less common than susceptibility genes. This is likely due to bacteriophages being able to infect the library strain efficiently and therefore it's unlikely that efficient resistance mechanisms for these phages are present. Nonetheless, putative resistance genes included *barA* for SPLA2 infection and *oxyR*, *rumA*,

nfi and *gtrB* for SPLA5c infection. However, the plaque assay was not able to confirm either *oxyR* or *rumA* as resistance genes for SPLA5c infection since their inactivation slightly reduced sensitivity to SPLA5c phage. This could be due the plaque assay treatment being a different assay to the TraDIS selection with increased exposure time, 18 hours, compared to 3 hours as well as being in solid media compared to liquid medium.

Comparison of host resistance and susceptibility genes across each SPLA phage did not identify a master regulator involved in phage infection which is likely due to genetic diversity of SPLA phages used (Figure 5). However, genes involved in LPS infection were shared between multiple phages as susceptibility genes for SPLA1a, SPLA1b and SPLA5c as well as resistance genes for SPLA5c. Since overlap in TraDIS hits, particularly between phages which are not using the same receptor are limited, these phages make suitable cocktail candidates as resistance to multiple phages is less likely due to the need for multiple mutations in different genes being required.

Phage cocktail selection should be based on a robust understanding of the infection process of the bacteriophage including the host genes involved in phage infection. Ideal candidate phages for applications should have limited resistance genes. However here we demonstrate that even very closely related bacteriophages can interact with their host differently. Both SPLA1a and SPLA5c are Seoulviruses which target components of the LPS, however, it is clear that the exact target is different due to differences in their TraDIS gene hits (Figure 6). In particular, *gtrB*, encoding a glucosyltransferase, was identified as a resistance gene for SPLA5c but not SPLA1a. *GtrB* was confirmed to provide resistance to SPLA5c since its inactivation lead to increased susceptibility to SPLA5c which resulted in

infection dynamics similar to SPLA1a infection (Figure 7). The mechanism of resistance is likely due to *gtr* operons generating changes in LPS structure which affect SPLA5c binding, but not SPLA1a. Therefore SPLA1a would make a better cocktail candidate as *gtr* operons are common in *Salmonella* and therefore resistance to SPLA5c would limit effectiveness (Davies et al., 2013).

Crucial to phage cocktail design is to avoid phages which work antagonistically together and instead use phages which work synergistically. Collateral sensitivity is a phenomenon observed commonly with antibiotic treatment where resistance to one antimicrobial results in increased resistance to second antimicrobial (Hall et al., 2009) . For example, resistance to aminoglycosides is often associated with increased susceptibility to beta lactams (Lázár et al., 2013). Additionally, bacteriophage treatment has been shown to increase susceptibility to antibiotics (Koderi Valappil et al., 2021). Of particular interest for the rationale design of phage cocktails was the observation that LPS biosynthesis genes acted as susceptibility factors for SPLA1a infection but contributed to resistance for SPLA5b infection. This was confirmed using isogenic mutant strains with *rfaL* and *rfaK* replaced with an *aph* gene. In this case, inactivation of LPS biosynthesis genes resulted in resistance to SPLA1a but increased susceptibility to SPLA5b. Natural occurring mutations which confer resistance when exposed to SPLA1a are likely to occur in TraDIS gene hits such as the mutation observed in pgm (Figure 11). When used together, we demonstrate that SPLA1a when used in combination with SPLA5b resulted in a 10 fold increase in effectiveness suggesting a collateral sensitivity dynamic when used as a cocktail. This highlights that SPLA5b may be an ideal cocktail candidate, despite not initially appearing useful due to limited infectivity (Chapter 4).

One of the greatest challenges to the successful application of bacteriophage-based therapies is the rapid emergence of bacterial resistance which would limit effectiveness of applications. Here we utilize a functional genomic approach to distinguish between phages which would be useful for downstream applications and those which would not be appropriate. Using this method, we distinguish between closely related phages, highlighting differences in their interactions with their hosts which impacts their effectiveness. Additionally, we show that particular phages can increase the effectiveness of others through a collateral sensitivity dynamic resulting in a more effective phage cocktail.

5. Application of *Salmonella* Bacteriophages in Food

5.1. Introduction to Chapter Six

Bacteriophages are a promising alternative to traditional antimicrobials for the control of pathogens within the food chain. Bacteriophages have been shown to be effective in reducing the prevalence of bacterial pathogens when directly applied to food products (Sukumaran et al., 2015, Anany et al., 2011). An important consideration for this phage application is the interaction between the bacteriophage and a food matrix as well as members of the food microbiota. Phages which are effective in liquid monoculture may not be as effective when other non-susceptible bacteria or food matrices are present. Nonetheless, an attractive trait of bacteriophage treatment is their specificity. In contrast to traditional antimicrobials, phages commonly only infect a small range of related bacterial strains or species whilst not directly affecting other members of the microbial community. This is of particular benefit to foods which contain a beneficial microbiota, such as fermented foods, as pathogens can be removed whilst leaving other bacteria unaffected.

The shift in consumer attitudes toward minimally processed food and away from the use of artificial preservatives, and regulations that control the use of antibiotics in animal husbandry has driven the search for natural alternatives to prevent the presence of harmful bacteria within food production. Phages are a potentially viable alternative; however public perception of bacteriophages remains a challenge and a potential barrier to their use (McCammon et al., 2023). The addition of viruses into food may not be well received by consumers, despite bacteriophages likely being highly abundant in food (Mahony and van Sinderen, 2022). To this end, many commercially available phage preparations specifically designed for use in food and feed processing have entered the market (Soffer et al., 2016, Yang et al., 2017a).

This chapteraimed to determine whether bacteriophages can prevent detection of *Salmonella* when directly applied to raw pet food products and whether the presence of phages will cause shifts in other members of the microbial community. In this chapter we investigated the viral communities present within food products using viromics and aimed to observe changes in abundance of SPLA phages consistent with phage infection.

5.2. Results

5.2.1. Determination of the limit of detection for *Salmonella* in Food Samples

The detection of pathogens within food samples requires a highly sensitive and accurate method. An ideal method should be able to detect the presence of 1 CFU present within the original 25g sample. Therefore, in order to establish a highly sensitive protocol for the detection of *Salmonella* within raw pet food samples, an adaption of the ISO accredited method for *Salmonella* isolation from food samples (ISO 6579:2002) was developed. In particular, serotyping for *Salmonella* identification was replaced by PCR (see methods section, 2.4). The rationale for this adaption was to improve sensitivity as well as throughput. Therefore, to determine the limit of detection for *Salmonella* in pet food products using this method, raw tripe samples were spiked with *Salmonella* Typhimurium strain SL1344 at varying concentrations prior to culture based isolation. Spiking was performed by pipetting adjusted *Salmonella* culture evenly over samples and incubating samples at room temperature for 30 minutes. Colony PCR was performed on selected colonies whose phenotype matched the expected appearance of *Salmonella* on selective plates. Amplification of a 440bp region of the 16S gene, as a positive control, and a 796bp region of the *InvA* gene suggested a positive *Salmonella* isolation (Figure 1). No doublet band was observed in samples spiked with *Salmonella* at a concentration of 0.1 CFU, and therefore the sample was deemed not to contain *Salmonella* (Lanes 1-3). However,

Salmonella was recovered from samples spiked with 1, 10 and 100 CFU. This suggests that the limit of detection using this protocol is between 0.1 and 1 CFU of *Salmonella* per 25 g of food sample.

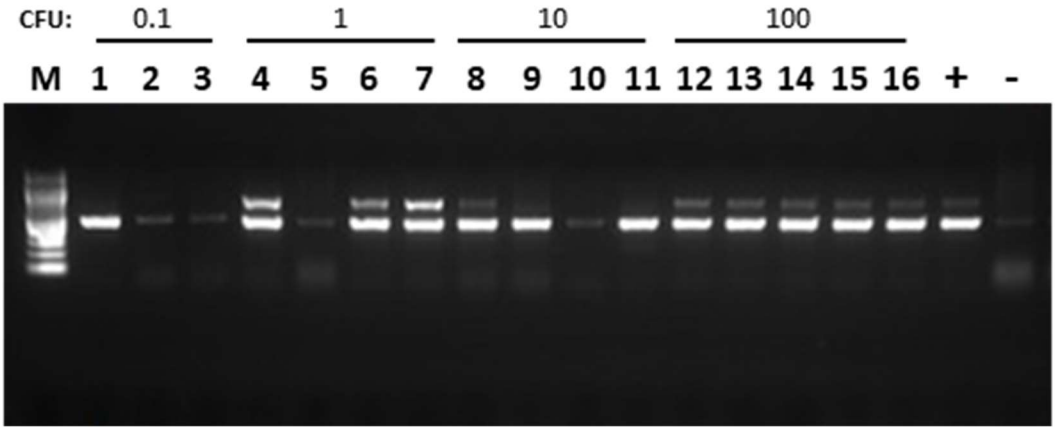


Figure 1 – Agarose gel electrophoresis of PCR products obtained from colony PCR of candidate *Salmonella* colonies using primers InvA-FW/RV and PB146/PB147. Lane M is 100bp ladder. Lanes 1-3 are colonies from samples spiked with 0.1 CFU. Lanes 4-7 are colonies from samples spiked with 1 CFU. Lanes 8-11 are colonies from samples spiked with 10 CFU. Lanes 12-16 are colonies from samples spiked with 100 CFU. Positive control (+) is *Salmonella* Typhimurium SL1344.

5.2.2. Optimisation of *Salmonella* and bacteriophage concentrations

In order to establish the optimal concentrations of *Salmonella* and bacteriophages to kill *Salmonella* in food samples and reduce the level of detection by culture-based enrichment, 10^3 and 10^5 CFU of *Salmonella* was spiked into 25 g pet food samples which were all part of the same product. The penta-phage cocktail (described in Chapter 3) was applied across the surface of spiked food samples at 10, 10^3, 10^5 and 10^7 PFU. *Salmonella* was not detected in the non-spiked negative control regardless of addition of phages (Table 1). *Salmonella* was recovered from all samples spiked with 10^3 CFU with the exception of a sample treated with 10^7 PFU of phage cocktail where *Salmonella* was unable to be detected. Notably, *Salmonella* was not detected in a sample spiked with 10^5 CFU of *Salmonella* and 10 or 10^7 PFU of phages. Since *Salmonella* could not be recovered in either

Salmonella spiked sample following the addition of 10^7 PFU of phages – 10^7 PFU and 10^3 CFU were chosen as optimal concentrations for further experiments.

Table 1 – Summary of detection of *Salmonella* in spiked food samples following the addition of a penta-phage cocktail at varying concentrations. Positive isolation (+) and negative isolation (-)

Salmonella (CFU)	Number of Phages (PFU)				
	Phage Buffer	10	10^3	10^5	10^7
Control	-	-	-	-	-
10^3	+	+	+	+	-
10^5	+	-	+	+	-

5.2.3. Application of the penta-phage cocktail lowers the frequency of *Salmonella* detection.

In order to assess the frequency of *Salmonella* detection in penta-phage treated raw petfood samples, twenty replicates of food samples spiked with 10^3 CFU *Salmonella enterica* ST4/74 and treated with 10^7 PFU phages. To allow the distinction between the isolation of the ST4/74 and other *Salmonella* potentially present in food samples, the strain was tagged with a kanamycin resistance selectable marker, *aphII*, in an intergenic region which does not affect gene expression of neighbouring genes (Kirkwood et al., 2021). Candidate *Salmonella* isolates were tested for kanamycin resistance by plating onto LB supplemented with kanamycin. In total, 145 candidate colonies were tested across forty samples and confirmation of positive *Salmonella* isolation was performed by PCR. In non-phage treated controls, *Salmonella* was recovered in all twenty samples (Figure 2). All *Salmonella* isolates were resistant to kanamycin suggesting recovery of the spiked strain from food samples. In samples treated with 10^7 PFU of the penta phage cocktail, only four samples tested positive for *Salmonella*, only three of which were kanamycin resistant.

Whole genome sequencing and serotype prediction revealed that the remaining isolate was *S*. Bovismorbificans, a serotype not used to isolate SPLA phages or test their host range (Chapter 4).

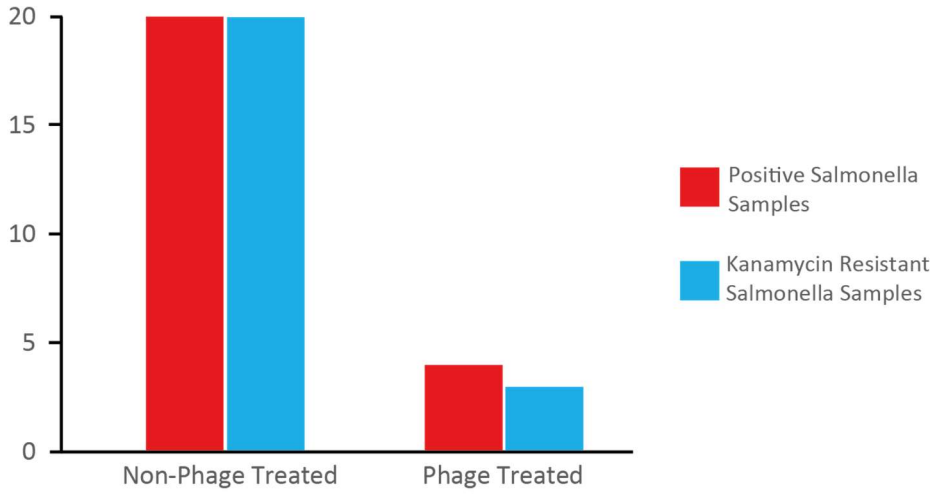


Figure 2 – Summary of detection of *Salmonella* in non-phage and phage treated spiked raw tripe samples. Red bars indicate the number of samples which *Salmonella* was detected (n=20). Blue bars indicate the number of samples where kanamycin resistant *Salmonella* was detected.

5.2.4. Bacterial metagenomics of phage treated enrichment cultures

The reduction in *Salmonella* detection in phage treated samples raised the question of how phage treatment affects *Salmonella* during cultured based enrichment, as well as effects on other bacteria present within food samples which are enriched during this process. To this end, we carried out shotgun metagenome sequencing of total DNA isolated from buffered peptone water enrichment cultures of phage and *Salmonella* spiked food samples (see methods section, 2.20) . Four experimental conditions were used; food spiked with *Salmonella* with or without addition of phage and food with or without addition of phage, each with five independent biological replicates. This allowed us to determine the effect of phage treatment on the microbiome and virome both when *Salmonella* was present and absence of *Salmonella*.

5.2.4.1. *Escherichia* and *Comamonas* are the dominant genera in enrichment cultures

To determine taxonomic assignment of microbial communities, the marker-based profiling tool MetaPhlAn was used on for all samples (Figure 3). This tool assigns taxonomy to shotgun metagenome reads based on a database of ~5.1 million clade specific marker genes. At the genera level, *Escherichia* was most abundant across all samples and groups with an average relative abundance of 33.5%. *Comamonas* was also very common in all samples with an average abundance of 32.1% across all samples. Both of these genera were likely highly abundant within the cow guts from which the samples originated. In samples spiked with *Salmonella* only, the average relative abundance of *Salmonella* was 5.1%. The addition of phages to samples spiked with *Salmonella* resulted in a decrease in average relative abundance to 0.003% consistent with non-spiked control groups.

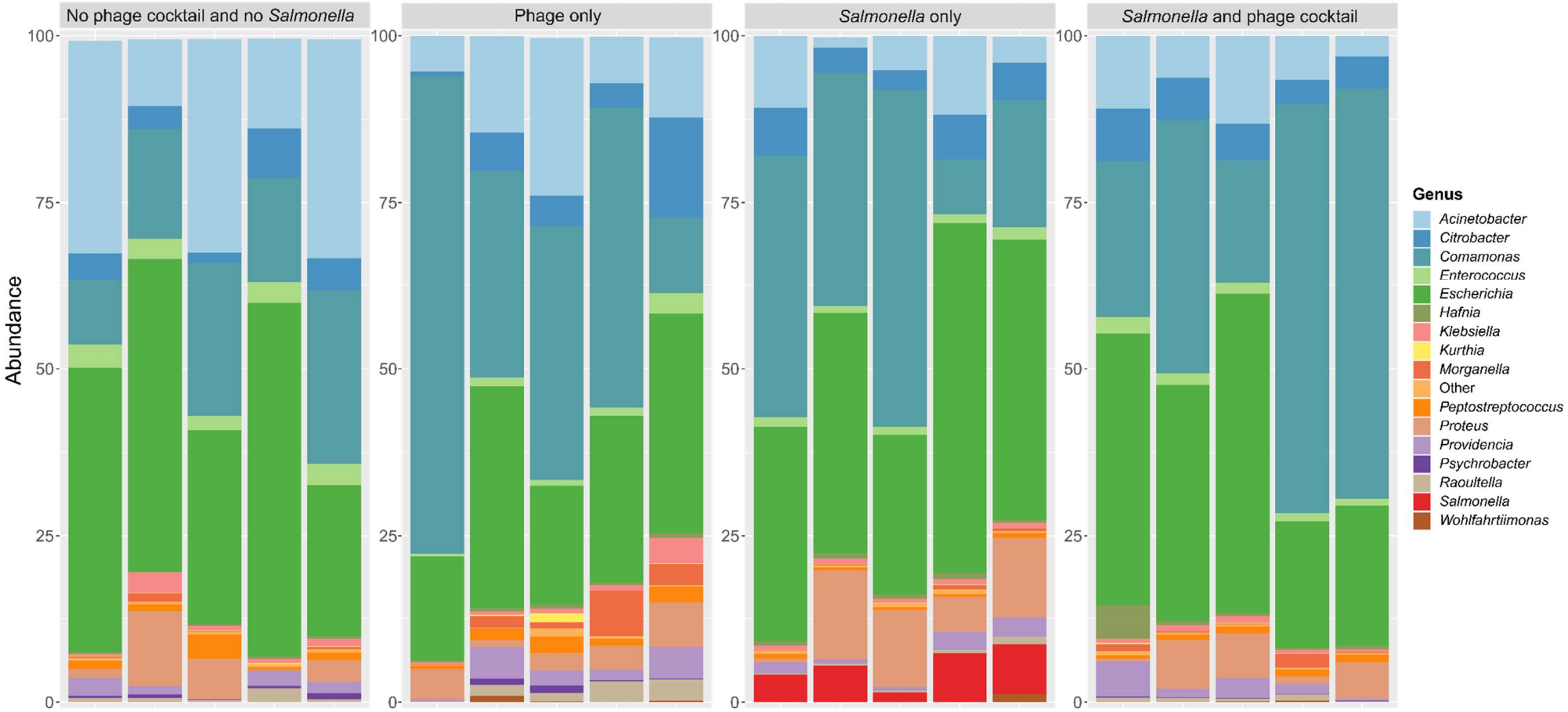
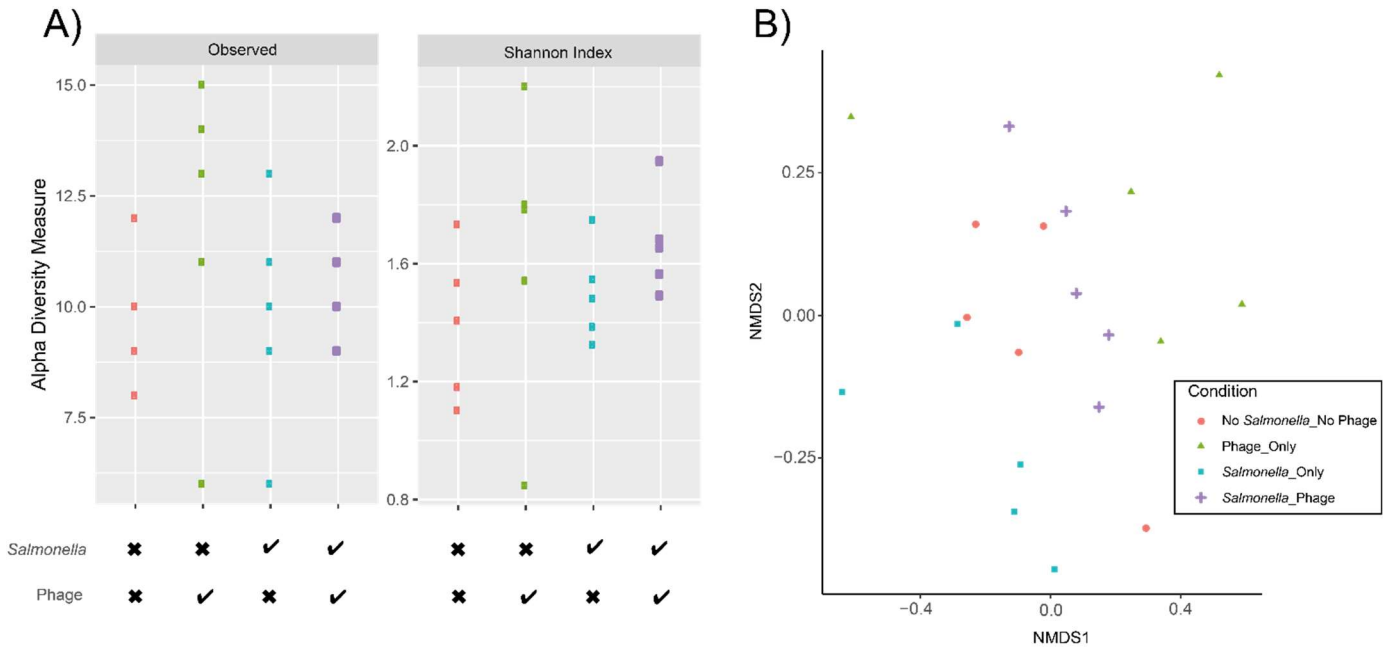


Figure 3 – Relative abundance of bacterial genera within enrichment samples of raw tripe spiked with *Salmonella* and/or bacteriophages. Taxonomic assignment of whole genome shotgun reads assigned by MetaPhlAn. Low abundance (>0.1%) are defined as Other (pale orange). *Salmonella* abundance presented in Red.

5.2.4.2. Bacterial alpha and beta diversity

In order to assess the diversity within each sample (alpha diversity), observed species diversity (richness) and Shannon index were calculated for all samples (Figure 4A). Following filtering of species whose total relative abundance, across all samples was below 0.5, 24 different bacterial species were detected, mainly consisting of member of the phyla *Proteobacteria* and *Firmicutes*. The observed number of species in each sample varied from 6 to 15. Shannon-Weiner diversity index (SI) values appeared to be slightly elevated in samples treated with only phages however SI remained low, with only one sample above 2, indicative of low diversity and/or an unequal distribution of abundance. To compare the overall effect of the addition of *Salmonella* and bacteriophages to food samples prior to enrichment, Bray-Curtis dissimilarity values were calculated and plotted using a nonmetric multidimensional scaling ordination (NMDS) (Figure 4B). Samples spiked with *Salmonella* without addition of bacteriophages (blue squares) appeared to cluster independently from other samples suggesting a distinct bacterial population compared to other samples. The use of a permutational multivariate ANOVA (PERMANOVA) revealed that this was not statistically significant, and the addition of Salmonella alone did not significantly affect diversity observed compared to other conditions (Figure 4C). The PERMANOVA did highlight a significant difference in beta diversity between samples spiked with Salmonella and phage compared to samples spiked with phage only, however this suggested a small effect size (R^2 value) and low significance (P-value) .



C)

Conditions	F	R^2	P
Salmonella and Phage (✚) vs No *Salmonella* No Phage (●)	2.2	0.22	0.113
Salmonella and Phage (✚) vs Phage only (▲)	2.7	0.25	0.046 *
Salmonella and Phage (✚) vs *Salmonella* only (■)	2.1	0.21	0.095
No *Salmonella* No Phage (●) vs Phage only (▲)	0.9	0.01	0.393
No *Salmonella* No Phage (●) vs *Salmonella* only (■)	1.1	0.12	0.323
Phage only (▲) vs *Salmonella* only (■)	2.2	0.22	0.123

Figure 4 – Bacterial alpha and beta diversity within raw tripe samples in enrichment cultures with bacteriophage treatment. A) Alpha diversity presented as observed diversity (left) and Shannon-Weiner diversity index right) for each sample condition. B) Beta diversity presented as Bray-Curtis dissimilarity values plotted using nonmetric multidimensional scaling (NMDS) ordination for each sample condition). C) Permutational multivariate ANOVA test of each condition

5.2.4.3. Bacteriophage treatment significantly reduces the abundance of *Salmonella* in food samples.

To test the hypothesis that phage treatment reduces the relative abundance of *Salmonella* within food samples, a Student t-test was used to compare the relative abundance of *Salmonella* in all test conditions (Figure 5a and b). Spiking samples with *Salmonella* dramatically increased the relative abundance of *Salmonella* within each sample compared to each other test condition (Figure 5a). The difference in relative abundance of *Salmonella* was significantly greater ($p<0.05$) in samples spiked with *Salmonella* only, compared to all other conditions (Figure 5a). It should be noted that *Salmonella* relative abundance was significantly higher when comparing samples treated with *Salmonella* and phages with control samples where no *Salmonella* was added (No *Salmonella* + no phage and phage only), this is likely due to *Salmonella* genomic DNA present from the lysis of spiked bacteria and overall relative abundance still remains very low.

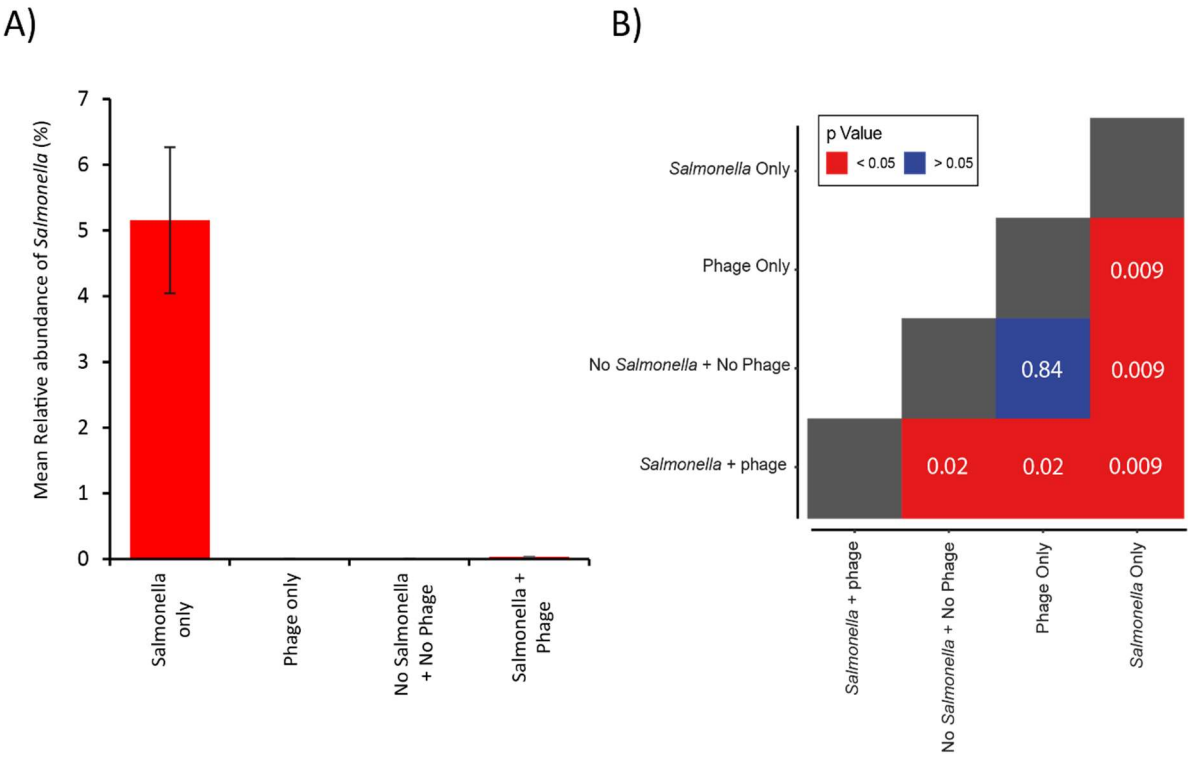


Figure 5 – Statistical comparison of relative abundance of Salmonella within raw tripe samples following addition of Salmonella and/or bacteriophages and enrichment culture. A) Mean relative abundance of Salmonella shotgun metagenomic reads present within cultures of each phage/*Salmonella* condition. Error bars represent standard error of the mean (SE) B) Matrix of statistical significance values (p-values) across five replicates calculated using a student t-test. No statistical significance ($p >0.05$) is presented in blue, statistical significance ($P < 0.05$) is presented in red.

5.2.4.4. Bacteriophage treatment does not significantly affect abundance of other bacterial species of the food microbiome.

To assess the effect of the addition of bacteriophages on relative abundance of other bacterial species found in food samples, a student t-test comparing each species present in phage only and no phage no *Salmonella* conditions was performed (Table 2). P values were corrected using Benjamini-Hochberg correction for multiple tests. No significant differences in the relative abundance of all 23 species were found. This suggested that the addition of bacteriophages to food samples alone did not change the relative abundance of each bacterial species.

Table 2 – Statistical comparison of relative abundance species present within raw tripe samples following addition of bacteriophages. Comparison is made across all five replicates. Difference in mean relative abundance is shown comparing no phage cocktail no Salmonella relative to phage only conditions.

	Difference in mean relative abundance	P Value
Acinetobacter baumannii	-0.315982	0.719
Acinetobacter lwoffii	1.329442	0.753
Acinetobacter variabilis	-5.459968	0.677
Citrobacter braakii	-0.300554	0.974
Citrobacter freundii	-0.003098	0.995
Comamonas kerstersii	1.108926	0.977
Enterococcus faecalis	0.278076	0.753
Enterococcus faecium	-0.016008	0.719
Escherichia coli	7.82688	0.677
Escherichia marmotae	0.086178	0.677
Hafnia paralvei	0.885732	0.677
Klebsiella pneumoniae	-0.561946	0.719
Kurthia gibsonii	-0.270254	0.677
Morganella morganii	-1.844142	0.677
Peptostreptococcus russellii	-0.706572	0.677
Proteus mirabilis	0.47509	0.954
Providencia alcalifaciens	-1.315392	0.677
Providencia rustigianii	0.874806	0.677
Providencia stuartii	0.02857	0.677
Psychrobacter sanguinis	-0.36671	0.677
Raoultella ornithinolytica	-1.29702	0.677
Vagococcus humatus	-0.106552	0.677
Wohlfahrtiimonas chitiniclastica	-0.177782	0.677

Since the addition of phage alone within did not significantly affect the relative abundance of other bacterial species, this raised the question of whether a similar affect would be observed when *Salmonella* was present in samples alongside phage treatment as potential amplification of phage may increase in phage concentration within the enrichment broth. Therefore, the analysis was repeated comparing the *Salmonella* only group, with the *Salmonella* and phage condition (Table 3). No significant differences in abundance of each species were detected, with the exception of *Enterococcus faecium* which significantly increased in relative abundance in *Salmonella* and phage spiked samples ($t = 17.1759$ $df = 4$, $SE = 0.025$). However relative abundance in both conditions remained very low, with *Enterococcus faecium* reads constituting less than 1% of total reads in each sample. Interestingly, the abundance of *Hafnia paralvei* was significantly lower in phage treated samples ($t = 4.0753$, $df = 4$, $SE = 0.122$), an effect only observed in *Salmonella* spiked samples. Previously, phage cocktail components were also shown to be slightly virulent against *Hafnia alvei* (Chapter 4).

Table 3 – Statistical comparison of relative abundance species present within raw tripe samples following addition of bacteriophages and *Salmonella*. Comparison is made across all five replicates. Difference in mean relative abundance is shown comparing *Salmonella* only relative to *Salmonella* and phage cocktail conditions.

	Difference in mean relative abundance	P Value
Acinetobacter baumannii	-8.977126	0.373
Acinetobacter lwoffii	-3.926106	0.598
Acinetobacter variabilis	-4.41907	0.373
Citrobacter braakii	0.625544	0.756
Citrobacter freundii	0.343746	0.316
Comamonas kerstersii	12.226244	0.373
Enterococcus faecalis	-0.883424	0.055
Enterococcus faecium	-0.431446	0.002
Escherichia coli	-1.539248	0.878
Escherichia marmotae	-0.043138	0.878
Hafnia paralvei	0.497876	0.034
Klebsiella pneumoniae	-0.317456	0.724
Kurthia gibsonii	-0.042586	0.190
Morganella morganii	-0.129568	0.756
Peptostreptococcus russellii	-0.953796	0.373
Proteus mirabilis	4.07916	0.452
Providencia alcalifaciens	0.089254	0.878
Providencia rustigianii	0.05385	0.912
Providencia stuartii	-0.108488	0.597
Psychrobacter sanguinis	-0.393348	0.180
Raoultella ornithinolytica	-0.306892	0.598
Vagococcus humatus	-0.05604	0.144
Wohlfahrtiimonas chitiniclastica	0.24539	0.598

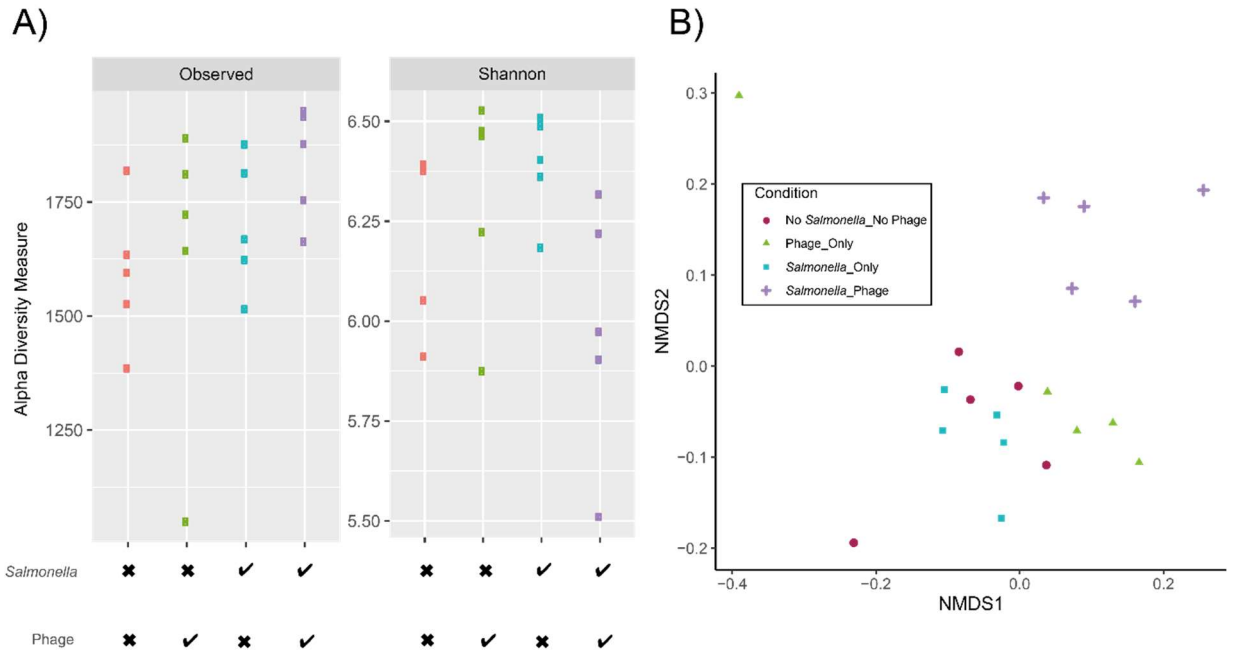
5.2.5. Viromics of food enrichment cultures

Metagenomic assembled genomes (MAGs) identified as viral were used to investigate the diversity of viruses, primarily bacteriophages, present within food samples and place them in the context of known viral genomes. Additionally, we also aimed to identify increases in abundance of bacteriophage cocktail components in enrichment cultures consistent with phage lytic activity.

5.2.5.1. Viral alpha and beta diversity

Across all samples, 3067 viral operational taxonomic units (vOTU) were assembled and passed quality filtering and dereplication. The filtered vOTUs ranged in size from 1500 bp

to 262,183 bp and had a mean length of 7921 bp. In order to understand the diversity within samples, alpha diversity metrics were calculated (Figure 6a). Observed diversity values corresponding to the number of vOTUs present in each sample were high in each condition. The addition of *Salmonella* or bacteriophages did not appear to lead to a difference in the number of vOTUs present. The Shannon Index for each condition was also above 5.8 for all samples suggesting high diversity, likely due to high species richness. Beta diversity was assessed using an NMDS plot of Bray Curtis dissimilarity values (Figure 6b). Samples where *Salmonella* and phage were added (Purple cross) appeared to cluster independently from all other samples suggesting a distinct viral population community structure from other samples. The use of a PERMANOVA revealed a significant shift in the virome of samples treated with Salmonella and bacteriophages (purple cross) compared to all other conditions (Figure 6c).



C)

Conditions	F	R^2	P
Salmonella and Phage (✚) vs No *Salmonella* No Phage (●)	4.8	0.38	0.013 *
Salmonella and Phage (✚) vs Phage only (▲)	4.9	0.38	0.011 *
Salmonella and Phage (✚) vs *Salmonella* only (■)	4.7	0.37	0.007 *
No *Salmonella* No Phage (●) vs Phage only (▲)	2.6	0.24	0.011 *
No *Salmonella* No Phage (●) vs *Salmonella* only (■)	1.5	0.16	0.086
Phage only (▲) vs *Salmonella* only (■)	3.3	0.29	0.007 *

Figure 6 – Viral alpha and beta diversity within raw tripe samples in enrichment cultures with bacteriophage treatment. A) Alpha diversity presented as observed diversity (left) and Shannon-Weiner diversity index right) for each sample condition. B) Beta diversity presented as Bray-Curtis dissimilarity values plotted using nonmetric multidimensional scaling (NMDS) ordination for each sample condition). C) Permutational multivariate ANOVA test of each condition

5.2.5.2. Characterisation of Tripe virome

To investigate the composition of the virome of raw tripe and to place assembled vOTUs in the context of known viral diversity, vContact2 was used to generate a network-based gene-sharing profile. vOTUs from all samples were clustered with viral diversity within the INPHARED database containing complete viral genomes from GenBank (Figure 7). This method has been shown to be an accurate tool for the taxonomic assignment of bacteriophages with similar viruses co-locating in clusters (Bin Jang et al., 2019). The vast majority of vOTUs present in the food samples clustered with unclassified viruses which do not currently have an assigned viral family from the latest International Committee on Taxonomy of Viruses (ICTV). Additionally, many vOTUs were found to cluster independently from viruses within GenBank suggesting that these vOTUS represent novel viruses with no related genomes in available databases. Interestingly, a single vOTU from food clustered with other *Inoviridae* phages. In contrast to many of the other phage families highlighted in this network, members of the *Inoviridae* family are single-stranded DNA viruses. This vOTU was 7.9kb in length encoding 12 genes consistent with the genome size range of other members of the *Inoviridae* family, and clustered with phages infecting *Ralstonia*. *Ralstonia* is a plant pathogenic bacterium which was not detected with metagenomic sequencing (Figure 3).

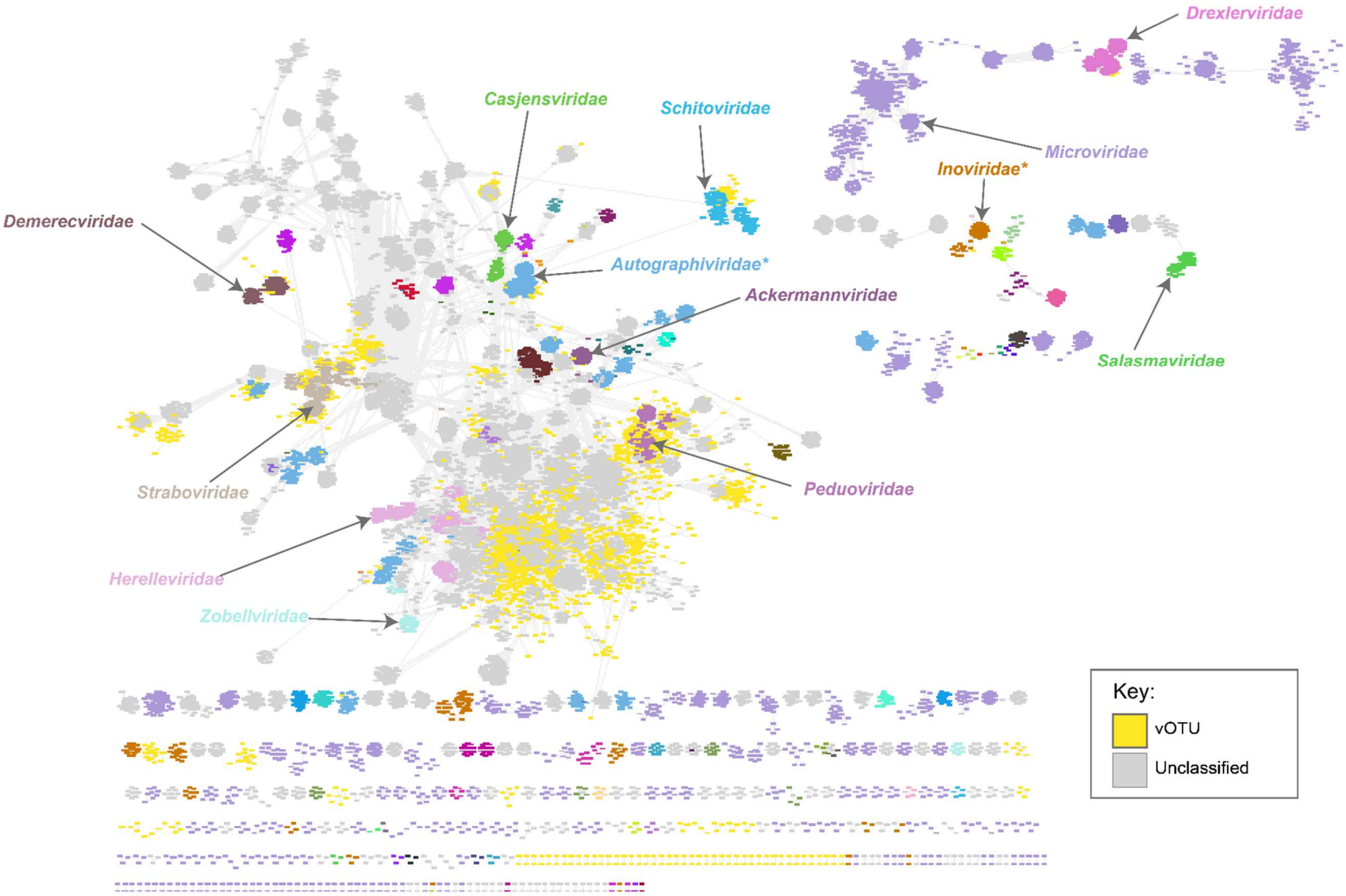


Figure 7 – Taxonomic analysis of vOTUs using vConTACT2 network analysis of vOTUs from Tripe samples spiked with Salmonella and Bacteriophages and a database of phage genomes extracted from GenBank (INPHARED). vOTUs from this study are coloured yellow. Selected viral families, currently recognised by the ICTV (May2023) are represented with other colours. Families annotated which are located in multiple locations within the network are designated with an asterisk (*).

5.2.5.2.1. Classification of vOTUs

To further investigate each vOTU present within tripe samples, virulent-temperate lifestyle predictions were made for each genome to estimate the dominant lifestyle of the tripe virome (Figure 8a). Majority of the vOTUs were predicted to be lytic, due to the absence of lysogeny genes, within their genomes. This demonstrates that lytic phages are likely to be dominant within raw tripe samples and suggests that they remain stable over the course of the enrichment process. Since many of the vOTUs appeared to be unrelated to previously isolated bacteriophages (Figure 7) we aimed to quantify the number of novel vOTU clusters detected (Figure 8b). A vOTU cluster was deemed to be novel if it did not contain or share edges with INPHARED phages within the network. The network predicted a total of 450 viral clusters contained tripe vOTUs, of which 378 were classified as novel, while the remaining 56 contained known viruses. This highlights that the diversity of the tripe virome is not currently reflected within publicly available databases.

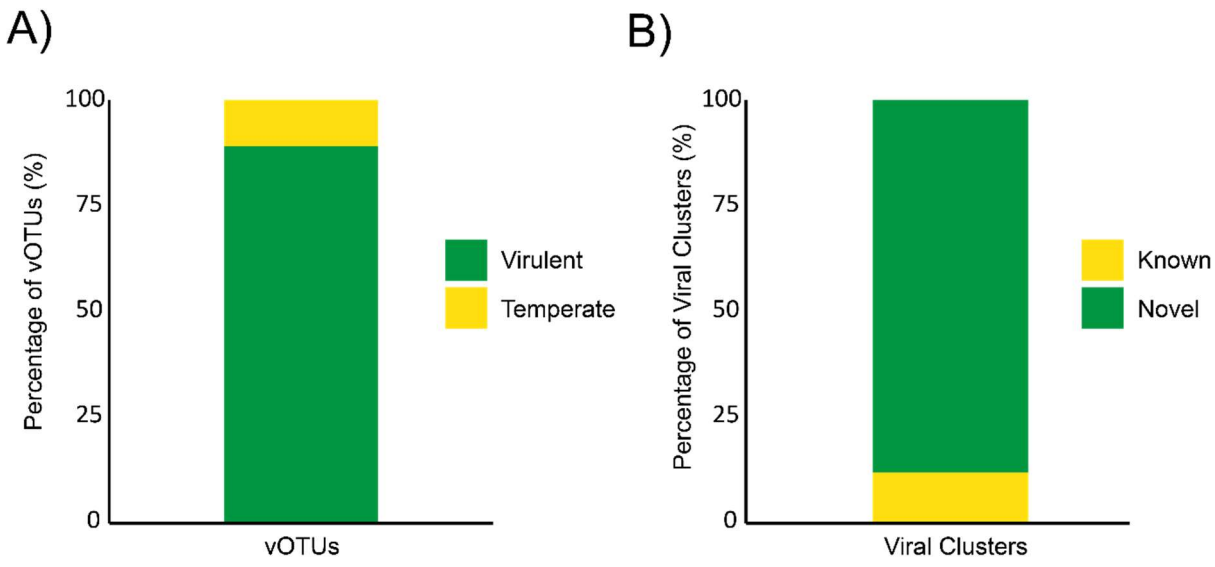


Figure 8 – Classification of vOTUs in raw tripe samples spiked with Salmonella and/or Bacteriophages. A) Lifestyle prediction of vOTUs by BACPHLIP. B) Quantification of known and novel viral clusters in vConTACT2 network.

5.2.5.3. Identification of SPLA phage blooms

Since *Salmonella* decreased in relative abundance in samples which had been treated with the penta phage cocktail, reads were mapped to genomes of cocktail components to determine changes in abundance of SPLA phages in the presence of *Salmonella*, indicative of phage lysis (phage blooms) (Figure 9). Mapping used a sequence identity threshold of 100%. Interestingly, in samples where the penta phage cocktail was not added, reads mapped to SPLA1a, SPLA4 and SPLA5b suggesting that similar phages are present within the tripe virome. No reads were mapped to SPLA9 or SPLA11 (both rosemountviruses) when the penta phage cocktail was not applied suggesting these phages or close relatives are not present within any of the tripe samples. In order to identify significant blooms of SPLA phages in the presence of *Salmonella*, phage only conditions were compared to *Salmonella* and phage conditions using a student t-test. Significant blooms were identified in all SPLA phages with the exception of SPLA4 ($P < 0.05$). In order to demonstrate that blooms were SPLA bacteriophages and not background members of the virome with high sequence similarity, no *Salmonella* and no phage conditions were compared to *Salmonella* only conditions. No significant changes in mapped reads was observed suggesting that the observed phage blooms were likely to be SPLA phages increasing in abundance and not similar phages present within samples.

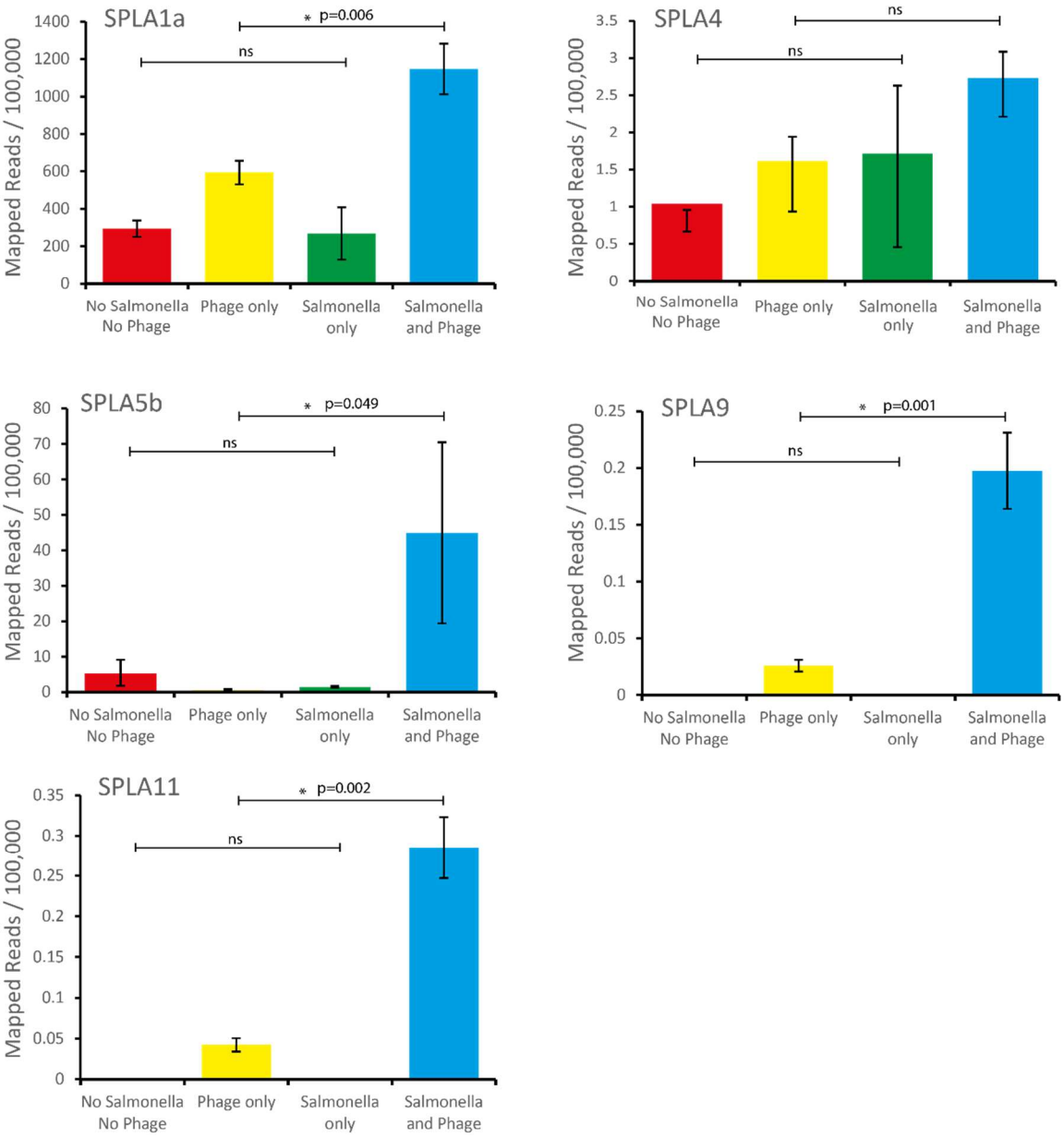


Figure 9 – Mean mapped reads per 100,000 reads of metagenomic sequencing mapped to penta phage cocktail component genomes. Mean reads per 100,000 is calculated using 5 replicates of each condition and corrected for library size. Error bars display standard error of the mean. Significant differences between Phage only and Salmonella/Phage conditions are highlighted with an asterisk (*).

5.3. Discussion

The antimicrobial activity of bacteriophages makes them a promising intervention method for the use directly in food to reduce the presence of foodborne pathogens. The detection of foodborne pathogens contaminating retail food is commonly carried out using culture-based techniques (Velusamy et al., 2010). These approaches use selective culture to increase the abundance of target bacteria which are later confirmed using methods such as serotyping, enzyme-linked immune sorbent assay (ELISA) or MALDI-TOF (Schrader et al., 2008, Verma et al., 2012, Wieser et al., 2012). The use of culture-based techniques is a critical tool in the detection of pathogens for food-based surveillance as it allows for highly accurate and reliable identification of pathogens. In the case of *Salmonella*, multiple incubations in different selective media are employed in order to enrich for *Salmonella* and increase the effectiveness of detection methods (Mooijman, 2018). An important consideration for the application of bacteriophages directly to food samples is that phages are likely to be present in enrichment cultures during routine pathogen surveillance. The antimicrobial activity of the applied phages is likely to be far higher during culture due to increased bacterial growth as well as liquid media and shaking that would result in increased phage-bacteria interactions (Sinha et al., 2018). Here, we show that treating with bacteriophages decreases the detection of *Salmonella* using a method with the same culture-based techniques as routine *Salmonella* detection methods (Figure 2). The antimicrobial activity of bacteriophages within the food sample prior to culture is not clear and therefore viable counts of *Salmonella* should be measured in order to determine this. However, if the antimicrobial activity occurred exclusively within culture, food samples containing viable *Salmonella* are likely to test negative which raises an issue with the use of bacteriophages in food production. A potential solution would be the application of

culture independent approaches, such as metagenomic sequencing, for food surveillance. However, these are currently not commonplace due to many factors including increased cost and reduced accuracy (Wang and Salazar, 2016). Nonetheless, if bacteriophages are to be used in food production, current surveillance methods are likely to be not appropriate for accurate pathogen detection.

A potential barrier to the use of phages is likely to be the production and delivery of the correct concentration of bacteriophage for the intended application. The use of phages in industrial processes such as food production is therefore likely to require production of bacteriophages in large volumes and high titres. Here we apply 10^7 PFU of bacteriophages per 25 g of raw tripe sample. Raw tripe product is commonly produced in quantities up to 2kg per product. Therefore 8 x 10^8 PFU would be required for this dose per product. Industrial production of bacteriophages at high titres can be expensive and therefore application to a low production cost product, such as raw pet food, is likely to reduce profit margins and may not be economically viable (Krysiak-Baltyn et al., 2018). Lower titres will need to be used that retain the desired antimicrobial activity whilst reducing phage production costs.

Metagenomic sequencing was used to assess whether phage treatment within food samples caused significant shifts within microbial populations. Enriching samples in aerobic conditions likely lead to a sample bias, towards fast growing aerobic bacteria, which could explain low diversity and low species richness observed (Figure 3). Since food samples used were tripe, which predominantly consists of ruminant stomach lining, many anaerobic bacteria which are likely abundant within samples were not enriched for. Nonetheless,

enriching for aerobic bacteria selected for fast growing aerobes, many of which are closely related to *Salmonella*, and therefore the most likely phage targets. Using this method was also consistent with culture-based techniques and allowed insight into phage induced population dynamics within enrichment culture.

The most common bacteria genera isolated from raw tripe samples were *Comamonas*, *Escherichia* and *Acinetobacter* (Figure 3). Bacteria of the genera *Comamonas* are fast growing Gram-negative bacteria, which likely explains their high abundance within enrichment cultures, with certain species such as *Comamonas kerstersii* known to cause human infection (Almuzara et al., 2017). *Comomanas* has previously been shown to be present within the rumen of cattle, as well as the human gut. (Carvalho et al., 2022, Wang et al., 2022). Additionally, *Escherichia* and *Acinetobacter* are both commonly found within the stomach lining and gut of cows which likely explains their presence within tripe enrichment cultures (Park and Kim, 2020). Many of the bacteria isolated, such as *Enterococcus faecium*, were commensal bacteria, particularly of cattle, and therefore are unlikely to pose a risk of to humans or pets. However opportunistic pathogens, such as *Acinetobacter baumannii*, were present within all samples which could pose a risk to humans preparing raw pet food diets as well as animals consuming them, particularly for immunocompromised individuals (McConnell et al., 2013).

The treatment of samples with a bacteriophage cocktail significantly reduced the relative abundance of spiked *Salmonella* to very low levels, comparable with unspiked control samples. This highlights the effectiveness of the penta phage cocktail in the presence of food matrix and food microbiota. Significant differences between untreated controls and

phage and *Salmonella* spiked samples were still observed however the relative abundance did not exceed 0.5% in any sample which could be due to presence of DNA from phage killed cells which would not be detected by culture based enrichment methods which rely on intact and viable cells.

An advantage of the use of phages over other antimicrobials is their specificity as non-susceptible bacteria should not be lysed by the bacteriophage cocktail. This is important for phage therapy as shifts in the human microbiota can be linked to disease such as inflammatory bowel disease (Cucchiara et al., 2009). It can also be important for their use in food as shifts in the microbiota could affect sensory characteristics of the product and well as food spoilage (Doulgeraki et al., 2012, Kazou et al., 2022). We demonstrate that the use of a phage cocktail in samples where the primary target, *Salmonella*, is not present, no significant changes in abundance of bacterial species are observed which could be due to the absence of susceptible hosts and the phage becoming inert (Table 2). However, in the presence of *Salmonella*, both *Enterococcus faecium* significantly increased in abundance whereas *Hafnia paralvei* significantly decreased in abundance (Table 3). It is not clear what causes these shifts. Rapid *Salmonella* lysis could have induced stress responses leading to decreases in abundance. Interestingly previous host range testing showed that SPLA phages do affect the growth of *afnia* species (Chapter 4). This effect may only have been observed in the presence of *Salmonella* due to an increase in phage titre from successful lysis.

Viral species richness was particularly high in all samples, regardless of the addition of *Salmonella* or bacteriophages (Figure 6). This could be due to high viral diversity within

tripe samples. However, this diversity could also be confounded by assembly of multiple incomplete fragments of the same viral genome. This could partially be avoided by filtering out smaller vOTUs, such as those which are below 10-20kb (Roux et al., 2017). However, this would exclude smaller bacteriophages leading to an underestimation of viral diversity (Moens et al., 2017, Van Doorslaer et al., 2018). An alternative to attempt to improve viral assemblies is to utilize the addition of long read metagenomics with short read sequence-based approaches. This hybrid approach has been shown to improve downstream viral prediction and viral diversity within samples whilst long read metagenomics alone has be shown to be inadequate for the accurate assembly and prediction of viral contigs (Cook et al., 2023). Nevertheless, the use of short read approaches utilised in this study identified 3067 vOTUs which were well dispersed amongst known bacteriophage sequences (Figure 7). Majority of vOTUs were clustered with phages currently unclassified at the family level, likely due to the abolition of the morphology based taxa, *Myoviridae*, *Siphoviridae* and *Podoviridae* leading to majority of phages currently being unclassified at family level (Turner et al., 2021, Turner et al., 2023).

Majority of vOTUs present within Tripe samples were predicted to be virulent phages (Figure 8a). The predictions, made using BACPHLIP, estimated bacteriophage lifestyle based on searching for temperate phage specific protein domains. An important consideration for this estimation is that absence of lysogeny genes may not indicate that a phage is virulent if the vOTU is incomplete. Therefore, this should only be treated as an estimation of the lifestyle composition of the tripe virome. Comparisons of viral clusters containing vOTUs from this study did not cluster with bacteriophage genomes within GenBank (Figure 8b). These novel viral clusters could relate to currently unknown viral diversity relating to

new phage taxa. Alternatively, these viral vOTUs could also be eukaryotic viruses which would not cluster with bacteriophage genomes used within the network.

Mapping of metagenome sequence reads to SPLA phages within the penta phage cocktail revealed that the tripe virome contained similar phages to SPLA1a, SPLA4 and SPLA5b, since read mapping was observed for unspiked samples. Since the difference in *Salmonella* abundance was still observed between these samples, this suggest that the presence of a phage alone is not enough to significantly reduce *Salmonella* contamination. The correct phage must be treated at the correct concentration, which is previously known to be virulent against the target in order to achieve the desired effect. It also highlights that since very similar phages, which were not distinguishable using highly stringent mapping, there should be little concern for the addition of phages to food samples. However significant phage blooms were observed for phages SPLA1a, SPLA5b, SPLA9 and SPLA11. Suggesting that these phages were able to replicate within the samples and are likely the cause of the decrease in *Salmonella* presence within phage spiked samples.

Overall, phages remain effective for the lysis of *Salmonella* even in the presence of food matrices and member of the food microbiota. Concerns over the addition of phages into food samples should be limited as the food samples already contain a rich and diverse virome, even containing phages with high similarity to phage cocktail components. However, routine use of bacteriophages in food processing is likely to require other considerations, such as alterations to current surveillance procedures, in order to maintain accurate testing methods critical for a safe food supply.

6. Discussion

The need for novel and effective control measures for bacterial pathogens in food production environments is of great importance. The application of control measures is integral to maintaining a safe supply of food and preventing the spread of bacterial infections. Whilst bacteriophages are a promising solution, there are still many important considerations and barriers which are preventing their use. One important consideration is determining where within a food production environment their application would be most effective. Phages have the potential to be used at many stages of food production, both pre-harvest and post-harvest. Pre-harvest applications could include the prophylactic treatment of livestock as well as treatment of crop plants. Post-harvest applications could include the direct addition to food products and well as disinfectant use within food production environments.

Preharvest applications would likely require the prophylactic use of phages in farm animals typically through supplementation of feed or drinking water (Ferriol-González and Domingo-Calap, 2021). This would prevent colonisation of livestock and also aim to prevent pathogen presence within food production factories. Phage delivery via drinking water would be an effective method which would be easy to monitor. However, stability over long periods of time and shelf life when stored as a liquid make this mode of delivery less attractive. One alternative is the addition of dried phages to feed pellets. Phages are able to be dried using a variety of methods such as lyophilisation and spray drying (Manohar and Ramesh, 2019, Chang et al., 2017). The supplementation of feed with spray dried phages has been shown to significantly reduce colonisation of *Salmonella* challenged piglets as well as no negative impact of pig gut microbiota (Thanki et al., 2022).

Perhaps the most simple and easy to implement application is their use for surface disinfection in conjunction with traditional chemical disinfection. Their application as sprays would ensure direct contact with pathogens and has previously been shown to be effective for the removal of *Pseudomonas aeruginosa* (Stachler et al., 2021). Whilst this approach could be effective in preventing cross contamination and could improve the effectiveness of cleaning procedures, the effect for the removal of pathogens already within food is likely to be limited. Additionally, routinely disinfecting factory equipment and all surfaces with bacteriophages is likely to require large volumes of bacteriophages which could be expensive to produce and may not be cost effective. In the case of raw pet food, the most effective application could be directly applying a bacteriophage cocktail as sprays for products which are minimally processed. Minimally processed products, such as those which are not minced, are likely only contaminated at the surface and therefore removal with bacteriophages should be easier. The application of sprays ensures equal coverage of the phage cocktail as well as a consistent dosage. The application of phages to food and feed may also benefit the consumer. Lytic bacteriophages ingested alongside a contaminating pathogen may also remain active within the gut preventing colonisation and infection. Further research could be carried out to test this hypothesis.

Typical protocols for the isolation of bacteriophages, including the methods commonly used within this study, utilise rich media and incubation temperatures for optimal bacterial growth. This is used as increased bacterial growth can lead to increased production of bacteriophages. Whilst this is appropriate for laboratory use, conditions within environments of the intend application usually vastly differ. This is an important consideration as phages which are virulent in laboratory conditions may not retain their

virulence in harsher conditions, such as those within a food production environment. Food production settings are typically much colder, and the availability of key nutrients is likely much scarcer. A potential way to overcome this downstream issue is to isolate phages at temperatures and conditions which resemble that of their intended use. When environmental conditions change, bacterial gene expression can also change which can alter the proteins and polymers found on their cell surface, such as cellulose (Castelijn et al., 2012, Kim et al., 2022). If these structures are used as phage receptors, the types of phages isolated may differ in these conditions leading to new phage discovery.

Perhaps the biggest barrier to the use of phages in food is the regulatory approval for their use. The exact regulatory guidelines for the use of bacteriophages for any application are yet to be universally defined. However, it is evident that the regulatory body responsible for defining these guidelines will depend on the given application. Phages used for therapy will likely need to adhere to guidelines of the Department for Health and Social Care (DHSC), the Medicines and Healthcare products Regulatory Agency (MHRA), the National Institute for Health and Care Excellence (NICE) and/or the National Institute for Health and Care Research (NIHR). Whereas, phage applications in food will likely need to be approved by the Food Standards Agency (FSA). Currently, some bacteriophage products are approved for use within food or have GRAS (Generally regarded as safe) status for use within the USA (Vikram et al., 2021). Currently no bacteriophage or commercially available bacteriophage products are approved at a European wide level for use in therapy or in food. The approval of bacteriophages for use is likely to be dependent on how their use is classified by law. If the use of bacteriophages is used for surface decontamination or food handling surfaces or directly onto products, they may require approval as a processing aid to produce food and

feed. Processing aids are substances or materials which are not consumed as a food ingredient alone, intentionally used to fulfil a technological purpose which may result in presence within the final product. Processing aids only require approval under EU food hygiene legislation if directly applied to products. If phages are demonstrated to have an ongoing effect when added to food, such as preventing future contamination, then they will likely require approval as a food additive. Food additives are defined as substances not normally consumed as a food alone which are intentionally added to food to fulfil a technological purpose which may affect characteristics of food not including substances added for maintaining nutritional qualities. A major distinction between processing aids and food additives is that there is no requirement for a processing aid to be included in product labelling. Nonetheless, it is currently unclear which category bacteriophages should be placed into which will likely affect the difficulty for their approval.

In principle, bacteriophages have the potential to be cheap to produce. They are naturally occurring, and their production is similar to microbial biotechnological processes which have been carried out for decades. However, their production for use in phage therapy, and also potentially within food, requires phage production to adhere to good manufacturing practice (GMP) standards. The same GMP standards which are required for the production of pharmaceuticals. This makes the production of designer or personalised phage cocktails, where phages have been specifically selected for their virulence against a particular bacterial strain, not economically viable. The only way for phages to be cost effective is to produce large batches of phage cocktails with very broad host range meaning they can be used to treat more patients, or to modify current GMP standards for the production of bacteriophages.

In order to better inform policy and legislation makers, it is clear that more work is required to determine the antimicrobial effect of bacteriophages directly within the food production environment. This study contributes greatly to the discover and in-depth characterisation of *Salmonella* phages, as well as providing evidence for their efficacy within a specific application in food. However more work is required to fully determine the optimal mode of delivery for phages in food processing environments. This should include aspects such as contact time, optimal temperatures, and delivery modes. In addition, aspects which could limit phage efficacy such as different food matrices or materials of food contact surfaces should be investigated. From this study, it is evident that efficacy on phage treatment is critically dependent on a well-designed combination of bacteriophages, with characterised lytic activity against the target bacterium which remains stable and efficacious in conditions for their intended use.

This thesis contributes to better understanding of how bacteriophages can be used in food processing environments, particularly in the production of raw pet food. The information surrounding raw pet food production gained is invaluable to the appropriate and effective application of bacteriophages in this setting. This information will be useful to Company P and other raw pet food producers to help better understand the presence and diversity of *Salmonella* circulating within raw pet food and help inform important intervention methods to help reduce prevalence. Additionally, the characterisation of twelve new *Salmonella* phages, with the potential to be used as antimicrobial is a useful finding. The methods used here to isolate, characterise, and formulate cocktails of phages should be used as a framework for the development of phage-based antimicrobials. This study has also demonstrated that a rationally designed cocktail can be effective when applied to food

for the reduction of *Salmonella*. The framework for the rationale design of bacteriophage cocktails, which encompasses much of the work of this thesis, is shown in Figure 1.

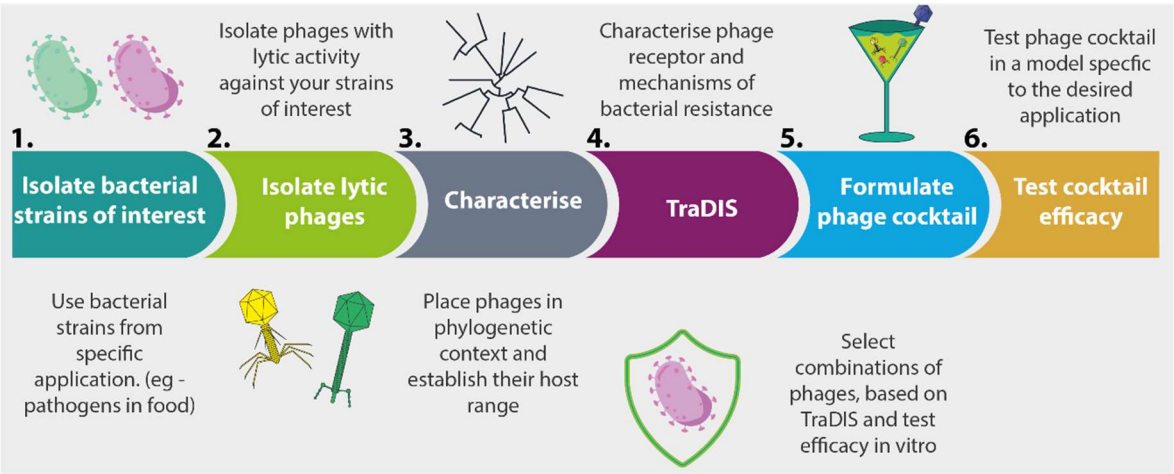


Figure 1 – Pipeline for the rationale design of bacteriophage cocktails for increased efficacy against bacterial pathogens.

7. References

ABEDON, S. T. 2019. Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? *Antibiotics (Basel, Switzerland),* 8**,** 182.

ACHESON, D. & HOHMANN, E. L. 2001. Nontyphoidal Salmonellosis. *Clinical Infectious Diseases,* 32**,** 263-269.

ACKE, E. 2018. Campylobacteriosis in dogs and cats: a review. *New Zealand Veterinary Journal. 66*(5), pp.221-228

ADRIAENSSENS, E. M., FARKAS, K., MCDONALD, J. E., JONES, D. L., ALLISON, H. E. & MCCARTHY, A. J. 2021. Tracing the fate of wastewater viruses reveals catchment-scale virome diversity and connectivity. *Water Research,* 203**,** 117568.

AJUEBOR, J., MCAULIFFE, O., O'MAHONY, J., ROSS, R. P., HILL, C. & COFFEY, A. 2016. Bacteriophage Endolysins and their Applications. *Science Progress,* 99**,** 183-199.

AL-SHAYEB, B., SACHDEVA, R., CHEN, L.-X., WARD, F., MUNK, P., DEVOTO, A., CASTELLE, C. J., OLM, M. R., BOUMA-GREGSON, K., AMANO, Y., HE, C., MÉHEUST, R., BROOKS, B., THOMAS, A., LAVY, A., MATHEUS-CARNEVALI, P., SUN, C., GOLTSMAN, D. S. A., BORTON, M. A., SHARRAR, A., JAFFE, A. L., NELSON, T. C., KANTOR, R., KEREN, R., LANE, K. R., FARAG, I. F., LEI, S., FINSTAD, K., AMUNDSON, R., ANANTHARAMAN, K., ZHOU, J., PROBST, A. J., POWER, M. E., TRINGE, S. G., LI, W.-J., WRIGHTON, K., HARRISON, S., MOROWITZ, M., RELMAN, D. A., DOUDNA, J. A., LEHOURS, A.-C., WARREN, L., CATE, J. H. D., SANTINI, J. M. & BANFIELD, J. F. 2020. Clades of huge phages from across Earth's ecosystems. *Nature,* 578**,** 425-431.

ALMUZARA, M., BARBERIS, C., VEIGA, F., BAKAI, R., CITTADINI, R., OCAMPO, C. V., SERENA, M. A., COHEN, E., RAMIREZ, M. & FAMIGLIETTI, A. 2017. Unusual presentations of *Comamonas kerstersii* infection. *New microbes and new infections,* 19**,** 91-95.

ALTAMIRANO, F. L. G. & BARR, J. J. 2019. Phage Therapy in the Postantibiotic Era. *Clinical Microbiology Reviews,* 32. pp.10-1128

ANANY, H., CHEN, W., PELTON, R. & GRIFFITHS, M. 2011. Biocontrol of Listeria monocytogenes and Escherichia coli O157: H7 in meat by using phages immobilized on modified cellulose membranes. *Applied and environmental microbiology,* 77**,** 6379-6387.

ANGELO, K. M., CHU, A., ANAND, M., NGUYEN, T.-A., BOTTICHIO, L., WISE, M., WILLIAMS, I., SEELMAN, S., BELL, R. & FATICA, M. 2015. Outbreak of Salmonella Newport infections linked to cucumbers—United States, 2014. *Morbidity and Mortality Weekly Report,* 64**,** 144.

APHA 2017. Salmonella in Livestock Production in GB, 2016. *https://www.gov.uk/government/publications/salmonella-in-livestock-production-in-great-britain-2016. Accessed:* March 2023

APHA 2022. Salmonella in animals and feed in Great Britain, 2022. Animal and Plant Health Agency. https://www.gov.uk/government/publications/salmonella-in-animals-and-feed-in-great-britain. Accessed: March 2023

ARNDT, D., GRANT, J. R., MARCU, A., SAJED, T., PON, A., LIANG, Y. & WISHART, D. S. 2016. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res,* 44**,** W16-21.

BAI, J., KIM, Y.-T., RYU, S. & LEE, J.-H. 2016. Biocontrol and Rapid Detection of Food-Borne Pathogens Using Bacteriophages and Endolysins. *Frontiers in Microbiology,* 7. p.474

BANKEVICH, A., NURK, S., ANTIPOV, D., GUREVICH, A. A., DVORKIN, M., KULIKOV, A. S., LESIN, V. M., NIKOLENKO, S. I., PHAM, S. & PRJIBELSKI, A. D. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology,* 19**,** 455-477.

BARKER, R. M. 1988. Abortive phage-infection and UV-protection markers on Col I plasmids from epidemic strains of Salmonella. *Journal of Medical Microbiology,* 25**,** 221-225.

BARNHART, B., COX, S. & JETT, J. 1976. Prophage induction and inactivation by UV light. *Journal of virology,* 18**,** 950-955.

BARQUIST, L., MAYHO, M., CUMMINS, C., CAIN, A. K., BOINETT, C. J., PAGE, A. J., LANGRIDGE, G. C., QUAIL, M. A., KEANE, J. A. & PARKHILL, J. 2016. The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. *Bioinformatics (Oxford, England),* 32**,** 1109-1111.

BATT, C. A. 2014. LISTERIA | Introduction. *In:* BATT, C. A. & TORTORELLO, M. L. (eds.) *Encyclopedia of Food Microbiology (Second Edition).* Oxford: Academic Press. 2, 1-23

BAWN, M., ALIKHAN, N.-F., THILLIEZ, G., KIRKWOOD, M., WHEELER, N. E., PETROVSKA, L., DALLMAN, T. J., ADRIAENSSENS, E. M., HALL, N. & KINGSLEY, R. A. 2020. Evolution of Salmonella enterica serotype Typhimurium driven by anthropogenic selection and niche adaptation. *PLOS Genetics,* 16**,** e1008850.

BAXA, U., STEINBACHER, S., MILLER, S., WEINTRAUB, A., HUBER, R. & SECKLER, R. 1996. Interactions of phage P22 tails with their cellular receptor, Salmonella O-antigen polysaccharide. *Biophysical Journal,* 71**,** 2040-2048.

BENSON, N. R. & ROTH, J. 1997. A Salmonella Phage-P22 Mutant Defective in Abortive Transduction. *Genetics,* 145**,** 17-27.

BERTANI, B. & RUIZ, N. 2018. Function and Biogenesis of Lipopolysaccharides. *EcoSal Plus,* 8.

BHUSHAN, K. 2017. BacteRiophage EXclusion (BREX): A novel anti-phage mechanism in the arsenal of bacterial defense system. *Journal of Cellular Physiology,* 233**,** 771-773.

BIN JANG, H., BOLDUC, B., ZABLOCKI, O., KUHN, J. H., ROUX, S., ADRIAENSSENS, E. M., BRISTER, J. R., KROPINSKI, A. M., KRUPOVIC, M., LAVIGNE, R., TURNER, D. & SULLIVAN, M. B. 2019. Taxonomic assignment of uncultivated prokaryotic virus genomes is enabled by gene-sharing networks. *Nat Biotechnol,* 37**,** 632-639.

BLANCO-MÍGUEZ, A., BEGHINI, F., CUMBO, F., MCIVER, L. J., THOMPSON, K. N., ZOLFO, M., MANGHI, P., DUBOIS, L., HUANG, K. D., THOMAS, A. M., NICKOLS, W. A., PICCINNO, G., PIPERNI, E., PUNČOCHÁŘ, M., VALLES-COLOMER, M., TETT, A., GIORDANO, F., DAVIES, R., WOLF, J., BERRY, S. E., SPECTOR, T. D., FRANZOSA, E. A., PASOLLI, E., ASNICAR, F., HUTTENHOWER, C. & SEGATA, N. 2023. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. *Nature Biotechnology*. pp.1-12.

BLASER, M. J. & NEWMAN, L. S. 1982. A Review of Human Salmonellosis: I. Infective Dose. *Reviews of Infectious Diseases,* 4**,** 1096-1106.

BOBAY, L. M., ROCHA, E. P. C. & TOUCHON, M. 2013. The adaptation of temperate bacteriophages to their host genomes. *Molecular Biology and Evolution,* 30. pp.737-751.

BOJANIĆ, K., MIDWINTER, A. C., MARSHALL, J. C., ROGERS, L. E., BIGGS, P. J. & ACKE, E. 2017. Isolation of Campylobacter spp. from Client-Owned Dogs and Cats, and Retail Raw Meat Pet Food in the Manawatu, New Zealand. *Zoonoses and Public Health,* 64. pp.438-449.

BOLDUC, B., JANG, H. B., DOULCIER, G., YOU, Z. Q., ROUX, S. & SULLIVAN, M. B. 2017. vConTACT: an iVirus tool to classify double-stranded DNA viruses that infect Archaea and Bacteria. *PeerJ,* 5**,** e3243.

BONDY-DENOMY, J., PAWLUK, A., MAXWELL, K. L. & DAVIDSON, A. R. 2013. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature,* 493**,** 429-U181.

BORGES, T. J., MORETTI, L. K., SILVA, M. M. N., TONDO, E. C. & PEREIRA, K. S. 2020. Salmonella sensitivity to sodium hypochlorite and citric acid in washing water of lettuce residues. *Journal of Food Safety,* 40**,** e12748.

BORTOLAIA, V., KAAS, R. S., RUPPE, E., ROBERTS, M. C., SCHWARZ, S., CATTOIR, V., PHILIPPON, A., ALLESOE, R. L., REBELO, A. R., FLORENSA, A. F., FAGELHAUER, L., CHAKRABORTY, T., NEUMANN, B., WERNER, G., BENDER, J. K., STINGL, K., NGUYEN, M., COPPENS, J., XAVIER, B. B., MALHOTRA-KUMAR, S., WESTH, H., PINHOLT, M., ANJUM, M. F., DUGGETT, N. A., KEMPF, I., NYKÄSENOJA, S., OLKKOLA, S., WIECZOREK, K., AMARO, A., CLEMENTE, L., MOSSONG, J., LOSCH, S., RAGIMBEAU, C., LUND, O. & AARESTRUP, F. M. 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy,* 75**,** 3491-3500.

BOSSI, L., FUENTES, J. A., MORA, G. & FIGUEROA-BOSSI, N. 2003. Prophage contribution to bacterial population dynamics. *Journal of Bacteriology,* 185**,** 6467-6471.

BRANCHU, P., BAWN, M. & KINGSLEY, R. A. 2018. Genome Variation and Molecular Epidemiology of Salmonella enterica Serovar Typhimurium Pathovariants. *Infection and Immunity,* 86. pp.10-1128

BRANCHU, P., CHARITY, O. J., BAWN, M., THILLIEZ, G., DALLMAN, T. J., PETROVSKA, L. & KINGSLEY, R. A. 2019. SGI-4 in monophasic Salmonella Typhimurium ST34 is a novel ICE that enhances resistance to copper. *Frontiers in microbiology***,** 1118.

BRIDIER, A., BRIANDET, R., THOMAS, V. & DUBOIS-BRISSONNET, F. 2011. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling,* 27**,** 1017-32.

BROADBENT, S. E., DAVIES, M. R. & VAN DER WOUDE, M. W. 2010. Phase variation controls expression of Salmonella lipopolysaccharide modification genes by a DNA methylation-dependent mechanism. *Mol Microbiol,* 77**,** 337-53.

BROEKER, N. K. & BARBIRZ, S. 2017. Not a barrier but a key: how bacteriophages exploit host's O-antigen as an essential receptor to initiate infection. *Molecular microbiology,* 105**,** 353-357.

BRUTTIN, A. & BRÜSSOW, H. 2005. Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. *Antimicrobial agents and chemotherapy,* 49**,** 2874-2878.

BULLEN, A., GRAHE, A., KASSOLIS, P. & SINGLETON, D. R. 2022. Complete Genome Sequence of a Novel Hafnia alvei Bacteriophage. *Microbiol Resour Announc,* 11**,** e0004922.

BURMEISTER, A. R., FORTIER, A., ROUSH, C., LESSING, A. J., BENDER, R. G., BARAHMAN, R., GRANT, R., CHAN, B. K. & TURNER, P. E. 2020. Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proceedings of the National Academy of Sciences,* 117**,** 11207-11216.

BUSHNELL, B. 2014. BBMap: a fast, accurate, splice-aware aligner. Lawrence Berkeley National Lab.(LBNL), Berkeley, CA (United States).

BUSTAMANTE, V. C. H., MART\'NEZ, L. C., SANTANA, F. J., KNODLER, L. A., STEELE-MORTIMER, O. & PUENTE, J. L. 2008. HilD-mediated transcriptional cross-talk between SPI-1 and SPI-2. *Proceedings of the National Academy of Sciences,* 105**,** 14591-14596.

BYUN, K.-H., HAN, S. H., YOON, J.-W., PARK, S. H. & HA, S.-D. 2021. Efficacy of chlorine-based disinfectants (sodium hypochlorite and chlorine dioxide) on Salmonella Enteritidis planktonic cells, biofilms on food contact surfaces and chicken skin. *Food Control,* 123**,** 107838.

CALLANAN, J., STOCKDALE, S. R., SHKOPOROV, A., DRAPER, L. A., ROSS, R. P. & HILL, C. 2020. Expansion of known ssRNA phage genomes: From tens to over a thousand. *Science Advances,* 6**,** eaay5981.

CALLOW, B. R. 1959. A new phage-typing scheme for Salmonella typhi-murium. *Epidemiology & Infection,* 57**,** 346-359.

CALOS, M. P. 2016. Phage Integrases for Genome Editing. *In:* CATHOMEN, T., HIRSCH, M. & PORTEUS, M. (eds.) *Genome Editing: The Next Step in Gene Therapy.* New York, NY: Springer New York.

CAMMACK, N. R., YAMKA, R. M. & ADAMS, V. J. 2021. Low Number of Owner-Reported Suspected Transmission of Foodborne Pathogens From Raw Meat-Based Diets Fed to Dogs and/or Cats. *Frontiers in Veterinary Science,* 8. p.741575

CAMPBELL, A. M. 1998. Prophages and Cryptic Prophages. *In:* DE BRUIJN, F. J., LUPSKI, J. R. & WEINSTOCK, G. M. (eds.) *Bacterial Genomes: Physical Structure and Analysis.* Boston, MA: Springer US.

CANCHAYA, C., FOURNOUS, G. & BRÜSSOW, H. 2004. The impact of prophages on bacterial chromosomes. *Molecular Microbiology. 53*(1), pp.9-18.

CAPELLA-GUTIÉRREZ, S., SILLA-MARTÍNEZ, J. M. & GABALDÓN, T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics,* 25**,** 1972-1973.

CARVAJAL, B. G., METHNER, U., PIEPER, J. & BERNDT, A. 2008. Effects of Salmonella enterica serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens. *Vaccine,* 26**,** 5423-5433.

CARVALHO, F. M. D., VALIATTI, T. B., SANTOS, F. F., SILVEIRA, A. C. D. O., GUIMARÃES, A. P. C., GERBER, A. L., SOUZA, C. D. O., CORSI, D. C., BRASILIENSE, D. M., CASTELO-BRANCO, D. D. S. C. M., ANZAI, E. K., BESSA-NETO, F. O., GUEDES, G. M. D. M., SOUZA, G. H. D. A. D., LEMOS, L. N., FERRAZ, L. F. C., BAHIA, M. D. N. M., VAZ, M. S. M., SILVA, R. G. B. D., VEIGA, R., SIMIONATTO, S., MONTEIRO, W. A. P., LIMA, W. A. D. O., KIFFER, C. R. V., PIGNATARI, A. C. C., CAYÔ, R., VASCONCELOS, A. T. R. D. & GALES, A. C. 2022. Exploring the Bacteriome and Resistome of Humans and Food-Producing Animals in Brazil. *Microbiology Spectrum,* 10**,** e00565-22.

CASTELIJN, G. A., VAN DER VEEN, S., ZWIETERING, M. H., MOEZELAAR, R. & ABEE, T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of Salmonella Typhimurium strains of different origin in high and low nutrient medium. *Biofouling,* 28**,** 51-63.

CHAN, B. K., ABEDON, S. T. & LOC-CARRILLO, C. 2013. Phage cocktails and the future of phage therapy. *Future Microbiology,* 8**,** 769-783.

CHANG, J. T., SCHMID, M. F., HAASE-PETTINGELL, C., WEIGELE, P. R., KING, J. A. & CHIU, W. 2010. Visualizing the structural changes of bacteriophage Epsilon15 and its Salmonella host during infection. *Journal of molecular biology,* 402**,** 731-740.

CHANG, R. Y., WONG, J., MATHAI, A., MORALES, S., KUTTER, E., BRITTON, W., LI, J. & CHAN, H. K. 2017. Production of highly stable spray dried phage formulations for treatment of Pseudomonas aeruginosa lung infection. *Eur J Pharm Biopharm,* 121**,** 1-13.

CHATTAWAY, M. A., PAINSET, A., GODBOLE, G., GHARBIA, S. & JENKINS, C. 2023. Evaluation of Genomic Typing Methods in the Salmonella Reference Laboratory in Public Health, England, 2012-2020. *Pathogens,* 12. p.223.

CHEN, B., AKUSOBI, C., FANG, X. & SALMOND, G. P. C. 2017. Environmental T4-Family Bacteriophages Evolve to Escape Abortive Infection via Multiple Routes in a Bacterial Host Employing “Altruistic Suicide” through Type III Toxin-Antitoxin Systems. *Frontiers in Microbiology,* 8. p.1006

CHEN, S., ZHOU, Y., CHEN, Y. & GU, J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *bioRxiv***,** 274100.

CHIOU, C.-S., CHEN, Y.-T., WANG, Y.-W., LIU, Y.-Y., KUO, H.-C., TU, Y.-H., LIN, A.-C., LIAO, Y.-S. & HONG, Y.-P. 2017. Dissemination of imcr-1/i -Carrying Plasmids among Colistin-Resistant Salmonella Strains from Humans and Food-Producing Animals in Taiwan. *Antimicrobial Agents and Chemotherapy,* 61(7). pp.10-1128.

CHOPIN, M.-C., CHOPIN, A. & BIDNENKO, E. 2005. Phage abortive infection in lactococci: variations on a theme. *Current Opinion in Microbiology,* 8**,** 473-479.

CLAVIJO, V., BAQUERO, D., HERNANDEZ, S., FARFAN, J. C., ARIAS, J., ARÉVALO, A., DONADO-GODOY, P. & VIVES-FLORES, M. 2019. Phage cocktail SalmoFREE® reduces Salmonella on a commercial broiler farm. *Poultry science,* 98**,** 5054-5063.

COBURN, B., GRASSL, G. A. & FINLAY, B. B. 2007. Salmonella, the host and disease: a brief review. *Immunology & Cell Biology,* 85**,** 112-118.

COOK, R., BROWN, N., REDGWELL, T., RIHTMAN, B., BARNES, M., CLOKIE, M., STEKEL, D. J., HOBMAN, J., JONES, M. A. & MILLARD, A. 2021. INfrastructure for a PHAge REference Database: Identification of Large-Scale Biases in the Current Collection of Cultured Phage Genomes. *PHAGE,* 2**,** 214-223.

COOK, R., BROWN, N., RIHTMAN, B., MICHNIEWSKI, S., REDGWELL, T., STEKEL, D. J., HOBMAN, J., CHEN, Y., NELSON, A. & SCANLAN, D. 2023. The long and short of it: Benchmarking viromics using Illumina, Nanopore and PacBio sequencing technologies. *bioRxiv***,** 2023.02. 12.527533.

COTA, I., SÁNCHEZ-ROMERO, M. A., HERNÁNDEZ, S. B., PUCCIARELLI, M. G., GARCÍA-DEL PORTILLO, F. & CASADESÚS, J. 2015. Epigenetic Control of Salmonella enterica O-Antigen Chain Length: A Tradeoff between Virulence and Bacteriophage Resistance. *PLoS genetics,* 11**,** e1005667-e1005667.

COWLEY, L. A., LOW, A. S., PICKARD, D., BOINETT, C. J., DALLMAN, T. J., DAY, M., PERRY, N., GALLY, D. L., PARKHILL, J., JENKINS, C., CAIN, A. K., FRASER, C. M. & COLLIER, R. J. 2018. Transposon Insertion Sequencing Elucidates Novel Gene Involvement in Susceptibility and Resistance to Phages T4 and T7 in Escherichia coli O157. *mBio,* 9**,** e00705-18.

CUCCHIARA, S., IEBBA, V., CONTE, M. P. & SCHIPPA, S. 2009. The microbiota in inflammatory bowel disease in different age groups. *Digestive Diseases,* 27.(3)

DĄBROWSKA, K. 2019. Phage therapy: What factors shape phage pharmacokinetics and bioavailability? Systematic and critical review. *Med Res Rev,* 39**,** 2000-2025.

DATSENKO, K. A. & WANNER, B. L. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences,* 97**,** 6640-6645.

DATTA, S., COSTANTINO, N. & COURT, D. L. 2006. A set of recombineering plasmids for gram-negative bacteria. *Gene,* 379**,** 109-115.

DAVIES, M. R., BROADBENT, S. E., HARRIS, S. R., THOMSON, N. R. & VAN DER WOUDE, M. W. 2013. Horizontally Acquired Glycosyltransferase Operons Drive Salmonellae Lipopolysaccharide Diversity. *PLOS Genetics,* 9**,** e1003568.

DAVIES, R. H., LAWES, J. R. & WALES, A. D. 2019. Raw diets for dogs and cats: a review, with particular reference to microbiological hazards. *J Small Anim Pract,* 60**,** 329-339.

DEMCZUK, W., SOULE, G., CLARK, C., ACKERMANN, H.-W., EASY, R., KHAKHRIA, R., RODGERS, F. & AHMED, R. 2003. Phage-based typing scheme for Salmonella enterica serovar Heidelberg, a causative agent of food poisonings in Canada. *Journal of clinical microbiology,* 41**,** 4279-4284.

DIRIENZO, J. M., NAKAMURA, K. & INOUYE, M. 1978. The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly, and functions. *Annual Review of Biochemistry,* 47**,** 481-532.

DOUBLET, B., BOYD, D., MULVEY, M. R. & CLOECKAERT, A. 2005. The Salmonella genomic island 1 is an integrative mobilizable element. *Molecular Microbiology,* 55**,** 1911-1924.

DOULGERAKI, A. I., ERCOLINI, D., VILLANI, F. & NYCHAS, G.-J. E. 2012. Spoilage microbiota associated to the storage of raw meat in different conditions. *International journal of food microbiology,* 157**,** 130-141.

DOWAH, A. S. A. & CLOKIE, M. R. J. 2018. Review of the nature, diversity and structure of bacteriophage receptor binding proteins that target Gram-positive bacteria. *Biophysical reviews,* 10**,** 535-542.

DUDA, R. L. & TESCHKE, C. M. 2019. The amazing HK97 fold: versatile results of modest differences. *Current Opinion in Virology.,36*, pp.9-16.

DUPUIS, M.-È., VILLION, M., MAGADÁN, A. H. & MOINEAU, S. 2013. CRISPR-Cas and restriction–modification systems are compatible and increase phage resistance. *Nature Communications,* 4**,** 2087.

DY, R. L., PRZYBILSKI, R., SEMEIJN, K., SALMOND, G. P. C. & FINERAN, P. C. 2014. A widespread bacteriophage abortive infection system functions through a Type IV toxin-antitoxin mechanism. *Nucleic acids research,* 42**,** 4590-4605.

EFSA 2014. EFSA explains zoonotic diseases - Salmonella. *EFSA explains zoonotic diseases*.

EL HAG, M., FENG, Z., SU, Y., WANG, X., YASSIN, A., CHEN, S., PENG, D. & LIU, X. 2017. Contribution of the csgA and bcsA genes to Salmonella enterica serovar Pullorum biofilm formation and virulence. *Avian Pathol,* 46**,** 541-547.

ENG, S.-K., PUSPARAJAH, P., AB MUTALIB, N.-S., SER, H.-L., CHAN, K.-G. & LEE, L.-H. 2015. Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science,* 8**,** 284-293.

FAGERLUND, A., HEIR, E., MØRETRØ, T. & LANGSRUD, S. 2020. Listeria Monocytogenes Biofilm Removal Using Different Commercial Cleaning Agents. *Molecules,* 25(4), p.792.

FEASEY, N. A., DOUGAN, G., KINGSLEY, R. A., HEYDERMAN, R. S. & GORDON, M. A. 2012. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *The Lancet,* 379**,** 2489-2499.

FELLOWS, P. J. 2017. Freeze drying and freeze concentration. *Food Processing Technology.*

FERRARI, R. G., PANZENHAGEN, P. H. N. & CONTE-JUNIOR, C. A. 2017. Phenotypic and Genotypic Eligible Methods for Salmonella Typhimurium Source Tracking. *Frontiers in Microbiology,* 8. p.2587

FERRIOL-GONZÁLEZ, C. & DOMINGO-CALAP, P. 2021. Phage Therapy in Livestock and Companion Animals. *Antibiotics (Basel),* 10 (5), p.559.

FIGUEIRA, R. & HOLDEN, D. W. 2012. Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology,* 158**,** 1147-1161.

FIGUEROA-BOSSI, N. & BOSSI, L. 2004. Resuscitation of a defective prophage in Salmonella cocultures. *Journal of Bacteriology,* 186(12), pp.4038-4041.

FIGUEROA-BOSSI, N., COISSAC, E., NETTER, P. & BOSSI, L. 1997. Unsuspected prophage-like elements in Salmonella typhimurium. *Molecular microbiology,* 25**,** 161-173.

FINLEY, R., RAID-SMITH, R. & WEESE, J. S. 2006. Human health implications of Salmonella-contaminated natural pet treats and raw pet food. *Clinical Infectious Diseases. 42*(5), pp.686-691

FINLEY, R., RIBBLE, C., ARAMINI, J., VANDERMEER, M., POPA, M., LITMAN, M. & REID-SMITH, R. 2007. The risk of salmonellae shedding by dogs fed Salmonella-contaminated commercial raw food diets. *Can Vet J,* 48**,** 69-75.

FORTERRE, P. & GAÏA, M. 2021. The Origin of Viruses. *Encyclopedia of Virology.*

FORTIER, L.-C. & SEKULOVIC, O. 2013. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence,* 4**,** 354-365.

FRIEDMAN, S. D., GENTHNER, F. J., GENTRY, J., SOBSEY, M. D. & VINJÉ, J. 2009. Gene Mapping and Phylogenetic Analysis of the Complete Genome from 30 Single-Stranded RNA Male-Specific Coliphages (Family Leviviridae). *Journal of Virology,* 83**,** 11233-11243.

FRYE, J. G. & JACKSON, C. R. 2013. Genetic mechanisms of antimicrobial resistance identified in Salmonella enterica, Escherichia coli, and Enteroccocus spp. isolated from U.S. food animals. *Front Microbiol,* 4**,** 135.

FUTOMA-KOŁOCH, B., BUGLA-PŁSKOŃSKA, G., DUDEK, B., DOROTKIEWICZ-JACH, A., DRULIS-KAWA, Z. & GAMIAN, A. 2019. Outer membrane proteins of Salmonella as potential markers of resistance to serum, antibiotics and biocides. *Current medicinal chemistry,* 26**,** 1960-1978.

GARCIA-RUSSELL, N., ELROD, B. & DOMINGUEZ, K. 2009. Stress-induced prophage DNA replication in Salmonella enterica serovar Typhimurium. *Infection, Genetics and Evolution,* 9.

GATHERER, D., DEPLEDGE, D. P., HARTLEY, C. A., SZPARA, M. L., VAZ, P. K., BENKŐ, M., BRANDT, C. R., BRYANT, N. A., DASTJERDI, A., DOSZPOLY, A., GOMPELS, U. A., INOUE, N., JAROSINSKI, K. W., KAUL, R., LACOSTE, V., NORBERG, P., ORIGGI, F. C., ORTON, R. J., PELLETT, P. E., SCHMID, D. S., SPATZ, S. J., STEWART, J. P., TRIMPERT, J., WALTZEK, T. B., DAVISON, A. J., SIDDELL, S. G., LEFKOWITZ, E. J., SABANADZOVIC, S., SIMMONDS, P., ZERBINI, F. M., SMITH, D. B. & VARSANI, A. 2021. ICTV virus taxonomy profile: Herpesviridae 2021. *Journal of General Virology,* 102.

GILL, M. A., RAFIQUE, M. W., MANAN, T., SLAEEM, S., RÖMLING, U., MATIN, A. & AHMAD, I. 2018. The cellulose synthase BcsA plays a role in interactions of Salmonella typhimurium with Acanthamoeba castellanii genotype T4. *Parasitol Res,* 117**,** 2283-2289.

GNOPO, Y. M. D., WATKINS, H. C., STEVENSON, T. C., DELISA, M. P. & PUTNAM, D. 2017. Designer outer membrane vesicles as immunomodulatory systems – Reprogramming bacteria for vaccine delivery. *Advanced Drug Delivery Reviews,* 114**,** 132-142.

GOERKE, C., KÖLLER, J. & WOLZ, C. 2006. Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in Staphylococcus aureus. *Antimicrobial agents and chemotherapy,* 50**,** 171-177.

GOLDFARB, T., SBERRO, H., WEINSTOCK, E., COHEN, O., DORON, S., CHARPAK-AMIKAM, Y., AFIK, S., OFIR, G. & SOREK, R. 2015. BREX is a novel phage resistance system widespread in microbial genomes. *The EMBO Journal,* 34**,** 169-183.

GORDILLO ALTAMIRANO, F. L. & BARR, J. J. 2019. Phage Therapy in the Postantibiotic Era. *Clinical Microbiology Reviews,* 32**,** e00066-18.

GREENFIELD, L. K. & WHITFIELD, C. 2012. Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. *Carbohydrate research,* 356**,** 12-24.

GREGORY, A. C., GERHARDT, K., ZHONG, Z.-P., BOLDUC, B., TEMPERTON, B., KONSTANTINIDIS, K. T. & SULLIVAN, M. B. 2022. MetaPop: a pipeline for macro- and microdiversity analyses and visualization of microbial and viral metagenome-derived populations. *Microbiome,* 10**,** 49.

GRIMONT, P. A. D. & WEILL, F. X. 2007. Antigenic formulae of the *Salmonella* serovars.9 th edition. *WHO Collaborating Center for Reference and Research on Salmonella. France: Institute Pasteur. 9*, pp.1-166.

GRYGORCEWICZ, B., CHAJECKA-WIERZCHOWSKA, W., AUGUSTYNIAK, A., WASAK, A., STACHURSKA, X., NAWROTEK, P. & DOLEGOWSKA, B. In-milk inactivation of Escherichia coli O157:H7 by the environmental lytic bacteriophage ECPS-6. *Journal of Food Safety*. *40*(2), p.e12747.

HALL, M. D., HANDLEY, M. D. & GOTTESMAN, M. M. 2009. Is resistance useless? Multidrug resistance and collateral sensitivity. *Trends in pharmacological sciences,* 30**,** 546-556.

HAN, K. H., CHOI, S. Y., LEE, J. H., LEE, H., SHIN, E. H., AGTINI, M. D., VON SEIDLEIN, L., OCHIAI, R. L., CLEMENS, J. D., WAIN, J., HAHN, J. S., LEE, B. K., SONG, M., CHUN, J. & KIM, D. W. 2006. Isolation of Salmonella enterica subspecies enterica serovar Paratyphi B dT+, or Salmonella Java, from Indonesia and alteration of the d-tartrate fermentation phenotype by disrupting the ORF STM 3356. *J Med Microbiol,* 55**,** 1661-1665.

HARRELL, J. E., HAHN, M. M., D'SOUZA, S. J., VASICEK, E. M., SANDALA, J. L., GUNN, J. S. & MCLACHLAN, J. B. 2021. Salmonella biofilm formation, chronic infection, and immunity within the intestine and hepatobiliary tract. *Frontiers in cellular and infection microbiology,* 10**,** 624622.

HAVELAAR, A. H., KIRK, M. D., TORGERSON, P. R., GIBB, H. J., HALD, T., LAKE, R. J., PRAET, N., BELLINGER, D. C., DE SILVA, N. R., GARGOURI, N., SPEYBROECK, N., CAWTHORNE, A., MATHERS, C., STEIN, C., ANGULO, F. J. & DEVLEESSCHAUWER, B. 2015. World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. *PLOS Medicine,* 12**,** e1001923-e1001923.

HEJAIR, H. M. A., ZHU, Y., MA, J., ZHANG, Y., PAN, Z., ZHANG, W. & YAO, H. 2017. Functional role of ompF and ompC porins in pathogenesis of avian pathogenic Escherichia coli. *Microbial Pathogenesis,* 107**,** 29-37.

HENROT, C. & PETIT, M. A. 2022. Signals triggering prophage induction in the gut microbiota. *Molecular Microbiology,* 118**,** 494-502.

HINKLEY, T. C., SINGH, S., GARING, S., LE NY, A.-L. M., NICHOLS, K. P., PETERS, J. E., TALBERT, J. N. & NUGEN, S. R. 2018. A phage-based assay for the rapid, quantitative, and single CFU visualization of E. coli (ECOR #13) in drinking water. *Scientific reports,* 8**,** 14630-14630.

HOCKENBERRY, A. J. & WILKE, C. O. 2021. BACPHLIP: predicting bacteriophage lifestyle from conserved protein domains. *PeerJ,* 9**,** e11396.

HOFER, B., RUGE, M. & DREISEIKELMANN, B. 1995. The superinfection exclusion gene (sieA) of bacteriophage P22: identification and overexpression of the gene and localization of the gene product. *Journal of Bacteriology,* 177**,** 3080-3086.

HOFER, U. 2016. The sting is in the phage's tail. *Nature Reviews Microbiology,* 14**,** 477-477.

HOLDEN, E. R., YASIR, M., TURNER, A. K., WAIN, J., CHARLES, I. G. & WEBBER, M. A. 2021. Massively parallel transposon mutagenesis identifies temporally essential genes for biofilm formation in Escherichia coli. *Microbial genomics,* 7**,** 000673.

HUA, Z., KORANY, A. M., EL-SHINAWY, S. H. & ZHU, M. J. 2019. Comparative Evaluation of Different Sanitizers Against Listeria monocytogenes Biofilms on Major Food-Contact Surfaces. *Front Microbiol,* 10**,** 2462.

HUMPHREY, T. 2004. Salmonella, stress responses and food safety. *Nature Reviews Microbiology,* 2**,** 504-509.

HUNT, M., MATHER, A. E., SÁNCHEZ-BUSÓ, L., PAGE, A. J., PARKHILL, J., KEANE, J. A. & HARRIS, S. R. 2017. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microb Genom,* 3**,** e000131.

ICTV (2023). *Current ICTV Taxonomy Release | ICTV*. [online] Available at: https://ictv.global/taxonomy. Accessed:March 2023

IIDA, S., STREIFF, M. B., BICKLE, T. A. & ARBER, W. 1987. Two DNA antirestriction systems of bacteriophage P1, darA, and darB: characterization of darA− phages. *Virology,* 157**,** 156-166.

ISLAM, M. S., ZHOU, Y., LIANG, L., NIME, I., LIU, K., YAN, T., WANG, X. & LI, J. 2019. Application of a Phage Cocktail for Control of Salmonella in Foods and Reducing Biofilms. *Viruses,* 11 (9), p.841..

ISO (2022) *ISO 6579:2002*. [online] ISO. Available at: https://www.iso.org/standard/29315.html. Accessed: March 2023

JAN, A. T. 2017. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Frontiers in microbiology,* 8**,** 1053-1053.

JOŃCZYK, E., KŁAK, M., MIĘDZYBRODZKI, R. & GÓRSKI, A. 2011. The influence of external factors on bacteriophages. *Folia microbiologica,* 56**,** 191-200.

KALYAANAMOORTHY, S., MINH, B. Q., WONG, T. K. F., VON HAESELER, A. & JERMIIN, L. S. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods,* 14**,** 587-589.

KARCZEWSKI, K. J., FRANCIOLI, L. C., TIAO, G., CUMMINGS, B. B., ALFÖLDI, J., WANG, Q., COLLINS, R. L., LARICCHIA, K. M., GANNA, A., BIRNBAUM, D. P., GAUTHIER, L. D., BRAND, H., SOLOMONSON, M., WATTS, N. A., RHODES, D., SINGER-BERK, M., ENGLAND, E. M., SEABY, E. G., KOSMICKI, J. A., WALTERS, R. K., TASHMAN, K., FARJOUN, Y., BANKS, E., POTERBA, T., WANG, A., SEED, C., WHIFFIN, N., CHONG, J. X., SAMOCHA, K. E., PIERCE-HOFFMAN, E., ZAPPALA, Z., O'DONNELL-LURIA, A. H., MINIKEL, E. V., WEISBURD, B., LEK, M., WARE, J. S., VITTAL, C., ARMEAN, I. M., BERGELSON, L., CIBULSKIS, K., CONNOLLY, K. M., COVARRUBIAS, M., DONNELLY, S., FERRIERA, S., GABRIEL, S., GENTRY, J., GUPTA, N., JEANDET, T., KAPLAN, D., LLANWARNE, C., MUNSHI, R., NOVOD, S., PETRILLO, N., ROAZEN, D., RUANO-RUBIO, V., SALTZMAN, A., SCHLEICHER, M., SOTO, J., TIBBETTS, K., TOLONEN, C., WADE, G., TALKOWSKI, M. E., AGUILAR SALINAS, C. A., AHMAD, T., ALBERT, C. M., ARDISSINO, D., ATZMON, G., BARNARD, J., BEAUGERIE, L., BENJAMIN, E. J., BOEHNKE, M., BONNYCASTLE, L. L., BOTTINGER, E. P., BOWDEN, D. W., BOWN, M. J., CHAMBERS, J. C., CHAN, J. C., CHASMAN, D., CHO, J., CHUNG, M. K., COHEN, B., CORREA, A., DABELEA, D., DALY, M. J., DARBAR, D., DUGGIRALA, R., DUPUIS, J., ELLINOR, P. T., ELOSUA, R., ERDMANN, J., ESKO, T., FÄRKKILÄ, M., FLOREZ, J., FRANKE, A., GETZ, G., GLASER, B., GLATT, S. J., GOLDSTEIN, D., GONZALEZ, C., GROOP, L., et al. 2020. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature,* 581**,** 434-443.

KARIUKI, S., GORDON, M. A., FEASEY, N. & PARRY, C. M. 2015. Antimicrobial resistance and management of invasive Salmonella disease. *Vaccine,* 33 Suppl 3**,** C21-C29.

KATOH, K., MISAWA, K., KUMA, K. I. & MIYATA, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic acids research,* 30**,** 3059-3066.

KAZOU, M., GAVRIIL, A., KALAGKATSI, O., PASCHOS, T. & TSAKALIDOU, E. 2022. The impact of different inoculation schemes on the microbiota, physicochemical and sensory characteristics of Greek Kopanisti cheese throughout production and ripening. *Microorganisms,* 11**,** 66.

KILONZO-NTHENGE, A. & MUKUNA, W. 2018. *Salmonella and antimicrobial resistance in fresh produce*, InTech Rijeka.

KIM, J.-W., DUTTA, V., ELHANAFI, D., LEE, S., OSBORNE, J. A. & KATHARIOU, S. 2012. A novel restriction-modification system is responsible for temperature-dependent phage resistance in Listeria monocytogenes ECII. *Appl. Environ. Microbiol.,* 78**,** 1995-2004.

KIM, S.-H., JYUNG, S. & KANG, D.-H. 2022. Comparative study of Salmonella Typhimurium biofilms and their resistance depending on cellulose secretion and maturation temperatures. *LWT,* 154**,** 112700.

KINGSLEY, R. A., MSEFULA, C. L., THOMSON, N. R., KARIUKI, S., HOLT, K. E., GORDON, M. A., HARRIS, D., CLARKE, L., WHITEHEAD, S., SANGAL, V., MARSH, K., ACHTMAN, M., MOLYNEUX, M. E., CORMICAN, M., PARKHILL, J., MACLENNAN, C. A., HEYDERMAN, R. S. & DOUGAN, G. 2009. Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome research,* 19**,** 2279-2287.

KIRKWOOD, M., VOHRA, P., BAWN, M., THILLIEZ, G., PYE, H., TANNER, J., CHINTOAN-UTA, C., BRANCHU, P., PETROVSKA, L., DALLMAN, T., HALL, N., STEVENS, M. P. & KINGSLEY, R. A. 2021. Ecological niche adaptation of Salmonella Typhimurium U288 is associated with altered pathogenicity and reduced zoonotic potential. *Communications Biology,* 4**,** 498.

KNECHT, L. E., VELJKOVIC, M. & FIESELER, L. 2019. Diversity and Function of Phage Encoded Depolymerases. *Front Microbiol,* 10**,** 2949.

KODERI VALAPPIL, S., SHETTY, P., DEIM, Z., TERHES, G., URBÁN, E., VÁCZI, S., PATAI, R., POLGÁR, T., PERTICS, B. Z. & SCHNEIDER, G. 2021. Survival comes at a cost: a coevolution of phage and its host leads to phage resistance and antibiotic sensitivity of pseudomonas aeruginosa multidrug resistant strains. *Frontiers in Microbiology,* 12**,** 783722.

KOEBNIK, R., LOCHER, K. P. & VAN GELDER, P. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Molecular Microbiology,* 37**,** 239-253.

KOLODZIEJEK, A. M. & MILLER, S. I. 2015. S almonella modulation of the phagosome membrane, role of S se J. *Cellular microbiology,* 17**,** 333-341.

KONG, Q., YANG, J., LIU, Q., ALAMURI, P., ROLAND, K. L. & CURTISS III, R. 2011. Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of Salmonella enterica serovar Typhimurium. *Infection and immunity,* 79**,** 4227-4239.

KOOHMARAIE, M., ARTHUR, T. M., BOSILEVAC, J. M., GUERINI, M., SHACKELFORD, S. D. & WHEELER, T. L. 2005. Post-harvest interventions to reduce/eliminate pathogens in beef. *Meat Science,* 71**,** 79-91.

KORTRIGHT, K. E., CHAN, B. K. & TURNER, P. E. 2020. High-throughput discovery of phage receptors using transposon insertion sequencing of bacteria. *Proceedings of the National Academy of Sciences,* 117**,** 18670-18679.

KRASOWSKA, A., BIEGALSKA, A., AUGUSTYNIAK, D., ŁOŚ, M., RICHERT, M. & ŁUKASZEWICZ, M. 2015. Isolation and characterization of phages infecting bacillus subtilis. *BioMed Research International,* 2015.

KRYSIAK-BALTYN, K., MARTIN, G. J. & GRAS, S. L. 2018. Computational modelling of large scale phage production using a two-stage batch process. *Pharmaceuticals,* 11**,** 31.

KUTTER, E. 2009. Phage Host Range and Efficiency of Plating. *In:* CLOKIE, M. R. J. & KROPINSKI, A. M. (eds.) *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions.* Totowa, NJ: Humana Press.

LABRIE, S. J., SAMSON, J. E. & MOINEAU, S. 2010. Bacteriophage resistance mechanisms. *Nature Reviews Microbiology,* 8**,** 317-327.

LAKSHMANAN, R. S., GUNTUPALLI, R., HU, J., PETRENKO, V. A., BARBAREE, J. M. & CHIN, B. A. 2007. Detection of Salmonella typhimurium in fat free milk using a phage immobilized magnetoelastic sensor. *Sensors and Actuators B: Chemical,* 126**,** 544-550.

LANGRIDGE, G. C., PHAN, M.-D., TURNER, D. J., PERKINS, T. T., PARTS, L., HAASE, J., CHARLES, I., MASKELL, D. J., PETERS, S. E. & DOUGAN, G. 2009. Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome research,* 19**,** 2308-2316.

LÁZÁR, V., PAL SINGH, G., SPOHN, R., NAGY, I., HORVÁTH, B., HRTYAN, M., BUSA-FEKETE, R., BOGOS, B., MÉHI, O. & CSÖRGŐ, B. 2013. Bacterial evolution of antibiotic hypersensitivity. *Molecular systems biology,* 9**,** 700.

LEMIRE, S., FIGUEROA-BOSSI, N. & BOSSI, L. 2011. Bacteriophage crosstalk: coordination of prophage induction by trans-acting antirepressors. *PLoS Genet,* 7**,** e1002149.

LENZ, J., JOFFE, D., KAUFFMAN, M., ZHANG, Y. & LEJEUNE, J. 2009. Perceptions, practices, and consequences associated with foodborne pathogens and the feeding of raw meat to dogs. *Canadian Veterinary Journal,* 50(6), p.637..

LI, D., LIU, C.-M., LUO, R., SADAKANE, K. & LAM, T.-W. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics,* 31**,** 1674-1676.

LI, H. & DURBIN, R. 2010. Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics,* 26**,** 589-595.

LI, J., SMITH, N. H., NELSON, K., CRICHTON, P. B., OLD, D. C., WHITTAM, T. S. & SELANDER, R. K. 1993. Evolutionary origin and radiation of the avian-adapted non-motile salmonellae. *Journal of Medical Microbiology,* 38(2), pp.129-139..

LI, J., ZHANG, H., NING, J., SAJID, A., CHENG, G., YUAN, Z. & HAO, H. 2019. The nature and epidemiology of OqxAB, a multidrug efflux pump. *Antimicrobial Resistance & Infection Control,* 8**,** 44.

LI, S., LI, Y., CHEN, H., HORIKAWA, S., SHEN, W., SIMONIAN, A. & CHIN, B. A. 2010. Direct detection of Salmonella typhimurium on fresh produce using phage-based magnetoelastic biosensors. *Biosensors and Bioelectronics,* 26**,** 1313-1319.

LIM, C., KO, J., JEON, D., SONG, Y., PARK, J., RYU, J. & LEE, D. W. 2019. Probing molecular mechanisms of M13 bacteriophage adhesion. *Communications Chemistry,* 2**,** 96.

LIU, C. G., GREEN, S. I., MIN, L., CLARK, J. R., SALAZAR, K. C., TERWILLIGER, A. L., KAPLAN, H. B., TRAUTNER, B. W., RAMIG, R. F. & MARESSO, A. W. 2020. Phage-Antibiotic Synergy Is Driven by a Unique Combination of Antibacterial Mechanism of Action and Stoichiometry. *mBio,* 11(4), pp.10-1128..

LIU, G., THOMSEN, L. E. & OLSEN, J. E. 2022. Antimicrobial-induced horizontal transfer of antimicrobial resistance genes in bacteria: a mini-review. *Journal of Antimicrobial Chemotherapy,* 77**,** 556-567.

LOOD, C., HAAS, P.-J., VAN NOORT, V. & LAVIGNE, R. 2022. Shopping for phages? Unpacking design rules for therapeutic phage cocktails. *Current Opinion in Virology,* 52**,** 236-243.

LOU, L., ZHANG, P., PIAO, R. & WANG, Y. 2019. Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. *Frontiers in Cellular and Infection Microbiology,* 9 p.270..

LOWDEN, P., WALLIS, C., GEE, N. & HILTON, A. 2015. Investigating the prevalence of Salmonella in dogs within the Midlands region of the United Kingdom. *BMC Veterinary Research,* 11 (1), pp.1-6.

LU, M.-J. & HENNING, U. 1994. Superinfection exclusion by T-even-type coliphages. *Trends in Microbiology,* 2**,** 137-139.

LU, M. J. & HENNING, U. 1989. The immunity (imm) gene of Escherichia coli bacteriophage T4. *Journal of Virology,* 63**,** 3472-3478.

MAGNET, S., COURVALIN, P. & LAMBERT, T. 1999. Activation of the cryptic aac(6')-Iy aminoglycoside resistance gene of Salmonella by a chromosomal deletion generating a transcriptional fusion. *J Bacteriol,* 181**,** 6650-5.

MAHON, B. E., PÖNKÄ, A., HALL, W. N., KOMATSU, K., DIETRICH, S. E., SIITONEN, A., CAGE, G., HAYES, P. S., LAMBERT-FAIR, M. A. & BEAN, N. H. 1997. An international outbreak of Salmonella infections caused by alfalfa sprouts grown from contaminated seeds. *Journal of Infectious Diseases,* 175**,** 876-882.

MAHONY, J. & VAN SINDEREN, D. 2022. Virome studies of food production systems: Time for 'farm to fork'analyses. *Current Opinion in Biotechnology,* 73**,** 22-27.

MAKELA, P. 1973. Glucosylation of lipopolysaccharide in Salmonella: mutants negative for O antigen factor 1221. *J Bacteriol,* 116**,** 847-856.

MANDAL, P. K., BALLERIN, G., NOLAN, L. M., PETTY, N. K. & WHITCHURCH, C. B. 2020. Bacteriophage infection produces membrane vesicles in Escherichia coli via both explosive cell lysis and membrane blebbing. *bioRxiv***,** 2020.12.23.424155.

MANNING, A. J. & KUEHN, M. J. 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiology,* 11**,** 258.

MANOHAR, P. & RAMESH, N. 2019. Improved lyophilization conditions for long-term storage of bacteriophages. *Scientific reports,* 9**,** 15242.

MANRIQUE, P., DILLS, M. & YOUNG, M. J. 2017. The human gut phage community and its implications for health and disease. *Viruses,* 9**,** 141.

MARCUS, S. L., BRUMELL, J. H., PFEIFER, C. G. & FINLAY, B. B. 2000. Salmonella pathogenicity islands: big virulence in small packages. *Microbes and Infection,* 2**,** 145-156.

MARTINEZ-ANTON, L., MARENDA, M., FIRESTONE, S. M., BUSHELL, R. N., CHILD, G., HAMILTON, A. I., LONG, S. N. & LE CHEVOIR, M. A. R. 2018. Investigation of the Role of Campylobacter Infection in Suspected Acute Polyradiculoneuritis in Dogs. *Journal of Veterinary Internal Medicine,* 32 (1), pp.352-360.

MARTÍNEZ-RUBIO, R., QUILES-PUCHALT, N., MARTÍ, M., HUMPHREY, S., RAM, G., SMYTH, D., CHEN, J., NOVICK, R. P. & PENADÉS, J. R. 2017. Phage-inducible islands in the Gram-positive cocci. *The ISME Journal,* 11**,** 1029-1042.

MAYOLA, A., IRAZOKI, O., MARTÍNEZ, I. A., PETROV, D., MENOLASCINA, F., STOCKER, R., REYES-DARIAS, J. A., KRELL, T., BARBÉ, J. & CAMPOY, S. 2014. RecA protein plays a role in the chemotactic response and chemoreceptor clustering of Salmonella enterica. *PLoS One,* 9**,** e105578.

MCCAMMON, S., MAKAROVS, K., BANDUCCI, S. & GOLD, V. 2023. Phage therapy and the public: Increasing awareness essential to widespread use. *Plos one,* 18**,** e0285824.

MCCONNELL, M. J., ACTIS, L. & PACHÓN, J. 2013. Acinetobacter baumannii: human infections, factors contributing to pathogenesis and animal models. *FEMS microbiology reviews,* 37**,** 130-155.

MCMURDIE, P. J. & HOLMES, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE,* 8**,** e61217.

MEGAHED, A., ALDRIDGE, B. & LOWE, J. 2019. Comparative study on the efficacy of sodium hypochlorite, aqueous ozone, and peracetic acid in the elimination of Salmonella from cattle manure contaminated various surfaces supported by Bayesian analysis. *PLOS ONE,* 14**,** e0217428.

MEHLENBACHER, S., CHURCHILL, J., OLSEN, K. E. & BENDER, J. B. 2012. Availability, Brands, Labelling and Salmonella Contamination of Raw Pet Food in the Minneapolis/St. Paul Area. *Zoonoses and Public Health,* 59 (7), pp.513-520.

MIKANATHA, N. M., SANDT, C. H., LOCALIO, A. R., TEWARI, D., RANKIN, S. C., WHICHARD, J. M., ALTEKRUSE, S. F., LAUTENBACH, E., FOLSTER, J. P., RUSSO, A., CHILLER, T. M., REYNOLDS, S. M. & MCDERMOTT, P. F. 2010. Multidrug-Resistant iSalmonella/i Isolates from Retail Chicken Meat Compared with Human Clinical Isolates. *Foodborne Pathogens and Disease,* 7**,** 929-934.

MIROLD, S., EHRBAR, K., WEISSMÜLLER, A., PRAGER, R., TSCHÄPE, H., RÜSSMANN, H. & HARDT, W. D. 2001. Salmonella host cell invasion emerged by acquisition of a mosaic of separate genetic elements, including Salmonella pathogenicity island 1 (SPI1), SPI5, and sopE2. *Journal of bacteriology,* 183**,** 2348-2358.

MITTRÜCKER, H.-W. & KAUFMANN, S. H. E. 2000. Immune response to infection with Salmonella typhimurium in mice. *Journal of Leukocyte Biology,* 67**,** 457-463.

MOENS, U., CALVIGNAC-SPENCER, S., LAUBER, C., RAMQVIST, T., FELTKAMP, M. C., DAUGHERTY, M. D., VERSCHOOR, E. J., EHLERS, B. & CONSORTIUM, I. R. 2017. ICTV virus taxonomy profile: Polyomaviridae. *Journal of General Virology,* 98**,** 1159-1160.

MOHAMMED, M. 2017. Phage typing or CRISPR typing for epidemiological surveillance of Salmonella Typhimurium? *BMC Research Notes,* 10**,** 578.

MOOIJMAN, K. A. 2018. The new ISO 6579-1: A real horizontal standard for detection of Salmonella, at last! *Food microbiology,* 71**,** 2-7.

MORARU, C., VARSANI, A. & KROPINSKI, A. M. 2020. VIRIDIC—A Novel Tool to Calculate the Intergenomic Similarities of Prokaryote-Infecting Viruses. *Viruses* [Online], 12(11), p.1268..

MORITA, M., FISCHER, C. R., MIZOGUCHI, K., YOICHI, M., ODA, M., TANJI, Y. & UNNO, H. 2002. Amino acid alterations in Gp38 of host range mutants of PP01 and evidence for their infection of an ompC null mutant of Escherichia coli O157:H7. *FEMS Microbiology Letters,* 216**,** 243-248.

MORSE, E. V., DUNCAN, M. A., ESTEP, D. A., RIGGS, W. A. & BLACKBURN, B. O. 1976. Canine salmonellosis: a review and report of dog to child transmission of Salmonella enteritidis. *American Journal of Public Health,* 66 (1), pp.82-83..

MOTTAWEA, W., DUCEPPE, M.-O., DUPRAS, A. A., USONGO, V., JEUKENS, J., FRESCHI, L., EMOND-RHEAULT, J.-G., HAMEL, J., KUKAVICA-IBRULJ, I., BOYLE, B., GILL, A., BURNETT, E., FRANZ,

E., ARYA, G., WEADGE, J. T., GRUENHEID, S., WIEDMANN, M., HUANG, H., DAIGLE, F., MOINEAU, S., BEKAL, S., LEVESQUE, R. C., GOODRIDGE, L. D. & OGUNREMI, D. 2018. Salmonella enterica Prophage Sequence Profiles Reflect Genome Diversity and Can Be Used for High Discrimination Subtyping. *Frontiers in microbiology,* 9**,** 836-836.

MUKHERJEE, N., NOLAN, V. G., DUNN, J. R. & BANERJEE, P. 2020. Exposures associated with non-typhoidal Salmonella infections caused by Newport, Javiana, and Mississippi serotypes in Tennessee, 2013–2015: a case-case analysis. *Pathogens,* 9**,** 78.

MUKHOPADHYAY, S. & RAMASWAMY, R. 2012. Application of emerging technologies to control Salmonella in foods: A review. *Food Research International,* 45**,** 666-677.

MÜLLER, A. J., HOFFMANN, C., GALLE, M., VAN DEN BROEKE, A., HEIKENWALDER, M., FALTER, L., MISSELWITZ, B., KREMER, M., BEYAERT, R. & HARDT, W. D. 2009. The S. Typhimurium Effector SopE Induces Caspase-1 Activation in Stromal Cells to Initiate Gut Inflammation. *Cell Host and Microbe,* 6 (2), pp.125-136..

MUNNOCH, S., WARD, K., SHERIDAN, S., FITZSIMMONS, G., SHADBOLT, C. T., PIISPANEN, J., WANG, Q., WARD, T., WORGAN, T. & OXENFORD, C. 2009. A multi-state outbreak of Salmonella Saintpaul in Australia associated with cantaloupe consumption. *Epidemiology & Infection,* 137**,** 367-374.

MUTTI, M. & CORSINI, L. 2019. Robust Approaches for the Production of Active Ingredient and Drug Product for Human Phage Therapy. *Frontiers in Microbiology,* 10 p.2289.

NEMSER, S. M., DORAN, T., GRABENSTEIN, M., MCCONNELL, T., MCGRATH, T., PAMBOUKIAN, R., SMITH, A. C., ACHEN, M., DANZEISEN, G., KIM, S., LIU, Y., ROBESON, S., ROSARIO, G., MCWILLIAMS WILSON, K. & REIMSCHUESSEL, R. 2014. Investigation of listeria, salmonella, and toxigenic escherichia coli in various pet foods. *Foodborne Pathogens and Disease,* 11(9), pp.706-709..

NEUERT, S., NAIR, S., DAY, M. R., DOUMITH, M., ASHTON, P. M., MELLOR, K. C., JENKINS, C., HOPKINS, K. L., WOODFORD, N., DE PINNA, E., GODBOLE, G. & DALLMAN, T. J. 2018. Prediction of Phenotypic Antimicrobial Resistance Profiles From Whole Genome Sequences of Non-typhoidal Salmonella enterica. *Frontiers in microbiology,* 9**,** 592-592.

NGUYEN, L.-T., SCHMIDT, H. A., VON HAESELER, A. & MINH, B. Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular biology and evolution,* 32**,** 268-274.

NISHIMURA, Y., YOSHIDA, T., KURONISHI, M., UEHARA, H., OGATA, H. & GOTO, S. 2017. ViPTree: the viral proteomic tree server. *Bioinformatics,* 33**,** 2379-2380.

NIU, Y. D., LIU, H., DU, H., MENG, R., SAYED MAHMOUD, E., WANG, G., MCALLISTER, T. A. & STANFORD, K. 2021. Efficacy of Individual Bacteriophages Does Not Predict Efficacy of Bacteriophage Cocktails for Control of Escherichia coli O157. *Frontiers in Microbiology,* 12 p.616712..

NOLIVOS, S., CAYRON, J., DEDIEU, A., PAGE, A., DELOLME, F. & LESTERLIN, C. 2019. Role of AcrAB-TolC multidrug efflux pump in drug-resistance acquisition by plasmid transfer. *Science,* 364**,** 778-782.

NORTH, O. I., SAKAI, K., YAMASHITA, E., NAKAGAWA, A., IWAZAKI, T., BÜTTNER, C. R., TAKEDA, S. & DAVIDSON, A. R. 2019. Phage tail fibre assembly proteins employ a modular structure to drive the correct folding of diverse fibres. *Nature Microbiology,* 4**,** 1645-1653.

NORWOOD, D. E. & GILMOUR, A. 2000. The growth and resistance to sodium hypochlorite of Listeria monocytogenes in a steady-state multispecies biofilm. *J Appl Microbiol,* 88**,** 512-20.

OFIR, G. & SOREK, R. 2018. Contemporary Phage Biology: From Classic Models to New Insights. *Cell,* 172**,** 1260-1270.

OKITSU, N., KAIEDA, S., YANO, H., NAKANO, R., HOSAKA, Y., OKAMOTO, R., KOBAYASHI, T. & INOUE, M. 2005. Characterization of ermB gene transposition by Tn1545 and Tn917 in macrolide-resistant Streptococcus pneumoniae isolates. *J Clin Microbiol,* 43**,** 168-73.

OTSUJI, N., SEKIGUCHI, M., IIJIMA, T. & TAKAGI, Y. 1959. Induction of phage formation in the lysogenic Escherichia coli K-12 by mitomycin C. *Nature,* 184**,** 1079-1080.

OWEN, S. V., WENNER, N., CANALS, R., MAKUMI, A., HAMMARLÖF, D. L., GORDON, M. A., AERTSEN, A., FEASEY, N. A. & HINTON, J. C. 2017a. Characterization of the Prophage Repertoire of African Salmonella Typhimurium ST313 Reveals High Levels of Spontaneous Induction of Novel Phage BTP1. *Front Microbiol,* 8**,** 235.

OWEN, S. V., WENNER, N., CANALS, R., MAKUMI, A., HAMMARLÖF, D. L., GORDON, M. A., AERTSEN, A., FEASEY, N. A. & HINTON, J. C. D. 2017b. Characterization of the Prophage Repertoire of African Salmonella Typhimurium ST313 Reveals High Levels of Spontaneous Induction of Novel Phage BTP1. *Frontiers in Microbiology,* 8 p.235..

OWEN, S. V., WENNER, N., DULBERGER, C. L., RODWELL, E. V., BOWERS-BARNARD, A., QUINONES-OLVERA, N., RIGDEN, D. J., RUBIN, E. J., GARNER, E. C., BAYM, M. & HINTON, J. C. D. 2021. Prophages encode phage-defense systems with cognate self-immunity. *Cell Host & Microbe,* 29**,** 1620-1633.e8.

PAL, C., BENGTSSON-PALME, J., RENSING, C., KRISTIANSSON, E. & LARSSON, D. G. 2014. BacMet: antibacterial biocide and metal resistance genes database. *Nucleic Acids Res,* 42**,** D737-43.

PAN, L., LI, D., SUN, Z., LIN, W., HONG, B., QIN, W., XU, L., LIU, W., ZHOU, Q., WANG, F., CAI, R., QIAN, M. & TONG, Y. 2021. First Characterization of a Hafnia Phage Reveals Extraordinarily Large Burst Size and Unusual Plaque Polymorphism. *Front Microbiol,* 12**,** 754331.

PARK, J. & KIM, E. B. 2020. Differences in microbiome and virome between cattle and horses in the same farm. *Asian-Australasian Journal of Animal Sciences,* 33**,** 1042.

PATEL, J. C. & GALÁN, J. E. 2005. Manipulation of the host actin cytoskeleton by Salmonella—all in the name of entry. *Current opinion in microbiology,* 8**,** 10-15.

PAVÓN, A. B. I. & MAIDEN, M. C. J. 2009. Multilocus Sequence Typing. *Methods in Molecular Biology.* Humana Press.

PETROVSKA, L., MATHER, A. E., ABUOUN, M., BRANCHU, P., HARRIS, S. R., CONNOR, T., HOPKINS, K. L., UNDERWOOD, A., LETTINI, A. A., PAGE, A., BAGNALL, M., WAIN, J., PARKHILL, J., DOUGAN, G., DAVIES, R. & KINGSLEY, R. A. 2016. Microevolution of Monophasic Salmonella Typhimurium during Epidemic, United Kingdom, 2005-2010. *Emerging infectious diseases,* 22**,** 617-624.

PETTY, N. K., EVANS, T. J., FINERAN, P. C. & SALMOND, G. P. C. 2007. Biotechnological exploitation of bacteriophage research. *Trends in Biotechnology,* 25**,** 7-15.

PICKARD, D., KINGSLEY, R. A., HALE, C., TURNER, K., SIVARAMAN, K., WETTER, M., LANGRIDGE, G. & DOUGAN, G. 2013. A genomewide mutagenesis screen identifies multiple genes contributing to Vi capsular expression in Salmonella enterica serovar Typhi. *J Bacteriol,* 195**,** 1320-6.

PREVELIGE, P. E. & CORTINES, J. R. 2018. Phage assembly and the special role of the portal protein. *Current Opinion in Virology,* 31**,** 66-73.

PRITCHARD, J. C., JACOB, M. E., WARD, T. J., PARSONS, C. T., KATHARIOU, S. & WOOD, M. W. 2016. Listeria monocytogenes septicemia in an immunocompromised dog. *Veterinary Clinical Pathology,* 45(2), pp.254-259..

PYE, H. V., THILLIEZ, G., ACTON, L., KOLENDA, R., AL-KHANAQ, H., GROVE, S. & KINGSLEY, R. A. 2023. Strain and serovar variants of Salmonella enterica exhibit diverse tolerance to food chain-related stress. *Food Microbiology,* 112**,** 104237.

RABSCH, W. 2007. Salmonella Typhimurium Phage Typing for Pathogens. *In:* SCHATTEN, H. & EISENSTARK, A. (eds.) *Salmonella: Methods and Protocols.* Totowa, NJ: Humana Press.

RAJKOVIC, A., TOMASEVIC, I., DE MEULENAER, B. & DEVLIEGHERE, F. 2017. The effect of pulsed UV light on Escherichia coli O157:H7, Listeria monocytogenes, Salmonella Typhimurium, Staphylococcus aureus and staphylococcal enterotoxin A on sliced fermented salami and its chemical quality. *Food Control,* 73**,** 829-837.

REYES-ROBLES, T., DILLARD, R. S., CAIRNS, L. S., SILVA-VALENZUELA, C. A., HOUSMAN, M., ALI, A., WRIGHT, E. R. & CAMILLI, A. 2018. Vibrio cholerae Outer Membrane Vesicles Inhibit Bacteriophage Infection. *Journal of Bacteriology,* 200**,** e00792-17.

ROBBINS, J. D. & ROBBINS, J. B. 1984. Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of Salmonella typhi. *Journal of infectious diseases,* 150**,** 436-449.

ROUX, S., EMERSON, J. B., ELOE-FADROSH, E. A. & SULLIVAN, M. B. 2017. Benchmarking viromics: an in silico evaluation of metagenome-enabled estimates of viral community composition and diversity. *PeerJ,* 5**,** e3817.

ROWLEY, G., SPECTOR, M., KORMANEC, J. & ROBERTS, M. 2006. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nature Reviews Microbiology,* 4**,** 383-394.

RYAN, E. M., ALKAWAREEK, M. Y., DONNELLY, R. F. & GILMORE, B. F. 2012. Synergistic phage-antibiotic combinations for the control ofiEscherichia coli/ibiofilmsiin vitro/i. *FEMS Immunology &amp Medical Microbiology,* 65**,** 395-398.

SALMOND, G. P. C. & FINERAN, P. C. 2015. A century of the phage: past, present and future. *Nature Reviews Microbiology,* 13**,** 777-786.

SAMPERS, I., HABIB, I., DE ZUTTER, L., DUMOULIN, A. & UYTTENDAELE, M. 2010. Survival of Campylobacter spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *International Journal of Food Microbiology,* 137 (2-3), pp.147-153..

SAVOLDI, A., CARRARA, E. & TACCONELLI, E. 2020. Gross national income and antibiotic resistance in invasive isolates: analysis of the top-ranked antibiotic-resistant bacteria on the 2017 WHO priority listauthors' response. *Journal of Antimicrobial Chemotherapy,* 75**,** 2018-2018.

SCHLESINGER, D. P. & JOFFE, D. J. 2011. Raw food diets in companion animals: a critical review. *Can Vet J,* 52**,** 50-4.

SCHMELCHER, M. & LOESSNER, M. J. 2014. Application of bacteriophages for detection of foodborne pathogens. *Bacteriophage,* 4**,** e28137-e28137.

SCHNAITMAN, C. A. & KLENA, J. D. 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiological Reviews,* 57**,** 655-682.

SCHRADER, K. N., FERNANDEZ-CASTRO, A., CHEUNG, W. K., CRANDALL, C. M. & ABBOTT, S. L. 2008. Evaluation of commercial antisera for Salmonella serotyping. *Journal of clinical microbiology,* 46**,** 685-688.

SCHWARZ, C., MATHIEU, J., LAVERDE GOMEZ, J. A., YU, P. & ALVAREZ, P. J. J. 2022. Renaissance for Phage-Based Bacterial Control. *Environmental Science and Technology,* 56 (8), pp.4691-4701..

SCHWECHHEIMER, C. & KUEHN, M. J. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nature reviews. Microbiology,* 13**,** 605-619.

SEED, K. D., LAZINSKI, D. W., CALDERWOOD, S. B. & CAMILLI, A. 2013. A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity. *Nature,* 494**,** 489-491.

SEEMAN, T. 2018. Shovill: faster SPAdes assembly of Illumina reads (v0. 9.0).

SEEMANN, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics,* 30**,** 2068-2069.

SERRA, D. O. & HENGGE, R. 2019. Cellulose in bacterial biofilms. *Extracellular Sugar-Based Biopolymers Matrices***,** 355-392.

SHABBIR, M. A. B., HAO, H., SHABBIR, M. Z., WU, Q., SATTAR, A. & YUAN, Z. 2016. Bacteria vs. Bacteriophages: Parallel Evolution of Immune Arsenals. *Frontiers in Microbiology,* 7 p.1292..

SHIN, H., LEE, J.-H., KIM, H., CHOI, Y., HEU, S. & RYU, S. 2012. Receptor diversity and host interaction of bacteriophages infecting Salmonella enterica serovar Typhimurium. PLOS ONE, e43392.

SILPHADUANG, U., MASCARENHAS, M., KARMALI, M. & COOMBES, B. K. 2007. Repression of intracellular virulence factors in Salmonella by the Hha and YdgT nucleoid-associated proteins. *Journal of bacteriology,* 189**,** 3669-3673.

SINGH, A. & YEMMIREDDY, V. 2021. Fate of Salmonella spp. in fresh-cut papaya (Carica papaya L.) at different storage temperature and relative humidity. *LWT,* 148**,** 111810.

SINHA, S., GREWAL, R. K. & ROY, S. 2018. Modeling bacteria–phage interactions and its implications for phage therapy. *Advances in applied microbiology.* (Vol. 103, pp. 103-141). Academic Press..

SITHOLE, T. R., MA, Y.-X., QIN, Z., WANG, X.-D. & LIU, H.-M. 2022. Peanut Butter Food Safety Concerns—Prevalence, Mitigation and Control of Salmonella spp., and Aflatoxins in Peanut Butter. *Foods,* 11**,** 1874.

SLAUCH, J. M., LEE, A. A., MAHAN, M. J. & MEKALANOS, J. J. 1996. Molecular characterization of the oafA locus responsible for acetylation of Salmonella typhimurium O-antigen: oafA is a member of a family of integral membrane trans-acylases. *Journal of bacteriology,* 178**,** 5904-5909.

SNYDER, L. 1995. Phage-exclusion enzymes: a bonanza of biochemical and cell biology reagents? *Molecular Microbiology,* 15**,** 415-420.

SOFFER, N., ABULADZE, T., WOOLSTON, J., LI, M., HANNA, L. F., HEYSE, S., CHARBONNEAU, D. & SULAKVELIDZE, A. 2016. Bacteriophages safely reduce Salmonella contamination in pet food and raw pet food ingredients. *Bacteriophage,* 6**,** e1220347.

SOLÍS, D., TORO, M., NAVARRETE, P., FAÚNDEZ, P. & REYES-JARA, A. 2022. Microbiological Quality and Presence of Foodborne Pathogens in Raw and Extruded Canine Diets and Canine Fecal Samples. *Frontiers in Veterinary Science,* 9 p.799710..

STACHLER, E., KULL, A. & JULIAN, T. R. 2021. Bacteriophage Treatment before Chemical Disinfection Can Enhance Removal of Plastic-Surface-Associated Pseudomonas aeruginosa. *Appl Environ Microbiol,* 87**,** e0098021.

STAMATAKIS, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* 30**,** 1312-1313.

STEHR-GREEN, J. K. & SCHANTZ, P. M. 1987. The impact of zoonotic diseases transmitted by pets on human health and the economy. *The Veterinary clinics of North America. Small animal practice. 17*(1), pp.1-15

STERN, A. & SOREK, R. 2011. The phage-host arms race: Shaping the evolution of microbes. *BioEssays,* 33**,** 43-51.

STONE, E., CAMPBELL, K., GRANT, I. & MCAULIFFE, O. 2019. Understanding and Exploiting Phage-Host Interactions. *Viruses,* 11**,** 567.

SUKUMARAN, A. T., NANNAPANENI, R., KIESS, A. & SHARMA, C. S. 2015. Reduction of Salmonella on chicken meat and chicken skin by combined or sequential application of lytic bacteriophage with chemical antimicrobials. *International journal of food microbiology,* 207**,** 8-15.

SUSSKIND, M. M., WRIGHT, A. & BOTSTEIN, D. 1971. Superinfection exclusion by P22 prophage in lysogens of Salmonella typhimurium: II. Genetic evidence for two exclusion systems. *Virology,* 45**,** 638-652.

TAGLIABUE, A. & RAPPUOLI, R. 2018. Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top. *Frontiers in immunology,* 9**,** 1068-1068.

TANNER, J. R. & KINGSLEY, R. A. 2018. Evolution of Salmonella within Hosts. *Trends in Microbiology. 26*(12), pp.986-998.

TASSINARI, E., BAWN, M., THILLIEZ, G., CHARITY, O., ACTON, L., KIRKWOOD, M., PETROVSKA, L., DALLMAN, T., BURGESS, C. M. & HALL, N. 2020. Whole-genome epidemiology links phage-mediated acquisition of a virulence gene to the clonal expansion of a pandemic Salmonella enterica serovar Typhimurium clone. *Microbial genomics,* 6(11).

TERZIAN, P., OLO NDELA, E., GALIEZ, C., LOSSOUARN, J., PÉREZ BUCIO, R. E., MOM, R., TOUSSAINT, A., PETIT, M.-A. & ENAULT, F. 2021. PHROG: families of prokaryotic virus proteins clustered using remote homology. *NAR Genomics and Bioinformatics,* 3**,** lqab067.

THANKI, A. M., BROWN, N., MILLARD, A. D. & CLOKIE, M. R. J. 2019. Genomic Characterization of Jumbo Salmonella Phages That Effectively Target United Kingdom Pig-Associated Salmonella Serotypes. *Front Microbiol,* 10**,** 1491.

THANKI, A. M., MIGNARD, G., ATTERBURY, R. J., BARROW, P., MILLARD, A. D. & CLOKIE, M. R. 2022. Prophylactic delivery of a bacteriophage cocktail in feed significantly reduces Salmonella colonization in pigs. *Microbiology spectrum,* 10**,** e00422-22.

TURNER, D., KROPINSKI, A. M. & ADRIAENSSENS, E. M. 2021. A Roadmap for Genome-Based Phage Taxonomy. *Viruses,* 13**,** 506.

TURNER, D., SHKOPOROV, A. N., LOOD, C., MILLARD, A. D., DUTILH, B. E., ALFENAS-ZERBINI, P., VAN ZYL, L. J., AZIZ, R. K., OKSANEN, H. M., PORANEN, M. M., KROPINSKI, A. M., BARYLSKI, J., BRISTER, J. R., CHANISVILI, N., EDWARDS, R. A., ENAULT, F., GILLIS, A., KNEZEVIC, P., KRUPOVIC, M., KURTBÖKE, I., KUSHKINA, A., LAVIGNE, R., LEHMAN, S., LOBOCKA, M., MORARU, C., MORENO SWITT, A., MOROZOVA, V., NAKAVUMA, J., REYES MUÑOZ, A., RŪMNIEKS, J., SARKAR, B. L., SULLIVAN, M. B., UCHIYAMA, J., WITTMANN, J., YIGANG, T. & ADRIAENSSENS, E. M. 2023. Abolishment of morphology-based taxa and change to binomial species names: 2022 taxonomy update of the ICTV bacterial viruses subcommittee. *Arch Virol,* 168**,** 74.

USMAEL, B., ABRAHA, B., ALEMU, S., MUMMED, B., HIKO, A. & ABDUREHMAN, A. 2022. Isolation, antimicrobial susceptibility patterns, and risk factors assessment of non-typhoidal Salmonella from apparently healthy and diarrheic dogs. *BMC Veterinary Research,* 18 (1), p.37.

VALEN, L. V. 1977. The Red Queen. *The American Naturalist,* 111**,** 809-810.

VAN BREE, F. P. J., BOKKEN, G. C. A. M., MINEUR, R., FRANSSEN, F., OPSTEEGH, M., VAN DER GIESSEN, J. W. B., LIPMAN, L. J. A. & OVERGAAUW, P. A. M. 2018. Zoonotic bacteria and parasites found in raw meat-based diets for cats and dogs. *The Veterinary record,* 182 (2), pp.50-50..

VAN DOORSLAER, K., CHEN, Z., BERNARD, H.-U., CHAN, P. K., DESALLE, R., DILLNER, J., FORSLUND, O., HAGA, T., MCBRIDE, A. A. & VILLA, L. L. 2018. ICTV virus taxonomy profile: Papillomaviridae. *Journal of General Virology,* 99**,** 989-990.

VAN GENT, M. & GACK, M. U. 2018. Viral Anti-CRISPR Tactics: No Success without Sacrifice. *Immunity,* 49**,** 391-393.

VELUSAMY, V., ARSHAK, K., KOROSTYNSKA, O., OLIWA, K. & ADLEY, C. 2010. An overview of foodborne pathogen detection: In the perspective of biosensors. *Biotechnology advances,* 28**,** 232-254.

VERMA, J., SAXENA, S. & BABU, S. G. 2012. ELISA-based identification and detection of microbes. *Analyzing Microbes: Manual of Molecular Biology Techniques.* (pp. 169-186). Berlin, Heidelberg: Springer Berlin Heidelberg..

VIKRAM, A., WOOLSTON, J. & SULAKVELIDZE, A. 2021. Phage biocontrol applications in food production and processing. *Current issues in molecular biology,* 40**,** 267-302.

VINNER, G. K., REZAIE-YAZDI, Z., LEPPANEN, M., STAPLEY, A. G., LEAPER, M. C. & MALIK, D. J. 2019. Microencapsulation of Salmonella-specific bacteriophage Felix O1 using spray-drying in a pH-responsive formulation and direct compression tableting of powders into a solid oral dosage form. *Pharmaceuticals,* 12**,** 43.

WALKER, P. J., SIDDELL, S. G., LEFKOWITZ, E. J., MUSHEGIAN, A. R., ADRIAENSSENS, E. M., ALFENAS-ZERBINI, P., DAVISON, A. J., DEMPSEY, D. M., DUTILH, B. E., GARCÍA, M. L., HARRACH, B., HARRISON, R. L., HENDRICKSON, R. C., JUNGLEN, S., KNOWLES, N. J., KRUPOVIC, M., KUHN, J. H., LAMBERT, A. J., ŁOBOCKA, M., NIBERT, M. L., OKSANEN, H. M., ORTON, R. J., ROBERTSON, D. L., RUBINO, L., SABANADZOVIC, S., SIMMONDS, P.,

SMITH, D. B., SUZUKI, N., VAN DOOERSLAER, K., VANDAMME, A. M., VARSANI, A. & ZERBINI, F. M. 2021. Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2021). *Archives of Virology,* 166 (9), pp.2633-2648..

WANG, F., HORIKAWA, S., HU, J., WIKLE, H., CHEN, I. H., DU, S., LIU, Y. & CHIN, B. 2017. Detection of Salmonella Typhimurium on Spinach Using Phage-Based Magnetoelastic Biosensors. *Sensors,* 17**,** 386-386.

WANG, F., WANG, D., HOU, W., JIN, Q., FENG, J. & ZHOU, D. 2019. Evolutionary Diversity of Prophage DNA in Klebsiella pneumoniae Chromosomes. *Frontiers in microbiology,* 10**,** 2840-2840.

WANG, X., KIM, Y., MA, Q., HONG, S. H., POKUSAEVA, K., STURINO, J. M. & WOOD, T. K. 2010. Cryptic prophages help bacteria cope with adverse environments. *Nature Communications,* 1**,** 147.

WANG, X., ZHANG, Z., LI, B., HAO, W., YIN, W., AI, S., HAN, J., WANG, R. & DUAN, Z. 2022. Depicting Fecal Microbiota Characteristic in Yak, Cattle, Yak-Cattle Hybrid and Tibetan Sheep in Different Eco-Regions of Qinghai-Tibetan Plateau. *Microbiol Spectr,* 10**,** e0002122.

WANG, Y. & SALAZAR, J. K. 2016. Culture-independent rapid detection methods for bacterial pathogens and toxins in food matrices. *Comprehensive Reviews in Food Science and Food Safety,* 15**,** 183-205.

WEESE, J. S., ROUSSEAU, J. & ARROYO, L. 2005. Bacteriological evaluation of commercial canine and feline raw diets. *Canadian Veterinary Journal,* 46 (6), p.513.

WEST, N. P., JUNGNITZ, H., FITTER, J. T., MCARTHUR, J. D., GUZMÁN, C. A. & WALKER, M. J. 2000. Role of phosphoglucomutase of Bordetella bronchiseptica in lipopolysaccharide biosynthesis and virulence. *Infect Immun,* 68**,** 4673-80.

WHEELER, N. E., GARDNER, P. P. & BARQUIST, L. 2018. Machine learning identifies signatures of host adaptation in the bacterial pathogen Salmonella enterica. *PLoS genetics,* 14**,** e1007333-e1007333.

WHICHARD, J. M., SRIRANGANATHAN, N. & PIERSON, F. W. 2003. Suppression of Salmonella Growth by Wild-Type and Large-Plaque Variants of Bacteriophage Felix O1 in Liquid Culture and on Chicken Frankfurters. *Journal of Food Protection,* 66**,** 220-225.

WIESER, A., SCHNEIDER, L., JUNG, J. & SCHUBERT, S. 2012. MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (mini review). *Applied microbiology and biotechnology,* 93**,** 965-974.

XIE, Y., WAHAB, L. & GILL, J. J. 2018. Development and Validation of a Microtiter Plate-Based Assay for Determination of Bacteriophage Host Range and Virulence. *Viruses,* 10 (4), p.189..

XU, Z., WANG, Y., CHATER, K. F., OU, H.-Y., XU, H. H., DENG, Z. & TAO, M. 2017. Large-scale transposition mutagenesis of Streptomyces coelicolor identifies hundreds of genes influencing antibiotic biosynthesis. *Applied and environmental microbiology,* 83**,** e02889-16.

YANG, S., SADEKUZZAMAN, M. & HA, S.-D. 2017a. Reduction of Listeria monocytogenes on chicken breasts by combined treatment with UV-C light and bacteriophage ListShield. *Lwt,* 86**,** 193-200.

YANG, X., BULLARD, B. R., GEORNARAS, I., HU, S., WOERNER, D. R., DELMORE, R. J., MORGAN, J. B. & BELK, K. E. 2017b. Comparison of the Efficacy of a Sulfuric Acid–Sodium Sulfate Blend and Lactic Acid for the Reduction of Salmonella on Prerigor Beef Carcass Surface Tissue. *Journal of Food Protection,* 80**,** 809-813.

YASIR, M., TURNER, A. K., BASTKOWSKI, S., BAKER, D., PAGE, A. J., TELATIN, A., PHAN, M.-D., MONAHAN, L., SAVVA, G. M. & DARLING, A. 2020. TraDIS-Xpress: a high-resolution whole-

genome assay identifies novel mechanisms of triclosan action and resistance. *Genome research,* 30**,** 239-249.

YUAN, Y. & GAO, M. 2017. Jumbo Bacteriophages: An Overview. *Frontiers in microbiology,* 8**,** 403-403.

ZGURSKAYA, H. I., KRISHNAMOORTHY, G., NTREH, A. & LU, S. 2011. Mechanism and Function of the Outer Membrane Channel TolC in Multidrug Resistance and Physiology of Enterobacteria. *Frontiers in microbiology,* 2**,** 189-189.

ZHANG, S., DEN BAKKER HENDRIK, C., LI, S., CHEN, J., DINSMORE BLAKE, A., LANE, C., LAUER, A. C., FIELDS PATRICIA, I. & DENG, X. 2019. SeqSero2: Rapid and Improved Salmonella Serotype Determination Using Whole-Genome Sequencing Data. *Applied and Environmental Microbiology,* 85**,** e01746-19.

8. Supplementary Materials

Table 1 – Summary of Bacterial Strains used for laboratory work in this study.

Strain Name	Genus	Species	Serovar	Source	Chapter Used	Ref	Accession
A20	*Salmonella*	*entercia*	Montevideo	Pork	3,4	This Study	SAMN35823516
B113	*Salmonella*	*entercia*	Panama	Beef	3,4	This Study	SAMN35823517
B34 c6	*Salmonella*	*entercia*	Mbandaka	Beef	3,4	This Study	SAMN35823518
b11 c12	*Salmonella*	*entercia*	Kedougou	Beef	3,4	This Study	SAMN35823519
B90	*Salmonella*	*entercia*	Infantis	Beef	3,4	This Study	SAMN35823520
B36 c10	*Salmonella*	*entercia*	Derby	Beef	3,4	This Study	SAMN35823521

B16 c28	*Salmonella*	*entercia*	Newport	Beef	3,4	This Study	SAMN35823522
B104	*Salmonella*	*entercia*	Enteritidis	Beef	3,4	This Study	SAMN35823523
A53	*Salmonella*	*entercia*	Typhimurium	Pork	3,4	This Study	SAMN35823524
S33_C6	*Salmonella*	*entercia*	Typhimurium	Food Factory	3,4	This Study	SAMN35823525
B8 c7	*Salmonella*	*entercia*	Typhimurium	Beef	3,4	This Study	SAMN35823526
JH4180	*Salmonella*	*entercia*	Typhimurium	Cattle	4,5,6	Rodwell et al 2021	SAMN36943032
LAHRS1	*Aeromonas*	*veronii*	NA	Retail Food	4	This Study	SAMN35823527
LAHRS2	*Citrobacter*	*freundii*	NA	Retail Food	4	This Study	SAMN35823528
LAHRS3	*Cronobacter*	*sakazakii*	NA	Retail Food	4	This Study	SAMN35823529
LAHRS4	*Enterobacter*	*cloacae*	NA	Retail Food	4	This Study	SAMN35823530
LAHRS5	*Escherichia*	*coli*	NA	Retail Food	4	This Study	SAMN35823531
LAHRS6	*Escherichia*	*fergusonii*	NA	Retail Food	4	This Study	SAMN35823532
LAHRS7	*Hafnia*	*alvei*	NA	Retail Food	4	This Study	SAMN35823533
LAHRS8	*Klebsiella*	*pneumoniae*	NA	Retail Food	4	This Study	SAMN35823534
LAHRS9	*Morganella*	*morganii*	NA	Retail Food	4	This Study	SAMN35823535
LAHRS10	*Providencia*	*rettgeri*	NA	Retail Food	4	This Study	SAMN35823536

LAHRS11	*Serratia*	*marcescens*	NA	Retail Food	4	This Study	SAMN35823537
SL476	*Salmonella*	*entercia*	Heidelberg	Turkey	4	Pye et al 2023	SAMN30870369
SL254	*Salmonella*	*entercia*	Newport	Cattle	4	Pye et al 2023	SAMN30870373
287/91	*Salmonella*	*entercia*	Gallinarum	Chicken	4	Pye et al 2023	SAMN30870372
SL480	*Salmonella*	*entercia*	Schwarzengrund	Human	4	Pye et al 2023	SAMN30870371
SL479	*Salmonella*	*entercia*	Kentucky	Human	4	Pye et al 2023	SAMN30870375
ST4/74	*Salmonella*	*entercia*	Typhimurium	Cattle	4,5,6	Rankin and Taylor 1966	GCA_000188735.1
S04698-09	*Salmonella*	*entercia*	Typhimurium	Cattle	4	Petrovska et al 2016	SAMEA7888680
01-2888	*Salmonella*	*entercia*	Typhimurium	Pigeon	4	Bawn et al 2020	SAMEA8668138
DT104	*Salmonella*	*entercia*	Typhimurium	NA	4	Bawn et al 2021	HF937208.1
03-715	*Salmonella*	*entercia*	Typhimurium	Pigeon	4	Bawn et al 2021	ERR028071
D23580	*Salmonella*	*entercia*	Typhimurium	Human	4	Bawn et al 2021	FN424405.1
4179-2001	*Salmonella*	*entercia*	Typhimurium	Bird	4	Bawn et al 2021	ERR028301
SO2882-06	*Salmonella*	*entercia*	Typhimurium	NA	4	This Study	SAMN36943031

S0767 6-03	*Salmonella*	*entercia*	Typhimuriu m	Bird	4	Bawn et al 2021	PRJEB345 99
S0196 0-05	*Salmonella*	*entercia*	Typhimuriu m	Pig	4	Bawn et al 2021	PRJEB345 97
SL134 4 WITS	*Salmonella*	*enterica*	Typhimuriu m	NA	6	Kirkwo od et al 2021	N/A

Table 2 – Summary of primers used in this study

Primer Name	Sequence (5' – 3')	Information
A-N701	CAAGCAGAAGACGGCATACGAGAT TCGCCTTAGTGACTGG CAAGCAGAAGACGGCATACGAGAT TCGCCTTAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATC TCGCGTTTTTCGTGC GCCGCTTCA	llumina adapter-specific sequencing primer customised for DNA tagmented with the MuSeek enzyme, used to sequence DNA from the TraDIS-Xpress experiments.
A-N702	CAAGCAGAAGACGGCATACGAGAT CTAGTACGGTGACTG GAGTTCAGACGTGTGCTCTTCCGAT CTCGCGTTTTTCGTG CGCCGCTTCA	llumina adapter-specific sequencing primer customised for DNA tagmented with the MuSeek enzyme, used to sequence DNA from the TraDIS-Xpress experiments.
A-N703	CAAGCAGAAGACGGCATACGAGAT TTCTGCCTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATC TCGCGTTTTTCGTGC GCCGCTTCA	llumina adapter-specific sequencing primer customised for DNA tagmented with the MuSeek enzyme, used to sequence DNA from the TraDIS-Xpress experiments.
A-S502	AATGATACGGCGACCACCGAGATC TACACCTCTCTATACA CTCTTTCCCTACACGACGCTCTTCCG ATCTCTGACCAGGC ATGCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S503	AATGATACGGCGACCACCGAGATC TACACTATCCTCTACA CTCTTTCCCTACACGACGCTCTTCCG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.

	ATCTCTGACCAGGC ATGCCAGGGTTGAGATGTG	
A-S505	AATGATACGGCGACCACCGAGATC TACACGTAAGGAGACA CTCTTTCCCTACACGACGCTCTTCCG ATCTCTGACCAGGC ATGCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S506	AATGATACGGCGACCACCGAGATC TACACACTGCATAACA CTCTTTCCCTACACGACGCTCTTCCG ATCTCTGACCAGGC ATGCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S507	AATGATACGGCGACCACCGAGATC TACACAAGGAGTAACA CTCTTTCCCTACACGACGCTCTTCCG ATCTTACCAGGCAT GCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S508	AATGATACGGCGACCACCGAGATC TACACCTAAGCCTACA CTCTTTCCCTACACGACGCTCTTCCG ATCTCCAGGCATGC CAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S510	AATGATACGGCGACCACCGAGATC TACACCGTCTAATACA CTCTTTCCCTACACGACGCTCTTCCG ATCTCTGACCAGGC ATGCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
A-S511	AATGATACGGCGACCACCGAGATC TACACTCTCTCCGACA CTCTTTCCCTACACGACGCTCTTCCG ATCTTACCAGGCAT GCCAGGGTTGAGATGTG	Transposon-specific customised sequencing primer for sequencing DNA from the TraDIS-Xpress experiments.
LA01_KO_btuB_Kan_FW	tactatcgatgaagcctgcggcatccttcttat attgtggatgctttacagtgtaggctggagctg cttcg	Primers for Recombination of btuB with Kanamycin Resistance cassette
LA02_KO_btuB_Kan_RV	tcgccgtcgcctcaatacctttaatgcgcgcct taccttcgttgtaatatcatatgaatatcctcct tagt	Primers for Recombination of btuB with Kanamycin Resistance cassette
LA03_KO_oxyR_Kan_FW	ttgctatgctacctatcgccatgaactatcgtg gcgacggaggatgaatagtgtaggctggagc tgcttcg	Primers for Recombination of oxyR with Kanamycin Resistance cassette

LA04_KO_oxyR_Kan_RV	ggggagtagtgtaataccactgcccgccgccaccatgttgcgcagcgtttcatatgaatatcctccttagt	Primers for Recombination of oxyR with Kanamycin Resistance cassette
LA05_KO_galE_Kan_FW	gatcggctttgctggcatccgcccaatacgccgggagatcgccgtcgcgggtgtaggctggagctgcttcg	Primers for Recombination of galE with Kanamycin Resistance cassette
LA06_KO_galE_Kan_RV	aggcttaacggagcgaattatgagagtattggttacaggtggtagcggttcatatgaatatcctccttagt	Primers for Recombination of galE with Kanamycin Resistance cassette
LA07_KO_rumA_Kan_FW	tcgaacagaaccattgattccagatgtcctgtgtgcgggaacatgtcgaggtgtaggctggagctgcttcg	Primers for Recombination of rumA with Kanamycin Resistance cassette
LA08_KO_rumA_Kan_RV	attacagaagataaaaagcaatttgcccgcgcacgcgtttcgcgccgtttcatatgaatatcctccttagt	Primers for Recombination of rumA with Kanamycin Resistance cassette
LA09_KO_rfaL_Kan_FW	agattcattaaagagactctgtctcatcccaaacctattgtggagaaaaggtgtaggctggagctgcttcg	Primers for Recombination of rfaL with Kanamycin Resistance cassette
LA10_KO_rfaL_Kan_RV	atgataccaatttgagcaatatcgacctgttcaaaattgccacgaacgatcatatgaatatcctccttagt	Primers for Recombination of rfaL with Kanamycin Resistance cassette
LA11_KO_bcsA_Kan_FW	atccgcggaagcccagcttcagaatatccagcaagctttccagcggtttagtgtaggctggagctgcttcg	Primers for Recombination of bcsA with Kanamycin Resistance cassette
LA12_KO_bcsA_Kan_RV	cttatcccgccggttagcgcgcgtttgagcgagcgctatcagggttaccgcatatgaatatcctccttagt	Primers for Recombination of bcsA with Kanamycin Resistance cassette
LA13_KO_yhjS_Kan_FW	tttatttctgtgctttcgctagtaaactgataaacagttaaaatagtgacgtgtaggctggagctgcttcg	Primers for Recombination of yhjS with Kanamycin Resistance cassette
LA14_KO_yhjS_Kan_RV	acagttccggcgacaataagcgcatctgcaccagctcggcgctgatttgtcatatgaatatcctccttagt	Primers for Recombination of yhjS with Kanamycin Resistance cassette
LA15_KO_rfbN_Kan_FW	gattgccaatgcttgcctaatttgtaacccaagaatttagcaaaggttgtgtgtaggctggagctgcttcg	Primers for Recombination of rfbN with Kanamycin Resistance cassette
LA16_KO_rfbN_Kan_RV	acattaattattcccacatataatgcagggtcgctttggcctaatgttctcatatgaatatcctcctagt	Primers for Recombination of rfbN with Kanamycin Resistance cassette

LA17_KO_0576_Kan_FW	ccgccgcccgttacccattggtggcggggaacattaattatacatgaatggtgtaggctggagctgcttcg	Primers for Recombination of ST4/74_0576 with Kanamycin Resistance cassette
LA18_KO_0576_Kan_RV	tggagcggaagggttgtgtcagatacgatcagcacatctctattaaagttcatatgaatatcctccttagt	Primers for Recombination of ST4/74_0576 with Kanamycin Resistance cassette
LA19_KO_wzzB_Kan_FW	ttaatgagaaattttacctgtcgtagccgaccaccatccggcaaagaagcgtgtaggctggagctgcttcg	Primers for Recombination of wzzB with Kanamycin Resistance cassette
LA20_KO_wzzB_Kan_RV	tagggtatctatgacagtggatagtaatacgtcttccgggcgtgggaacgcatatgaatatcctccttagt	Primers for Recombination of wzzB with Kanamycin Resistance cassette
LA21_ck_btuB_FW	cgcgtactatcgatgaagcc	Primers to Check for Successful recombination of btuB
LA22_ck_btuB_RV	aatccaccgacgccggaatc	Primers to Check for Successful recombination of btuB
LA23_ck_oxyR_FW	tcgccatgaactatcgtggc	Primers to Check for Successful recombination of oxyR
LA24_ck_oxyR_RV	accatggcggaagctt	Primers to Check for Successful recombination of oxyR
LA25_ck_galE_FW	taacggcgatgcggatgatc	Primers to Check for Successful recombination of galE
LA26_ck_galE_RV	cgcatctttgttatgctatg	Primers to Check for Successful recombination of galE
LA27_ck_rumA_FW	taagggaccaggcctaccga	Primers to Check for Successful recombination of rumA
LA28_ck_rumA_RV	atgggacaattagggtcaca	Primers to Check for Successful recombination of rumA
LA29_ck_rfaL_FW	gactctgtctcatcccaaac	Primers to Check for Successful recombination of rfaL
LA30_ck_rfaL_RV	aacgcgctgataccgtaata	Primers to Check for Successful recombination of rfaL
LA31_ck_bscA_FW	tcatcgcattatcatcattg	Primers to Check for Successful recombination of bcsA
LA32_ck_bcsA_RV	tgcgggcgacaaaacgtccg	Primers to Check for Successful recombination of bcsA
LA33_ck_yhjS_FW	gctaaatctgatgcgtttta	Primers to Check for Successful recombination of yhjS

LA34_ck_yhjS_RV	gcacgatatcgctgatggtc	Primers to Check for Successful recombination of yhjS
LA35_ck_rfbN_FW	ctacctgtgccgccagccat	Primers to Check for Successful recombination of rfbN
LA36_ck_rfbN_RV	tggttggcggaatcaaaata	Primers to Check for Successful recombination of rfbN
LA37_ck_0576_FW	cttcttgctaagaaacctga	Primers to Check for Successful recombination of ST474_0576
LA38_ck_0576_RV	tcgttggtgtattcaatagg	Primers to Check for Successful recombination of ST474_0577
LA39_ck_wzzB_FW	aaccgggcaatgcccggttt	Primers to Check for Successful recombination of wzzB
LA40_ck_wzzB_RV	atggctacactgtctccagct	Primers to Check for Successful recombination of wzzB
LA47_KO_yfdH_Kan_FW	aggaacattatttccaaatattaatttatcaataatcatccatgcaccgtgtgtaggctggagctgcttcg	Primers for Recombination of yfdH (gtrB) with Kanamycin Resistance cassette
LA48_KO_yfdH_Kan_RV	aattaacaaaatagctgcatctgatccgctcgttattccgctttcgtttaccatatgaatatcctcctttagt	Primers for Recombination of yfdH (gtrB) with Kanamycin Resistance cassette
LA55_ck_yfdH_FW	GCG AAA GCT CTC TTA GGG CA	Primers to Check for Successful recombination of yfdH (gtrB)
LA56_ck_yfdH_RV	AGC TAT CAG CCT GAT ATG CGG	Primers to Check for Successful recombination of yfdH (gtrB)
P-Tn5Cm-01	P-CTGTCTCTTATACACATCTTCTAGACAACC	5' Phosphorylated for Chloramphenicol transposon amplification
P-Tn5Cm-04	P-CTG TCT CTT ATA CAC ATC TGA CGC	5' Phosphorylated for Chloramphenicol transposon amplification
rfaK_HR1_F	GGGCTACTGAGACCCAGGAAAACCCGATCAAGGGCTAC	Amplification of homologous regions for golden gate cloning
rfaK_HR1_R	GGGGGTCTCGCTCCAAACGCGCTGATACCGTAATAAGT	Amplification of homologous regions for golden gate cloning
rfaK_HR2_F	GGGCGCTTGAGACCTAGTTAACTTTATAGAAAAAGAAATGTTATAAAAAAAAGAAATGCGTGCC	Amplification of homologous regions for golden gate cloning

rfaK_HR2_R	GGGGGTCTCGTCGTCATCGGGTATT GCTCTTGCCATAC	Amplification of homologous regions for golden gate cloning
16s_FW (PB146)	CTCTTGCCATCAGATGTGCC	Detection of Salmonella by PCR (Positive Control)
16s_RV (PB147)	TCACCGCTACACCTGGAATT	Detection of Salmonella by PCR (Positive Control)
InvA_FW	CGGTGGTTTTAAGCGTACTCTT	Detection of Salmonella by PCR
InvA_RV	CGAATATGCTCCACAAGGTTA	Detection of Salmonella by PCR