# CHARACTERIZATION OF *SALMONELLA* ENTERITIDIS BACTERIOPHAGES AND EVALUATION OF PHAGE DELIVERY SYSTEMS TO INCREASE PHAGE SURVIVAL IN SIMULATED DIGESTIVE SYSTEM AND CHICKENS

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# Characterization of *Salmonella* Enteritidis Bacteriophages and Evaluation of Phage Delivery Systems to Increase phage Survival in Simulated Digestive System and Chickens

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**A thesis submitted in fulfillment of the requirements for the degree of Master of Science in Medical Microbiology of the Jomo Kenyatta University of Agriculture and Technology**

**2023**

## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature……. Date…….

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This thesis has been submitted for examination with our approval as university supervisors

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## DEDICATION

To my dad who made great sacrifices to make me who I am today, for his advice and mentorship. To my siblings for moral support all through my studies. God bless you all

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AMR** | Anti-Microbial Resistance |
| **ANOVA** | Analysis of Variance |
| **API** | Analytical Profile Index |
| **APTS** | Aminopropyl-triethoxysilane |
| **ATCC** | American Type Culture Collection |
| **ATP** | Adenosine Triphosphate |
| **BSL2** | Biosafety Level 2 |
| **BPW** | Buffered Peptone Water |
| **$CaCl_2$** | Calcium Chloride |
| **CGIAR** | Consortium of International Agricultural Research Centres |
| **CFU** | Colony Forming Unit |
| **CRISPR** | Clustered Regularly Interspaced Short Palindromic Repeats |
| **cGIT** | Chicken Gastrointestinal Tract |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **FCE** | Feed Conversion Efficiency |
| **GIT** | Gastro-Intestinal Tract |

| | |
|---|---|
| **HACCP** | Hazard Analysis Critical Control Point |
| **HCL** | Hydrochloric Acid |
| **HMSN** | Hollow Mesoporous Silica Nanoparticles |
| **IACUC** | Institutional Animal Care and Use Committee |
| **ILR** | International Livestock Research Institute |
| **iNTS** | Invasive Non-Typhoidal *Salmonella* |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **Kb** | kilobases |
| **MDR** | Multi Drug-Resistant |
| **ML** | Millilitre |
| **MSNs** | Mesoporous Silica Nanoparticles |
| **NACOSTI** | National Commission for Science, Technology, and Innovation |
| **NaOH** | Sodium Hydroxide |
| **NTS** | Non-Typhoidal *Salmonella* |
| **PCR** | Polymerase Chain Reaction |
| **PFU** | Plaque-forming unit |
| **RFLP** | Restriction fragment length polymorphism |
| **SFB** | Selenite Fecal Broth |

| | |
|---|---|
| **SGF** | Simulated Gastric Fluid |
| **SIF** | Simulated Intestinal Fluid |
| **Spp** | Species |
| **SPIs** | Salmonella Pathogenicity Islands |
| **SVs** | Silica Vesicles |
| **TAE** | Tris Acetate Ethylenediamine tetraacetic acid |
| **TMOS** | Tetramethyl Orthosilicate |
| **TSB** | Tryptic Soy Broth |
| **TSI** | Triple Sugar Iron |
| **WHO** | World Health Organisation |

## ABSTRACT

*Salmonella enterica* serovar enteritidis, a multidrug resistant strain, is one of the leading causes of foodborne illness in the globe. Humans mostly contract this non-typhoidal *Salmonella* serovar by eating tainted poultry flesh and other poultry items. For lowering the prevalence of multi-drug resistant non-typhoidal *Salmonella* in chicken farms, bacteriophages are an alternative to antibiotics. Phages with a stronger prophylactic or therapeutic potential may be able to survive the harsh conditions of the gastrointestinal tract, which have a low pH, high temperatures, and several digestive enzymes. Using various pH-adjusted medium, incubation temperatures, and simulated gastric and intestinal fluids, this study examined the host range, identification, and stability of 10 distinct *Salmonella* enteritidis phages isolated from Kenyan chicken farms. Additionally, their capacity to survive in Kenya's water sources—including rivers, boreholes, rainwater, and tap water—was evaluated. Additionally tested was the capacity of silica vesicles to adsorb/encapsulate, release, and safeguard phages in artificial stomach juice. Finally, 3-day old broiler chicks were used to assess their capacity for survival in vivo (24). On seven different strains of *Salmonella* enteritidis, all phages showed a wider host range and were relatively stable for 12 hours at pH values between 5 and 9 and temperatures between 25 °C and 42 °C. After 3 hours of incubation at pH 3, a viral titre decreases of up to 3 logs was seen. Phages remained stable in simulated stomach fluid for 20 minutes before losing their ability to infect. For up to two hours, phages remained largely stable in simulated intestinal fluid. *Salmonella* growth was significantly inhibited by phages in pH 2 and pH 3-adjusted media as well as in simulated gastric fluid at pH 2.5, but this effect was less pronounced in simulated intestinal fluid at pH 8. The other studied waters had just a minor impact on the phages, but river water had the greatest negative impact. The adsorption/encapsulation efficiencies of the three silica vesicles (SV 100, SV 140, and SV 140-C18) were 57.4%, 60%, and 90%, respectively. They were able to shield phages in stomach fluid for an hour and had modest, steady phage release rates up until day 4. SV 140-C18 had the lowest log reduction of 4 logs PFU/ml. Both SVs 100 and 140 lost six logs of PFU/ml decrease. Up until day 8 following inoculation, silica vesicle-encapsulated phages in the chickens displayed larger phage titres than non-encapsulated phages; however, until day 28 there was no discernible difference. On day 28, SV-encapsulated phages K28 and K11 had the highest titres. These findings imply that some of these phages may have a chance of surviving in living organisms and may be given orally by drinking water and survive the digestive system to avoid salmonellosis. The 10 *Salmonella* Enteritidis phages can be investigated for phage release and protection in people and other hosts, including chickens, where non-typhoidal *Salmonella* can be decolonized in vivo. SV 140-C18 should also be evaluated for phage release and protection in humans.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

Salmonellosis with non-typhoidal typhus brought on by a drug-resistant (MDR) *S.* Enteritidis, a type of *Salmonella enterica*, is one of the leading causes of foodborne illnesses worldwide. (Balasubramanian *et al.*, 2019). In these conditions, where there are no other viable treatment choices, the prevalence of MDR infections is a major cause for concern and presents a significant challenge to available management and treatment alternatives (Sulis *et al.,* 2022). Over 78 million foodborne illnesses are caused by *S.* Enteritidis globally (Murray *et al.,* 2022) (Figure 1.1). Non-typhoidal *Salmonella* (NTS) infections kill a disproportionately high number of people in Africa, particularly in impoverished metropolitan areas. Up to 39% of community-acquired bloodstream infections and 37.7% of poultry deaths in Sub-Saharan Africa are brought on by it (Gezmu et al., 2021; Hedman et al., 2020). According to reports from Kenya, MDR invasive NTS (iNTS) infections affect 10.8% of children and 5.8% of adults, respectively (Gilchrist & maclennan, 2019; Muthumbi *et al.*, 2015).

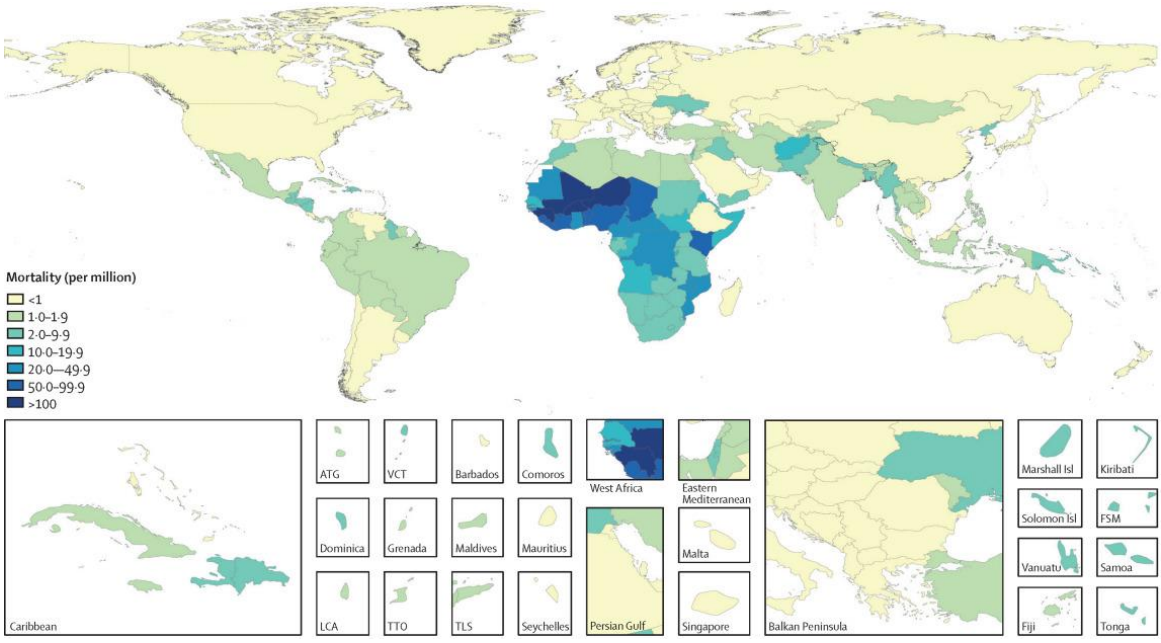


**Figure 1.1: Mortality rates (per million) from invasive non-typhoidal *Salmonella* disease by 2017.**

Source: (Stanaway *et al.*, 2019)

The poultry industry constitutes a significant sector of agriculture in Kenya through the provision of bioavailable protein and employment (Omiti & Okuthe, 2008). However, poultry poses a potential source of zoonotic diseases such as NTS, which are mostly foodborne transmitted (Figure 1.2). Poultry, especially broilers are well-known reservoirs of NTS serovars, many of which can infect humans, for example *S.* Enteritidis and Typhimurium and have been determined to be the source for approximately 58% of foodborne *Salmonella sp* (Stanaway *et al.,* 2019). Farm cleaning, feed evaluation, prebiotics and probiotics usage, and the use of antibiotics at sub-therapeutic levels in asymptomatic birds are all used to control NTS at the farm level in an effort to reduce transmission to people and increase output and productivity of poultry (Barua *et al.,* 2014; Brown *et al.,* 2013; mccarron *et al.,* 2015; Singh *et al*., 2010). Antibiotic use to prevent disease and encourage growth in chicken has resulted in a variety of pathogenic microorganisms, including NTS serovars, developing antimicrobial resistance (Costa *et al.,* 2017). Therefore, it is necessary to look for more options for treating these. illnesses

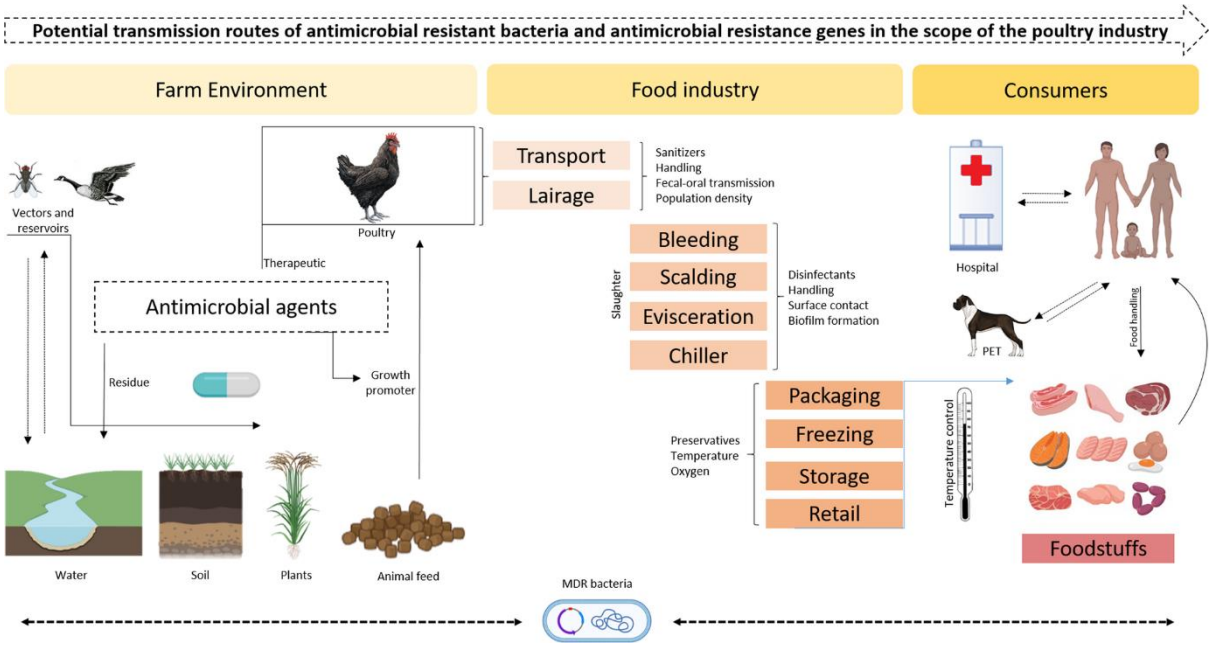


**Figure 1.2: Routes via which bacteria and genes for antibiotic resistance could spread within the context of the chicken industry.**

Source: (Monte *et al.*, 2019)

Bacteriophages are a possible replacement for the usage of antibiotics in poultry. Phage therapy, the use of bacteriophages to kill or otherwise control the bacterial population in infected hosts, is a possible alternative to antibiotics, or at least, as a supplementary approach for the treatment of *Salmonella* infections(Górski *et al.,* 2020). Bacteriophages have several properties that render them suitable for use in *Salmonella* control: in general, they are highly specific, they do not cross-species or genus barriers, they are self-replicating and self-limiting, and ubiquitous (N. Liu *et al*., 2020). Their specificity to target bacteria is attributed to the binding of the bacteriophages to host cell surface receptors such as pili, flagella, porins, efflux pumps, or sugar moieties in lipopolysaccharides (Pham-Khanh *et al*., 2019). The result of infection by a lytic phage is the ultimate lysis of bacteriophage-infected bacteria. These phages are described as having a lytic activity as compared to temperate phages that integrate their genetic material into the host genome and lay dormant till the environmental condition triggers the lytic cycle(Kortright *et al*., 2019; Wan *et al*., 2021) (Figure 1.3)

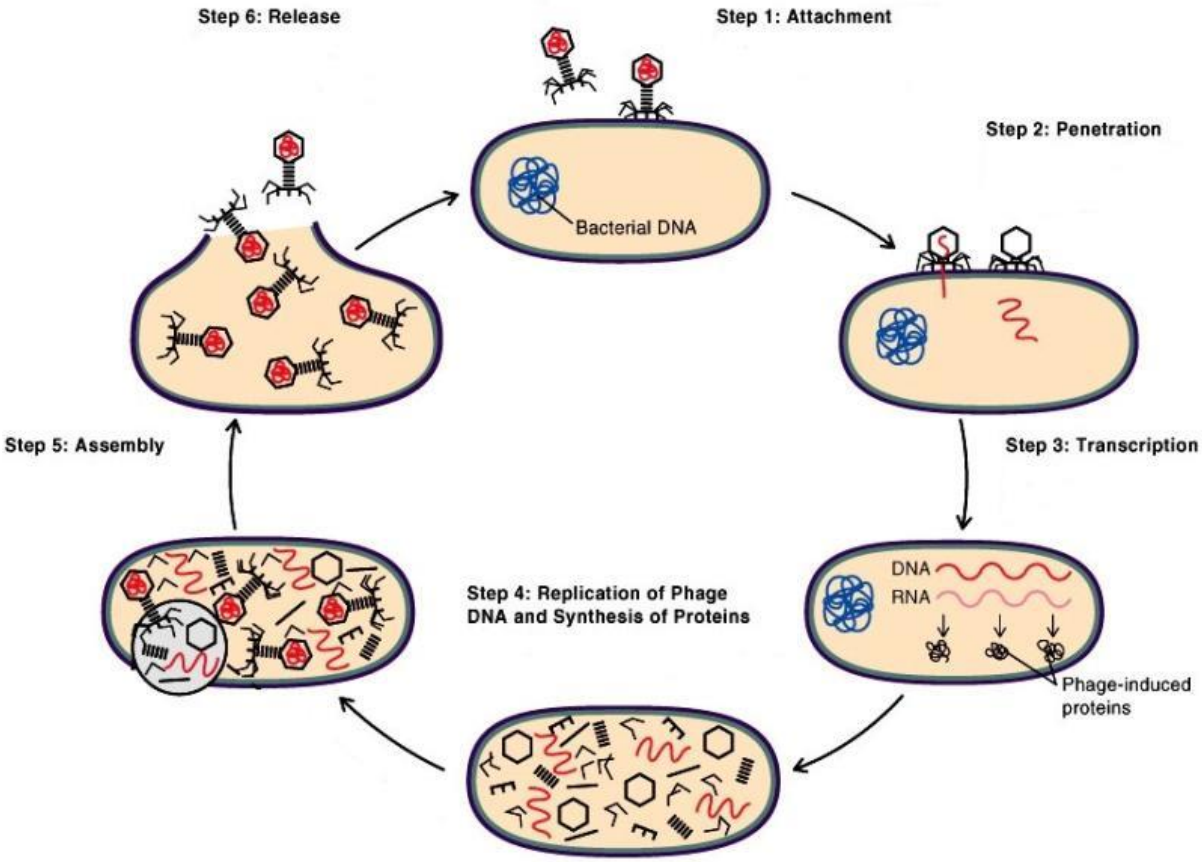



**Figure 1.3: General replication cycle of bacteriophages**

Source:(Chiang *et al*., 2019)

As with pharmaceutical drug delivery, targeting phages to the site of infection remains a hurdle for efficient therapy. Phages face a setback of neutralization by gastric acids and enzymes during the gastrointestinal tract (GIT)-transit to the infection site in small intestines, this renders a huge percentage of phages inactive (Chatain *et al*., 2014; Międzybrodzki *et al.*, 2017). Phages that survive in these harsh conditions are likely to reach the infection site and eliminate NTS. Strategies have been proposed to make phages acid-tolerant during the stomach transit, which includes encapsulation of phages using various elements such as silica, lipids, and alginate, use of genetically modified phages and addition of antacids e.g., $cacl_2$ (Abdelsattar *et al*., 2019a; Barros *et al*., 2020; Lorenzo-Rebenaque *et al*., 2022; Silva Batalha *et al.*, 2021; Soto *et al*., 2018). However, there is limited information

regarding the effectiveness of most of the strategies, which requires further research to ensure the high efficacy of these strategies.

### 1.2 Statement of the Problem

Oral phage administration is considered as the possible therapeutic option that is safe and most efficient to target *S*. Enteritidis in the GIT as compared to other routes (Stanford *et al*., 2010; Tang *et al*., 2015; Vinner *et al*., 2019). The effectiveness of phage therapy depends on the availability of phages at the site of infection. However, most phages are highly sensitive to the acidic environment in the stomach, which significantly reduces the phage titters, rendering phage therapy ineffective and unable to reach the infection site in the required amount. Studies have shown that most phage titres in stomach gastric acids reduce by 72% within 15 minutes and 99% after 45 minutes (Parker *et al*., 2016). Recently three main strategies have been suggested to improve the survivability of phages from the stomach acid, namely the use of antacids e.g. $CaCl_2$ to neutralize the acid and pave the way for phages to transit through the acidic region of the GIT, encapsulation of phages using natural and synthetic compounds such as alginate, lipids and modified silica, that will protect phages from the acid during the stomach transit, and the use of genetically modified phages that display lipids on their surfaces, thereby protecting the phages from the harsh acid conditions (Gomez-Garcia *et al*., 2021; Lorenzo-Rebenaque *et al*., 2022; Tang *et al*., 2015).

However, despite advances in strategies for protecting phages from harsh acidic conditions, up to date, there is still limited information regarding their efficacy in protecting phages from the harsh gastrointestinal conditions. There has also not been any report of the method that simultaneously resolves both the acid sensitivity issue and the limited intestinal residence time of phages to achieve optimum results. As such, there is a need for more research to improve the efficacy of the strategies for protecting phages from the harsh stomach acidic conditions.

### 1.3 Justification of the Study

The MDR NTS serovar Enteritidis is among the priority infectious organisms on the WHO priority 1 list, for research and development of new antibiotics and other alternatives such as the use of bacteriophages (WHO, 2017). Phage therapy has been recognized as a powerful technology holding tremendous potential to combat increasingly MDR bacterial infections caused by *S. enterica* serovars which currently have limited therapeutic options (Kortright *et al*., 2019). Bacteriophages, infect and lyse bacteria without any apparent noxious effect on mammalian cells. This characterizes them as safe due to their abilities to specifically target bacterial host cells and self-replicate in nature. Phages that are likely to survive the harsh GIT conditions, are likely to reach the infection site in required concentration and will be able to eliminate the *S*. Enteritidis population. Physiological characterisations of phages ensure the selection of most stable phages that are likely to survive the harsh GIT environment which consists of fluctuating pH, temperature, enzymes, microbiome, and continuous peristaltic movement of the gut.

Aiding the selection of phages using characterisation, the use of encapsulation materials also helps to protect phages from the harsh gastrointestinal environment to the infection site. One of the promising materials is the mesoporous silica nanoparticles/ vesicles (MSNs). The MSNs can adsorb a range of different types and sizes of molecules onto their external and internal surfaces via electrostatic interactions. Particularly, MSNs are a popular and preferred choice for compounds delivery given their flexible and desirable properties such as high drug loading capacity, tuneable pore size and volume, ease-of-functionalization, and biocompatibility. MSNs have mesopores between 2 and 50 nm pore size. The external and internal surfaces can be modified with additional chemical compounds to increase phage adsorption to the MSNs (Cademartiri *et al*., 2010; Mody *et al*., 2013; Selvarajan *et al*., 2020). MSNs have not been extensively used to test for phage survival and as a delivery mechanism for phages, but rather they have been tested for adoption and immobilization of phages. Testing the ability of SVs to adsorb, protect and release phages will ensure that phages are protected from the harsh environment and delivered to the site of infection in required concentrations.

### 1.4 Research Questions

1. What is the host range *Salmonella enterica* serovar Enteritidis specific phages and at which temperature and pH they are?
2. What is the growth kinetics of *Salmonella enterica* serovar Enteritidis specific phages in rich media and simulated digestive environment?
3. What is the phage binding capacity, rate of release and the phage protective capabilities of mesoporous silica vesicles in simulated digestive environment and in chickens?

### 1.5. Objectives

#### 1.5.1 General Objective

To characterize *Salmonella enterica* serovar Enteritidis bacteriophages and evaluate phage delivery system to increase phage survival in the chicken simulated digestive environment and in chickens.

#### 1.5.2. Specific Objectives

1. To determine the host range, thermal and pH stable values of *Salmonella enterica* serovar Enteritidis bacteriophages.
2. To determine the growth kinetics of *Salmonella enterica* serovar Enteritidis bacteriophages in rich media and simulated digestive system.
3. To determine the phage binding capacity, rate of release and the phage protective capability of mesoporous silica vesicles in simulated digestive system, and in chickens.

### 1.6 Hypotheses

1. Phages that are more stable at low pH (1.5-5) and high temperature (40-50°C) will survive longer in chicken (chicken stomach normal pH = 2.5-3, optimum temperature between 40.6° and 41.7°C).

2. Encapsulation material more stable in low pH (1.5-3.5) and low phage release rate will increase phage survival and prolong residence time of phages in the gut.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction to Non Typhoidal Salmonellosis

Salmonellosis is a bacterial disease caused by *Salmonell*a *sp*., a facultative anaerobic gram-negative rod bacterium, it has two distinct species: *S. Enterica* and *S. Bongeri. S. Enterica* has six species namely, *enterica, salamae, arizonae, diarizona, houtenae,* and *indica*. *S. Enterica* has more than 2600 serovars of which most infect both humans and animals (Antunes *et al*., 2016; Pegues *et al.,* 2006). Some of the host-specific serovars *S.* Gallinarum and *S.* Pullorum are responsible for fowl typhoid and pullorum disease respectively in chickens. Both diseases present with watery foul-smelling diarrhoea, which is the most prominent clinical sign in the young bird, lethargy, anorexia, vomiting, fever, abdominal pain, which is evident through the restlessness of the birds, within 4 to 72 hours post-infection (Figure 2.1) (Foley *et a*l., 2013; Singh *et al*., 2010). In humans, invasive nontyphoidal *Salmonella* (iNTS) serovars such as Enteritidis and Typhimurium are responsible for gastroenteritis, which takes place 12-48 hours post-infection, is followed by watery mucoid diarrhoea, fever, vomiting, abdominal pains, and nausea. iNTS which causes bacteraemia leads to various conditions based on the organs affected (Eguale *et al*., 2015; Kariuki *et al*., 2006; Mohan *et al.*, 2019). Consumption of contaminated poultry and poultry products is the major source of NTS serovars from humans (Iannetti *et al.*, 2020).

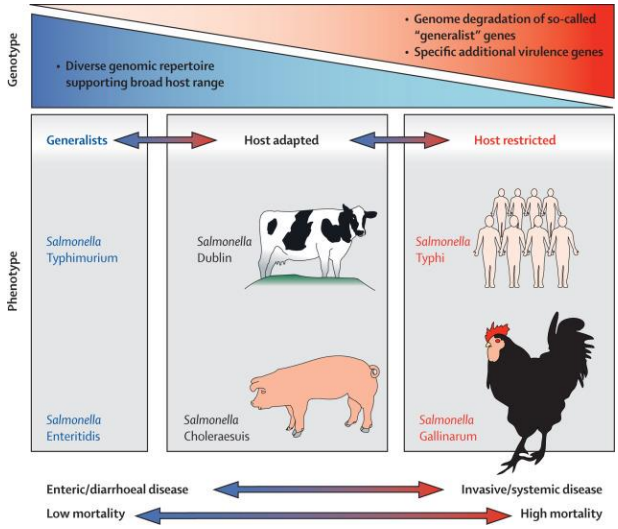



**Figure 2.1: Host restricted/specific and generalist/non-host specific *Salmonella* serovars.**

Source: (Feasey *et al*., 2012)

Preventing and controlling contamination of foodborne and zoonotic *Salmonella sp*. Remains a considerable challenge in commercial and backyard chicken farming systems in developing countries. *Salmonella sp*. Can be transmitted vertically from the breeder stock to the young birds through the hatchery, horizontally through contact among birds within the house, use of contaminated feed, and farm personnel can introduce the pathogens (Khan *et al.,* 2018). Therefore, good farming practices are essential for controlling poultry zoonoses at the farm level. Currently, probiotics, prebiotics, and antibiotics are used to control *Salmonella*. Continuous use of these antibiotics as growth promoters has contributed to the formation of antimicrobial-resistant strains of *Salmonella sp*. It is estimated that globally, 77,500 deaths occur per year due to multi-drug resistant (MDR) NTS serovars, which are very difficult to treat with very few therapeutic options (Mathew *et al*., 2020; Murray *et al.,* 2022). This problem has resulted in a renowned search for alternatives to antibiotics to control *Salmonella* infections.

## 2.2 Bacteriophages are a promising alternative to the use of antibiotics to control *Salmonella.*

Bacteriophages are viruses that specifically infect bacteria. They were first co-discovered by Fredrick Twort in 1915 and by Félix d' Hérelle in 1917. Like other viruses, bacteriophages do not have the machinery for metabolism (ATP production) and protein synthesis (by ribosomes), they only contain the genetic material of which they rely on the host for multiplication, thus they are obligate parasites (Casey *et al.*, 2018; Clokie *et al.*, 2011). They are present in large quantities wherever their host is found, in the soil, water, sewages, and gastrointestinal tract (GIT). In the absence of the host, they can still maintain their infectivity for many years (Merikanto *et al.,* 2018). Bacteriophages are classified based on the morphological structure, genome type, host organism, and life cycle. Based on the life cycle they are categorized as lytic and lysogenic (temperate) phages. Lytic phages are highly virulent and have the potential of being used for phage therapy. They attach to the host receptors, release their genetic material into the host cell, adapt their machinery to make more copies of the phages, and exit the host through lysis, within minutes to hours (Figure 2.2). Temperate phages integrate their genome into the host genome to form a prophage. Environmental factors causing stress to the host cell can lead to prophage triggering the lytic cycle (Garin-Fernandez & Wichels, 2020; Hyman, 2019).

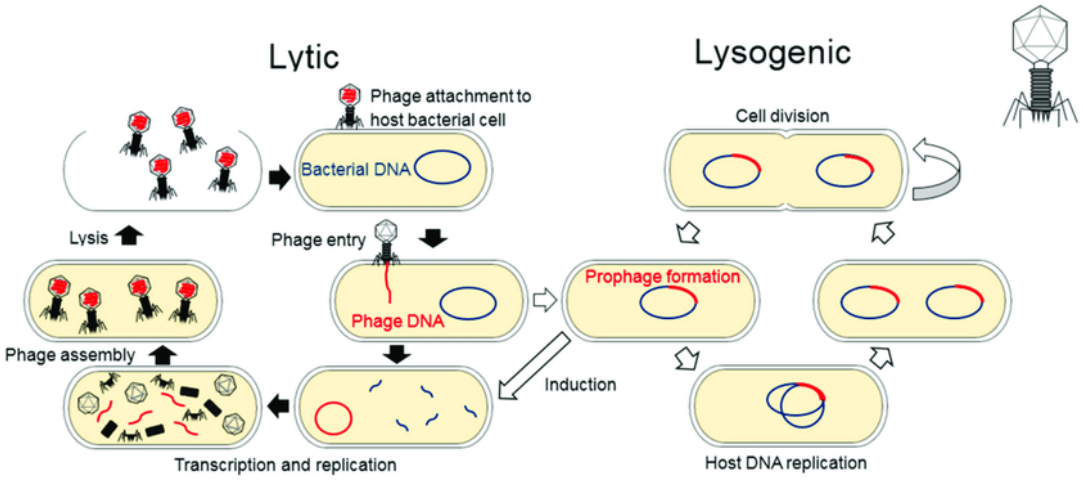



**Figure 2.2: Bacteriophage lifecycle showing lytic and the lysogenic cycles.**

Source: (batinovic *et al.,* 2019)

**2.3 Biological Characteristics of Phages**

## 2.3 1 pH and Temperature

Phages exhibit unique characteristics that aid in survival in the environment they are located in. They have varying stability to pH, temperature, and organic compounds because of the phenotypic expression that helps them resist harsh environmental conditions. Kim *et al*., (2020) demonstrated that *Salmonella* bacteriophages isolated from chicken breast muscles were stable between pH 5 and 11, and at the temperature range of 60 to 65 ℃ for 1 hour. Yan *et al*., (2020) reported that the rate of survival of *Salmonella* phage LPSEYT is almost 100% over a temperature range of 30 to 60 ℃ and the pH of 3 and 11. The novel *Salmonella* specific phage vb_sens_SE1 isolated from wastewater treatment plant indicated that it is highly stable between 20℃ and 50℃ and at the optimum pH of 7-8 (Lu *et al*., 2020). Huang et al., (2018) reported that *Salmonella* phage LPST10 isolated from different food matrices was stable from 30℃ to 60℃, and at a pH range of 3-13, and the decrease in the concentration of the phage at this range was less than 10%, however at 70℃ decrease after 30 minutes. No phage present was found at the pH <3 and >13. O'Flynn *et al.*,( 2006) demonstrated that *Salmonella* phages st104a and st104b survive at a pH value of 2.5 in the porcine stomach.

### 2.3.2 Phage Host Range

The bacteriophage host range is categorized into narrow (monovalent) and broad host range (multivalent). The narrow host phages complete their life cycle with one host, while the broad host range can complete their life cycle with more than one intra and inter specie hosts (Figure 2.3). Horizontal transfer of genes between different bacterial species and broad host range phages, enables them to express receptor binding proteins that recognize receptors from different hosts (de Jonge *et al*., 2019). Islam *et al*., (2019) reported that novel *Salmonella* phages LPSTLL, LPST94, and LPST153 from biofilms, were able to lyse *S. enterica* serovars Typhimurium, Enteritidis, Dublin, Choleraesuis, Paratyphi B, Pullorum, Javiana, Anatum and

Kentucky, and subs. *Arizonae*. Santos *et al*., (2010) isolated and characterized a multivalent *Salmonella* phage PVP-SE1, which was able to lyse 13 *Salmonella* serovars, *Escherichia coli* (K5, N9), and *Enterobacter amnigenus* CECT 4078 (ATCC 33072). According to Jung *et al.*,( 2017) *Salmonella* Typhimurium KCCM 40253 (KACC), *S.* Typhimurium ATCC 19585 (ATCC), *S.* Typhimurium ATCC 19585 (ATCCCIP), and clinically isolated antibiotic-resistant *S.* Typhimurium CCARM 8009 were all susceptible to the high lytic capacity of phage P22 (CCARM). Atterbury *et al.*, (2007) isolated *Salmonella* phages 10, 25, 27, 28, 36, 37, 51, 92 104, and 151 from broiler chickens, and the host range was determined with 70 *Salmonella* isolates. Phage 10 had the widest lytic spectra of *S. enterica* serovars Amsterdam, Derby, Enteritidis, Java, Orion, Stanley, Typhi, and Typhimurium. Duc *et al.*,( 2020) isolated A wide host range phage PS5 from food matrices that managed to lyse *S.* Enteritidis, *S.* Typhimurium, and *E. coli* O157: H7.

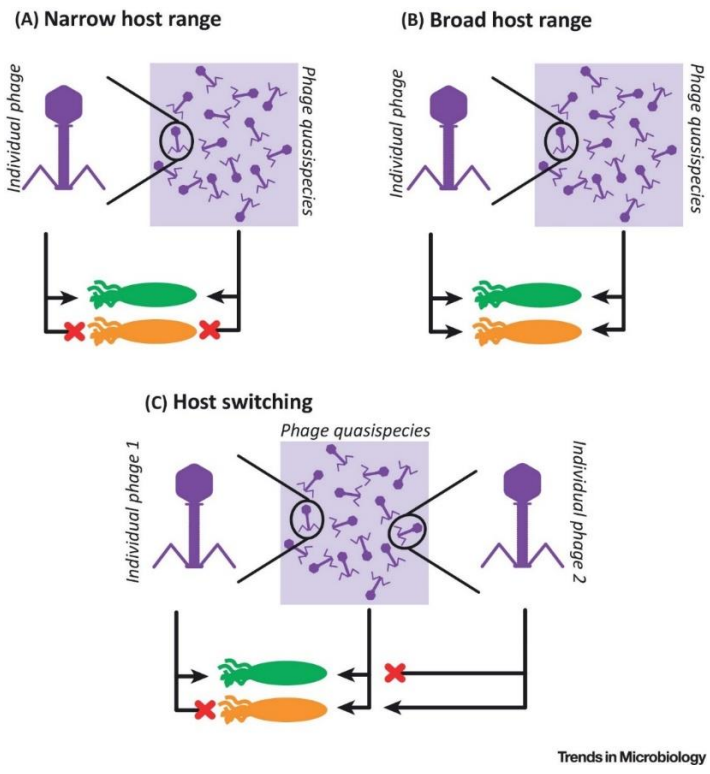



**Figure 2.3: Subdivision of Bacteriophages Based on Host Range**

Source: (de Jonge *et al*., 2019)

## 2.2. Phage Therapy

Since their discovery, bacteriophages were continuously used for treatment of local and systematic infections in Eastern Europe despite being abandoned for antibiotics by most Western countries. Currently, they are used across the globe to treat MDR bacteria such as *Acinetobacter baumannii,* and *Pseudomonas aeruginosa.* A combination of different phages with broad-spectrum activity can be potentially beneficial against different bacterial strains. According to Naghizadeh (2019), the use of combined phage therapy is more effective than the use of single phage due to the broad host range and the synergistic effect which helps mitigate the development of bacterial resistance against phages. Phage therapy of NTS serovars has demonstrated that the use of wide host range phage cocktails reduces biofilms and enhance *Salmonella* control. Clevigo (2019) reported that SalmoFREE®, a commercialized *Salmonella* phage cocktail given to broiler chickens through drinking water, demonstrated high efficacy and innocuity at the production scale upon assessment of cloacal swabs. Atterbury *et al*.(2007), reported that *S. enterica* serovars Enteritidis and Typhimurium specific phages successfully reduced the *Salmonella sp*. cecal colonization by ≥4.2 $\log_{10}$ CFU and ≥2.19 $\log_{10}$ CFU respectively, within 24 hours compared with controls in chickens. Clavijo *et. al,*(2019) observed that the use of SalmoFREE® controls the incidence of *Salmonella sp*. and does not affect the animals nor the production parameters, demonstrating its efficacy and innocuity at the production scale. By observing a significant decrease in bacterial counts (0.92-5.12 log10 CFU/sample) and an increase in phage titers (0-2.96 log10 PFU/sample) that were seen in the various food matrices tested, Huang *et al*. (2018) showed that phages LPST10, LPST18, and LPST23 were highly efficient in infecting S. Typhimurium ATCC 14028.

### 2.4 Phage Encapsulation Technology to Enhance Efficiency

Phages are effective in eliminating gastrointestinal bacterial infections like salmonellosis. However, stomach hydrochloric acid, bile salt, fluctuating temperature, and pancreatic enzymes limit phage activity (Ly-Chatain, 2014). Phage encapsulation is an excellent technology to protect them from these environmental

factors. Phage encapsulation involves packaging phages in nanovesicles made of different natural and synthetic materials, deliver them through an oral route (Figure 2.4). These nanovesicles deliver phages at varying infection sites in the intestines (Choińska-Pulit *et al*., 2015). Phage encapsulation as proven to assist in protection of phages, and achieving the objective of the therapy. Soto *et al*.,(2018) demonstrated that 80.6% of alginate encapsulated *S.* Enteritidis phage f3αse maintained the viability at pH 3 and 60 ℃ for 10 hours in a water flow system. Boggione *et al*., (2017) encapsulated UFV-AREG1 bacteriophage with alginate-calcium microspheres using a microfluidic device and demonstrated that 82.1% of phages retained the stability and efficacy for 21 days in the gel matrix. Ma *et al*., (2016) encapsulated *S.* Typhimurium phage Felix_O1 (FO1) with alginate-calcium microspheres and delivered orally in broiler chicks. Following a single oral dose of $10^9$ plaque-forming unit (PFU), the majority were detected in faeces after 4 hours, with low levels up to 12 hours. Colom *et al*.,(2015) used lipid-based nanovesicles to encapsulate *Salmonella* phages UAB_Phi20, UAB_Phi78, and UAB_Phi87 for oral delivery in chickens. Free phage titter was reduced by 7.8 log units while encapsulated phages were only reduced by 3 units after passing through the chicken GIT. Despite such advances with phage encapsulation technology, there is still limited information regarding the efficacy of these strategies to effectively protect phages from the harsh gastrointestinal conditions. There has also not been any report of the method that simultaneously resolves both the acid sensitivity issue and the limited intestinal residence time of phages, as such, there is a need for more research to improve the strategies.

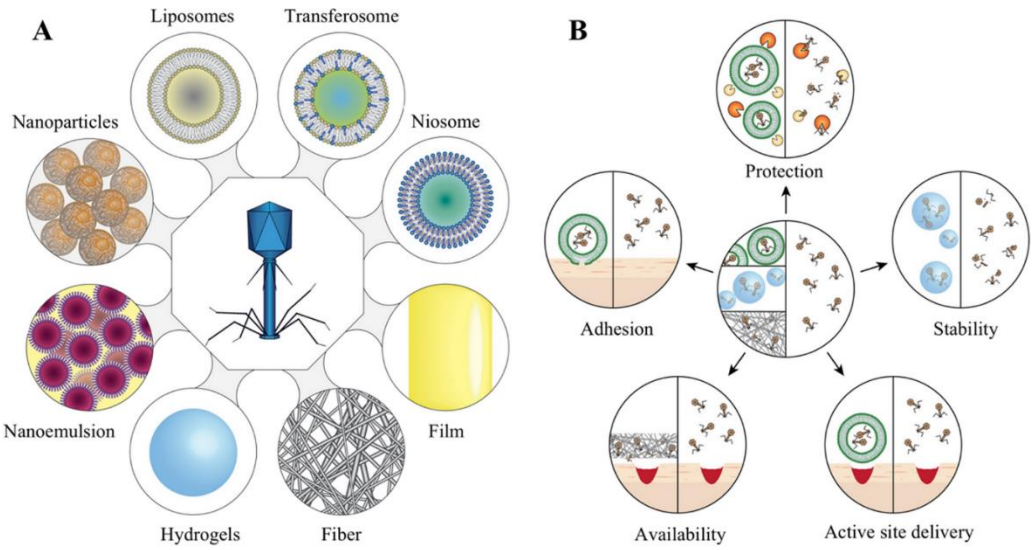



**Figure 2.4: Methods and advantages of phage encapsulation for therapy**

**A) Phage encapsulation methods. B) Benefits of encapsulating phages for therapy versus the deployment of freely diffusing phages**.

Source: (Wu *et al*., 2021)

### 2.5 The use of Mesoporous Silica Vesicles for Phage Delivery

Silica nanoparticles can be used to protect phage virions against inactivation throughout the gastrointestinal tract and control their release. Silica nanoparticles can adsorb a range of different types and sizes of molecules onto their external and internal surfaces via electrostatic interactions (Selvarajan *et al*., 2020). There are many different types of silica nanoparticles, such as the conventional non-porous silica nanoparticles, mesoporous silica nanoparticles (MSNs), hollow mesoporous silica nanoparticles (HMSN), and core-shell silica, either with or without surface modification (Figure 2.5) (Mody *et al.*, 2013). Particularly, MSNs are a popular and preferred choice for compounds delivery given their flexible and desirable properties such as high drug loading capacity, tuneable pore size and volume, ease-of-functionalization, and biocompatibility. MSNs have mesopores between 2 and 50 nm pore size (Mody *et al.,* 2014). The external and internal surfaces can be modified with additional chemical compounds to increase phage adsorption to the MSNs (Yu *et al*., 2012). MSNs have not been extensively used to test for phage survival and as a

delivery mechanism for phages. But rather they have been tested for adoption and immobilization of phages. Previous studies demonstrated that normal 50 nm SVs successfully adsorb phages up to 2.5 logs PFU/ml (Bone *et al*., 2018). Modified SVs with tetramethyl orthosilicate (TMOS), poly (ethylene glycol), aminopropyl-triethoxysilane (APTS), Karstedt's platinum catalyst, and glacial acetic acid, demonstrated increased phage adsorption by about 3.5 log PFU/ml compared to non-modified SVS whose adsorption was only by 2 logs PFU/ml (Argyo *et al*., 2014). Amino functionalized MSNs chemisorption at maximum adsorption conditions on 1 mm particles, yielded 16 functional phages per particle, which is 2.5 times more than by the physisorption method (Mody *et al*., 2015).

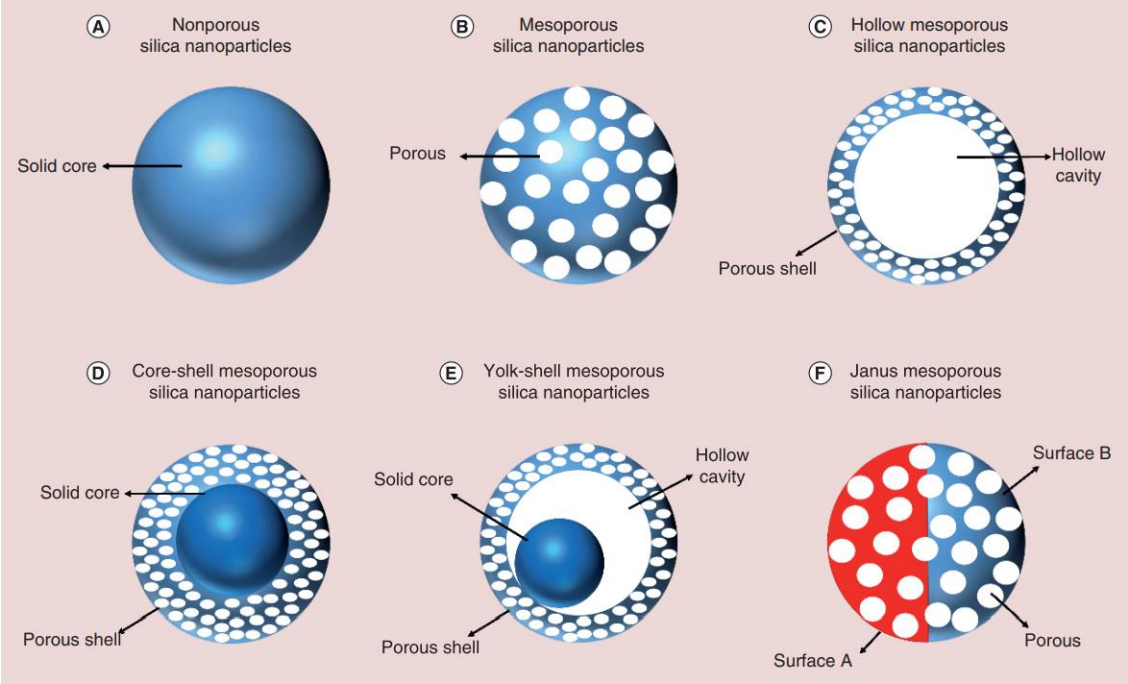



**Figure 2.5: Different types of silica nanoparticles commonly used for biomedical applications.**

Source: (Arriagada *et al*., 2019)

### 2.6 The Summary and the Research Gap

Therefore, the purpose of this work was to characterize 10 distinct *S.* Enteritidis-specific phages that have previously been isolated from chicken farms and

slaughterhouses in the counties of Nairobi, Kiambu, and Machakos. The phage's endurance at various thermal (25 °C to 60 °C) and pH (1 to 12) settings, and their growth kinetics in SGF and SIF, were evaluated. It was also determined how long they lasted in various water sources. The ability of phages to connect to functionalized SVs and keep the phages surface-bound in an active, infectious condition was tested. Then, it was determined how quickly the SVs released phages and how well they protected phages in SGF. A scoring system was used to score the 10 phages according to how long they could survive under the previously mentioned conditions to determine which ones had the best chance of working in chicken. Three phages were chosen based on the scoring system, SVs were used to encapsulate them, and 3-day-old chickens were used to test the phage's viability. These results offer useful information for choosing phages for therapy, creating phage delivery systems for bacterial recognition or inactivation, and shielding phages from the hostile gastrointestinal environment.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1. Study area

The International Livestock Research Institute's Biosafety Level 2 (BSL 2) laboratory was used for biological phage characterization and in vitro phage therapy tests, while the Animal Isolation Unit was used for the in vivo study (ILRI). the field-collected bacterial isolates and bacteriophage lysates of *Salmonella enterica* serovar Enteritidis from an earlier investigation. A global research alliance for future food security, CGIAR includes ILRI as one of its research centres. It prioritizes eradicating poverty, boosting food and nutrition security, and expanding the use of natural resources and ecosystem services (ILRI, 2020).

## 3.2. Study design

The study design was a randomized, placebo-controlled, experimental study. The birds were randomly allocated to 6 groups which received silica-encapsulated, and non-encapsulated phages. The control group received phosphate buffer solution (placebo).

## 3.3 Study population

### 3.3.1. Bacteriophage isolates

*Salmonella enterica* serovar Enteritidis positive bacteriophage stored at 4°C in the Biosafety Level 2 (BSL 2) laboratory, previously collected from Nairobi and Kiambu counties, Kenya. This study was an objective, part of a main project that aims to use bacteriophages as a One Health approach for the replacement of antibiotics, and reduction of drug resistant nontyphoidal *Salmonella*, in poultry farms in Kenya (**Ref: ILRI-IREC2019-08/1, ILRI-RC012 19/IBC/012/CR and NACOSTI/P/19/94777/28167**)

Laboratory Animals

A cohort of 3 days old, commercially purchased local broiler chickens (*Gallus gallus domesticus)* were used (**Ref: ILRI-IACUC2021-31**)

### 3.3.2. Inclusion Criteria

- Phages that specifically infect *S.* Enteritidis serovar
- Phages stable at pH value between 1.5 and 9, and temperature between 37℃ and 50℃
- Healthy birds upon physical and clinical examination

### 3.3.3. Exclusion Criteria

- Phages that lose their viability (lytic capacity) along the purification process
- Phages with opaque plaques upon being spotted on *S.* Enteritidis
- Broiler chickens that have reached the end point i.e., extreme laboured breathing, fever (>43°C), fowl smelling diarrhoea and anorexia, were humanely euthanised.

### 3.4 Sampling techniques

Purposive sampling technique was carried out to select phages for the study, that show clear plaques on S. Enteritidis, do not lose viability during the phage purification process, are stable between 1-9 pH value and 37-50°C temperature. Broiler chickens were selected of the same age, weight, ideally the same body size, and without any clinical signs related to *Salmonella*.

### 3.5 Sample Size Determination

The control randomized trial followed the sample size estimation for the clinical trial, (Sakpal, 2010).

Level of significance = 5%, Power = 80%, Type of test = two-sided

Formula of calculating sample size is:

$$n = 2 \times \frac{\left(Z_{\frac{\alpha}{2}} + Z_{1-\beta}\right)^2}{d^2}\ \sigma^2$$

Where:

n = sample size required in each group (6 groups)

$\sigma^2$ = mean change in phage concentration to week 2 in Phage given groups = 5

$d^2$ = mean change in phage concentration from baseline to week 2 in placebo groups = 3

= clinically significant difference = 0.5

ó = standard deviation = 1.195

$Z_{\alpha/2}$: This depends on level of significance, for 5% this is 1.96

$Z_{1-\beta}$: This depends on power, for 80% this is 0.84

n= 2× $[1.96+0.84/3^2]^2( 5^2)$

n= (4 x 6) groups

**n = 24 chickens**

**3.6 Study Variables**

The pH values of media (TSB, SGF & SIF), temperature, water source and time of exposure were the independent variables, while the phage concentration and optical density were the dependent variables in the study. Encapsulated and non-encapsulated *S.* Enteritidis specific phages were exposed to different pH values of rich media (TSB), simulated digestive fluids (SGF & SIF), different temperature values, and water from different sources. The remaining phage concentrations for all phages were observed at different times of exposure. In chickens, both encapsulated

and non-encapsulated *S.* Enteritidis specific phages were given orally, and their concentrations were observed from the cloacal swabs and faeces.

### 3.7 Laboratory Procedures

#### 3.7.1 Bacteria strains Used

The *S.* Enteritidis strains used in this study were obtained by taking 1 g of chicken feces from farms in Kiambu and Nairobi counties, inoculating it in 10 ml of Tryptic Soy Broth (TSB), and allowing it to incubate for an entire night at 37 °C. The Selenite Fecal Broth (SFB) (Oxoid, Ireland) was then added to 5 ml of this combination, and it was incubated for 24 hours at 37 °C. The samples were then cultured on *Salmonella-Shigella* Agar, Brilliance Green *Salmonella* Agar, and XLT-4 (Oxoid, Ireland), then streaked on MacConkey agar media in Oxoid, Ireland (Oxoid, Ireland). The isolates were biochemically identified using the Triple Sugar Iron agar (TSI) (Oxoid, Ireland), Urea hydrolysis test agar (Oxoid, Ireland), motility indole-lysine media (Oxoid, Ireland), and biomérieux API test strips (biomérieux, France) to confirm the identity of *Salmonella*. Additionally, the isolates were serotyped using *Salmonella* polyvalent O and H antisera (*Salmonella* Agglutinating Serum, Remel Europe Ltd, Cambridge, UK). *Inva* PCR and CRISPR typing were used to confirm all *Salmonella* Enteritidis strains (Table 1) (Gunasegaran *et al*., 2011; Nair *et al*., 2015).

#### 3.7.2 Phages Used

The phages were obtained by introducing chicken farm feces into tryptic soy broth (TSB). Then 5 ml of the filtered supernatants was placed on Tryptic Soy Agar (TSA) plates that contained 200 ml of *S.* Enteritidis and 5 ml of soft agar (10 mm CaCl2, 0.7% agar) following overnight incubation at 42 °C and filtering (0.45 m Minisart® single-use filter unit, Sigma-Aldrich). The plates were then checked for cell lysis or phage plaques after 6 hours of incubation at 42 °C. Five rounds of plaque purification were used to purify the phage, with each round selecting one plaque at random. (Huang, Virk, *et al.*, 2018; Nyachieo *et al*., 2021).

### 3.7.3 Host Range of *Salmonella* Enteritidis-specific phages

The tropism of purified isolates was determined to identify phages that have specific activity toward *S.* Enteritidis strains (labeled IL-RI K1 to ILRI K63, indicating the place of isolation [ILRI] and the country of origin [Kenya]. In summary, 5 ml of the filtered supernatants were placed on TSA plates along with 200 ml of *S.* Enteritidis and 5 ml of soft agar (10 mm $CaCl_2$, 0.7% agar). After 6 hours at 42 °C, the plates were examined for signs of cell lysis or phage plaques. Five rounds of plaque purification were used to purify the phage, with each round selecting one plaque at random (Huang, Virk, *et al*., 2018; Nyachieo *et al*., 2021)

### 3.7.4 Restriction Fragment Length Polymorphism (RFLP)

By removing phages that were extremely closely related to each other using the EcoRV enzyme, phage selection was evaluated using restriction fragment length polymorphism (RFLP) analysis. First, phage DNA was isolated using the Phage DNA Isolation Kit (Norgen Biotek Corp, Thorold, Canada) in accordance with the manual. After that, 20 liters of the mixture including 1 gram of purified phage DNA, 1 liter of restriction enzyme, 2 liters of Green Buffer (FastDigest), and nuclease-free water were incubated at 37 ℃ for two hours. The phage DNA fragments were broken up by enzymatic digestion and then separated by electrophoresis in a 0.85% agarose gel in the TAE buffer (40X Tris-acetate-EDTA, Promega) at 50V/cm. As a size marker, the Biolabs TM 1kb DNA Ladder was applied. (Maszewska *et al*., 2016; Sharma *et al*., 2017).

### 3.7.5 Phage stability in pH-adjusted media

The pH of TSB was altered by either adding 1 N of NaOH or 1 N of HCL until the desired pH was achieved to test the stability of the phage at various pH levels (1, 2, 3, 4, 5, 6, 7, 8, 9, and 12). Then, 900 ml of TSB with an adjusted pH was mixed with 100 ml of phage ($8.9 \times 10^8$ PFU/ml), and the mixture was incubated at 42 °C for 12 hours. Following that, serial dilutions were performed, and PFU/ml was calculated using the double-layer method. Selected pH levels were utilized to gauge the drop in phage titres over the first three hours (3, 4, 9). Briefly, 900 ml of TSB with an

adjusted pH was mixed with 100 l of each phage lysate before being incubated at 42 °C for three hours. Using the double-layer method, phage titres were measured at 0, 0.5, 1, 2, and 3 hours, respectively (Duc *et al*., 2020).

### 3.7.6 Bacteriophage stability in simulated gastric and intestinal fluids

As previously described, phage stability was investigated in simulated SGF and SIF. SGF (Reagecon co. DBC12-250) and SIF (Reagecon co. DB13-121) had their pH values altered to 2.5 and 8, respectively. These are the ideal pH levels for the small intestine (pH 8) and genuine stomach (pH 2.5) of chickens. The solutions were amended by adding 1N of NaOH or 1N of HCL. A total of 900 ml of SGF and SIF were mixed with 100 ml of each phage lysate at a concentration of 8.9 x $10^8$ PFU/ml to evaluate the rate of phage persistence. This mixture was then incubated at 42 °C for three hours. Using the double-layer method, phage titres were tested at 0, 0.5, 1, 2, and 3 hours. (Abdelsattar *et al*., 2019; Silva Batalha *et al*., 2021).

### 3.7.7 Phage thermal stability

The stability of the 10 *S*. Enteritidis phages was tested at 25 ℃, 30 ℃, 37 ℃, 42 ℃, 50 ℃ and 60 ℃ as previously described (Huang, Virk, et al., 2018; A. Liu et al., 2020; F. Tang et al., 2019; Yan et al., 2020). Briefly, 100 µl of each S. Enteritidis phage were incubated overnight at various temperatures at a titre of 8.9 x $10^8$ PFU/ml. Additionally, phage titres were evaluated at 0, 0.5, 1, 2, and 3 hours. The PFU per ml were then calculated using the double-layer method following serial dilution.

### 3.7.8 Phage persistence in different water sources

A river that flows through the ILRI campus (1.2706° S, 36.7240° E), rain from Kangundo, Nairobi (1.3056° S, 37.3453° E), a borehole from the ILRI farm, and the tap in the ILRI laboratory were all used to collect water samples. The waters were separated into three groups after collection: autoclaved, filtered, and raw. Following water treatments, 900 µl of water was mixed with 100 l of each phage (adjusted to 4.5 x $10^{10}$ PFU/ml), and the mixture was then incubated at 37 °C. After 12, 24, and

48 hours, phage spot tests were performed. *S.* enteritidis Sal 568 was used as the host, and 20 µl of the material was taken, serially diluted, and spotted on TSA plates. The double-layer method was used to calculate PFUs per millilitre. ( Gundy *et al.,* 2009; Pinon & Vialette, 2019).

### 3.7.9 *Salmonella* eradication by phages in pH-adjusted medium

The pH of TSB was altered by either adding 1N of NaOH or 1N of HCL until the desired pH was attained to test the impact of pH on the phage's ability to suppress *Salmonella sp* (2, 3, and 8). A 4.5 x $10^7$ PFU/ml adjustment was made to all phage titters. The $10^6$ colony-forming units (CFU)/ml of *Salmonella* strain Sal 568 were obtained after 2 hours of exponential growth at 42 degrees Celsius. Then, 1 ml of the bacterial culture and 10 µl of the phage lysates were combined and incubated at 42 °C for 15 minutes. A pH-adjusted 1 ml of TSB was used to resuspend the phage-infected cell pellet after the combination was centrifuged at 7,000 g for 2 minutes. Optical density ($OD_{600nm}$) was then read at 0, 0.5, 1, 2, 3, and 4 hours, as described elsewhere (Clavijo *et al*., 2019; Larock *et al*., 2015).

### 3.7.10 Control of *Salmonella* by phages in Simulated Gastric Fluid and Simulated Intestinal Fluid

The pH of SGF and SIF were changed to pH 2.5 and pH 8, respectively, by either adding 1N of NaOH or 1N of HCL until the desired pH was reached, to test the impact of SGF and SIF on phage's ability to suppress *Salmonella sp*. A 4.5 x 107 PFU/ml adjustment was made to all phage titters. Briefly, 106 CFU/ml of the *Salmonella* Enteritidis strain Sal568 were obtained after 2 hours of exponential growth at 42 °C. Then, 1 ml of the bacterial culture was mixed with 10 µl of the phage lysates, and the mixture was incubated for 15 minutes at 42 °C. Following a 2-minute centrifugation at 7,000 g, the mixture was then resuspended in 1 ml of SGF or SIF with the phage-infected cell pellet. Optical density ($OD_{600nm}$) was then read at 0, 0.25, 0.5, 0.75, 1, 2, 3, and 4 hours, as described by others (Zaczek-Moczydłowska *et al*., 2020).

### 3.7.11 Amplification of phages in simulated gastric fluid

Previously reported methodologies were employed with minimal modifications to investigate the impact of SGF on phage titters after replication (Ramirez *et al*., 2018; Zaczek-Moczydłowska *et al*., 2020). Briefly, initial phage titters were adjusted to 2.1 x $10^7$ PFU/ml and SGF that had been pH adjusted (pH 2.5) was utilized. At 42 °C, *Salmonella* Enteritidis strain Sal568 was cultivated exponentially for two hours to a concentration of $10^6$ CFU/ml. Then, 1 ml of the bacterial culture was mixed with 10 µl of the phage lysate, and the mixture was incubated for 15 minutes at 42 °C. The bacterial pellets were then resuspended in 1 ml of SGF after the combination had been centrifuged at 7,000 g for 2 minutes. The mixture was shaken at 200 rpm while being incubated at 42 °C. The mixtures were centrifuged at 7,000 g for 2 minutes every 15 minutes to concentrate the phage-infected cells while collecting 20 µl of the supernatant to perform a double-layer phage titter assay on TSA plates. The mixture's volume was kept constant by adding 20 µl of SGF. This process was repeated 30, 45, and 60 minutes after the incubation period.

### 3.7.12 Synthesis and Characterization of Silica Vesicles

The SVs used in this study were acquired from Professor Neena Mitter (Mitter, 2022). The SV 100, SV 140, and SV 140-$C_{18}$ were the three SVs that were deployed. A two-step process for creating SVs has been previously explained. Briefly, 30 g of pH 4.7 NaAc-HAc buffer solution ([NaAc] = [HAc] = 0.40 M) were dissolved in 0.852 g of $Na_2SO_4$ and 0.5 g of $EO_{39}BO_{47}EO_{39}$ [commercial name B50-6600, where EO is poly (ethylene oxide) and BO is poly (butylene oxide) [Dow Company] under vigorous stirring overnight to form a homogeneous solution at 10℃. The solution was then given 3.33 g of tetraethyl orthosilicate (TEOS) with constant stirring for 24 hours at 10°C. In step two, the reaction mixture was heated hydrothermally at 140°C for an additional 24 hours in an autoclave. The SV-containing precipitate was filtered, repeatedly washed in deionized water to remove the salts that had been added, dried in the air, and then calcined at 550°C in a muffle furnace (Carbolite) for five hours. 48 mg of calcined SVs were mixed with 6 ml of toluene (Sigma Aldrich) in a 50 ml flask to alter SVs with octadecyl (-$C_{18}$) groups. After swirling the mixture

for 6 hours at 110°C, 0.12 ml of n-octadecyl-trimethoxysilane (Sigma-Aldrich) was added to the mixture, which was then agitated for another 12 hours at 110°C. The SVs were recovered by centrifugation, thoroughly cleaned with ethanol and toluene, and then dried inside a fume hood at room temperature (Lacasta *et al*., 2021; Mody *et al*., 2013).

### 3.7.13 Silica Vesicles resuspension

Resuspension of SVs was carried out as previously described. Briefly, while under a sterile laminar flow, an empty 50 ml Falcon tube was weighed. A portion of the lyophilized SV was placed into the weighted 50 ml tube. The tube with the SV substrate was weighed again to know how many SVs were in it. The content was resuspended with PBS (under the laminar flow) to have a suspension of 10 mg/ml. The content was mixed for 15 minutes in the sonicator bath while checking every 5 minutes if the suspension is homogenizing(Lacasta *et al*., 2021; Mody *et al*., 2013).

### 3.7.14 Binding of phages to functionalized SV particles

Phage physisorption, also known as electrostatic binding to SVs, was carried out as previously explained. Briefly, 10 µl of phage stock ($10^{10}$ PFU/ml) were combined with 50 µl of 10 mg/ml silica particles. Shaking was done while the mixture was incubated at 37 °C all night. The titer of non-immobilized phage particles in the supernatant was then calculated using the spot assay after centrifuging the phage and modified silica particle combination at 5000g for 2 minutes. After that, the phage-SV pellet was re-suspended in 50 ml of PBS buffer and centrifuged once more for 1 minute at 5000 g. When the pellet could not be easily re-suspended, a pipette tip was used to disrupt it, and the washing steps were repeated three times (Bone *et al*., 2018; Cademartiri *et al*., 2010).

### 3.7.15 The phage release rate from functionalized particles

As previously mentioned, the rate of SV-bound phage release was carried out. Briefly, 10 liters of phage stock (1010 PFU/ml) were combined with 50 µl of 10 mg/ml silica particles. Shaking was done while the mixture was incubated at 37 °C

all night. The titer of non-immobilized phage particles in the supernatant was then calculated using the spot assay after centrifuging the phage and modified silica particle combination at 5000g for 2 minutes. After that, the phage-SV pellet was re-dissolved in 50 l of PBS buffer and incubated at 37 °C while being shaken. The titer of non-immobilized phage particles in the supernatant was then evaluated using the spot assay after centrifuging the phage and modified silica particle combination at 5000g for 2 minutes every 12 hours (Bone *et al*., 2018; Cademartiri *et al*., 2010).

### 3.7.16 Phage detection on functionalized particles in Simulated Gastric Fluid

The previously altered technique was applied to assess the capacity of functionalized SVs to safeguard bound phages in SGF. Briefly, 10 liters of phage stock ($10^{10}$ PFU/ml) were combined with 50 liters of 10 mg/ml silica particles. Shaking was done while the mixture was incubated at 37 °C all night. The titer of non-immobilized phage particles in the supernatant was then calculated using the spot assay after centrifuging the phage and modified silica particle combination at 5000g for 2 minutes. After that, the phage-SV pellet was re-dissolved in 50 µl of pH 2.5 SGF and incubated at 37 °C while being shaken. 20 µl of the content were serially diluted and tested on TSA plates every 15 minutes (Bone *et al*., 2018; Cademartiri *et al*., 2010).

### 3.7.17 *In vivo* Stability of Encapsulated and Non encapsulated phages

The goal was to determine the survival nature of SV-encapsulated and non-encapsulated S. Enteritidis specific phages in chickens. Three days old chicken were purchased from Kenchic (Kenchic, 2022). The chicks were vaccinated at hatchery against Newcastle disease virus and infectious Bronchitis virus. On arrival of the birds at the ILRI Animal Isolation Unit, cloacal swabs and fecal samples were collected and tested for *Salmonella*-phages, using standard microbiology and molecular techniques explained above. After a week of acclimation, the birds were randomly allocated into individual pens with wood shavings in floor at a temperature of 30°C in a house provided with ventilation, and drinking water and feed were provided ad libitum. On day 0 of the experiment, all birds were orally gavaged individually with 1ml ( K1 = 8.95 X $10^{10}$ PFU/ml , K11= 7.84 X $10^{10}$ PFU/ml, K47=

9.52 X$10^{10}$ PFU/ml) of either SV-encapsulated or non-encapsulated phages, as shown in table 3.1. The control group was given PBS. The presence-absence of phages was measured by cloacal swabs and fecal collection at days 0, 1,2, 3, 5, 7, 9, 12, 14, 16, 21 and 28, and checked for the presence and concentration of bacteriophages using a double layer method (Lorenzo-Rebenaque *et al*., 2021; Vaz *et al.*, 2020; Wernicki *et al*., 2017).

Table 3.1: Phage allocation used in the animal study.

| **Chicken ID** | **Phage Given** | **Encapsulation** |
|---|---|---|
| A1 | PBS | Non |
| A2 | PBS | Non |
| A3 | PBS | Non |
| A4 | K11 (SV) | SV- Encapsulation |
| A5 | K11(SV) | SV- Encapsulation |
| A6 | K47 (SV) | SV- Encapsulation |
| A7 | K1 | Non |
| A8 | PBS | Non |
| A9 | K1 | Non |
| A10 | K47 (SV) | SV- Encapsulation |
| A11 | K47 | Non |
| A12 | K1 (SV) | SV- Encapsulation |
| A13 | PBS | Non |
| A14 | K11 | Non |
| A15 | K1 | Non |
| A16 | K47 (SV) | SV- Encapsulation |
| A17 | K1 (SV) | SV- Encapsulation |
| A18 | K47 | Non |
| A19 | K11 | Non |
| A20 | PBS | Non |
| A21 | K11 | Non |
| A22 | K47 | Non |
| A23 | K1 (SV) | SV- Encapsulation |
| A24 | K11(SV) | SV- Encapsulation |

### 3.8 Data analysis

To ascertain the variations in means among different phages and time points as well as after exposure to various pH and temperature values, a two-way analysis of variance (ANOVA) was performed. Additionally, it was used to compare phage means both before and after the phage binding procedure with SVs. To evaluate the phage amplification in pH-adjusted media, SGF, and SIF, as well as to calculate phage survival in various water sources, a straightforward linear regression model was applied. It was also used to assess the importance of phages in terms of release rate and SVs' capacity to shield phages in SGF and chickens. The GraphPad Prism software, version 9.2.0, was used to conduct the statistical analyses. Each statistical analysis was considered significant if the P value was less than or equal to 0.05. Phage experiments were conducted twice with triplicate values.

### 3.9 Data Management

All the data was recorded in Microsoft excel 365 and stored in the CGIAR OneDrive.

# CHAPTER FOUR

# RESULTS

## 4.1. Determination of *Salmonella* Enteritidis Specific bacteriophages thermal & pH stable values and the host range.

### 4.1.1. Isolated Enteritidis Specific Bacteriophages and their Host Range

While only 10% of the samples from the visited farms had *Salmonella sp*. strains, 75% contained a total of 600 *Salmonella* phages. After being tested against a panel of 16 *Salmonella* strains from the Enteritidis, Heidelberg, and Kentucky serovars that were recovered from the same chicken farms, 63 (10.5%) of the purified phages were able to infect and lyse at least one of the 16 *Salmonella* strains.

**Table 4.1: Typing of *Salmonella* Strains used in this study.**

| | Sal 16 | Sal 73 | Sal 157 | Sal 172 | Sal 177 | Sal 181 | Sal 182 | Sal 187 | Sal 188 | Sal 192 | Sal 194 | Sal 312 | Sal 568 | Sal 569 | Sal 571 | Sal 572 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Inva* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Group O (A-S) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Poly H (Phase 1 & 2) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Group D (9) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| CRISPR 1 | E | E | C | K | E | K | K | H | H | C | C | E | E | E | K | E |
| CRISPR 2 | E | E | H | K | E | K | K | H | H | H | H | E | E | E | K | E |

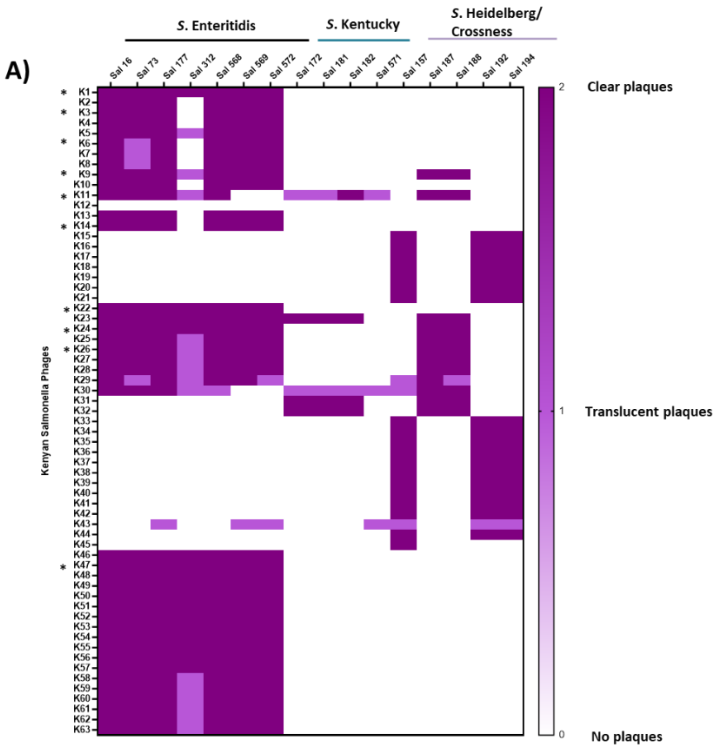
E: Enteritidis, C: Crossness, H: Heidelberg, K: Kentucky.

On the six *S.* enteritidis serovars (Sal 16, Sal 73, Sal 177, Sal 568, Sal 569, and Sal 572), a total of 39 (5%) phages were able to display plaques, however some phages failed to do so on serovar sal 312. *S.* Kentucky and S. Heidelberg serovars may be infected and destroyed by four (10.2%) of the *S.* Enteritidis phages (K11, K23, K30, and K43). The remaining 20% of the phages had translucent plaques, indicating that

they are lysogenic/template phages, whereas the remaining 80% of the phages displayed clear plaques, indicating that they are lytic phages (Figure 4.1A).

### 4.1.2. RFLP analyses for *Salmonella* Phages

The DNA digestion with restriction enzyme EcoRV revealed different patterns with the band sizes ranging from 100MB to 1KB (Figure 4.1B). All the sample were efficiently digested by the enzymes. A total of 10 different phages were chosen for further characterisation using host range and RFLP tests (Table 4.2).



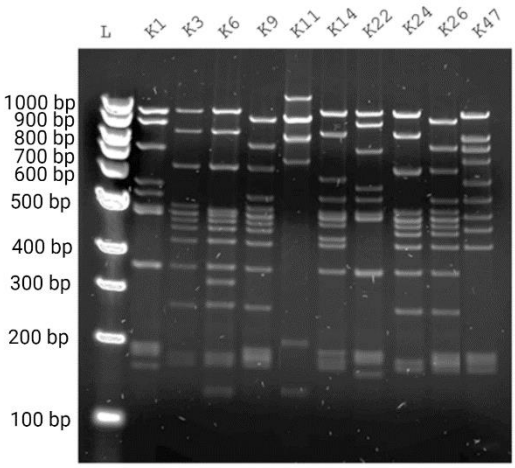



**Figure 4.1:Variations in the genomic DNA of *Salmonella* phage hosts and restriction fragment length polymorphism (RFLP)**

**A)** The tropism of 63 *Salmonella sp*. phages for the Enteritidis, Kentucky, and Heidelberg serovars is displayed on a heatmap. The selected phages are denoted by an asterisk (*). **B)** Ten DNA profiles were discovered by gel electrophoresis of DNA from the genomes of Kenyan *S.* Enteritidis phages that had been EcoRV-digested.

**Table 4.2: Phages and *S.* Enteritidis serovars identity based on area of sample collection**

| Phages | Original *Salmonella* Strain | Poultry Farm (PF)/Slaughterhouse (SH) | Region | Remarks |
|---|---|---|---|---|
| ILRI_K1 | Sal16 | PF_16 | Kiambu (Peri-Urban) | *Salmonella* isolated |
| ILRI_K3 | Sal16 | SH_6 | Nairobi (Urban) | *Salmonella* absent |
| ILRI_K6 | Sal16 | SH_7 | Nairobi (Urban) | *Salmonella* absent |
| ILRI_K9 | Sal16 | PF-58 | Nairobi (Urban) | *Salmonella* absent |
| ILRI_K11 | Sal73 | SH_1 | Kiambu (Peri-Urban) | *Salmonella* isolated |
| ILRI_K14 | Sal73 | PF_33 | Kiambu (Peri-Urban) | *Salmonella* isolated |
| ILRI_K22 | Sal177 | PF_16 | Kiambu (Peri-Urban) | *Salmonella* isolated |
| ILRI_K24 | Sal177 | SH_6 | Nairobi (Urban) | *Salmonella* absent |
| ILRI_K26 | Sal177 | SH_6 | Nairobi (Urban) | *Salmonella* absent |
| ILRI_K47 | Sal312 | SH_6 | Nairobi (Urban) | *Salmonella* absent |

### 4.1.3. Preliminary Whole Genome Sequencing for *Salmonella* Phages

Preliminary whole genome sequencing data analyses indicate that these are novel phages, not hitherto isolated (Table 4.3).

**Table 4.3: Phage genomes from Kenya and reported phage genomes from the NCBI public database have comparable nucleotides.**

| | Most Similar Kenyan Phage Genome | | Most Similar Phage Genome from the NCBI Public Database | | |
|---|---|---|---|---|---|
| | Phage Name | Nucleotide Similarity % (Aligned Nucleotide %) | Phage Name | NCBI Accession Number | Nucleotide Similarity % (Aligned Nucleotide %) |
| ILRI_K1 | ILRI_K22 | 99.99 (100%) | *Salmonella* phage wast | MT074451.1 | 93.72% (90%) |
| ILRI_K3 | ILRI_K24 | 99.79% (100%) | *Salmonella* phage wast | MT074451.1 | 92.18% (90%) |
| ILRI_K6 | ILRI_K24 | 98.23% (97%) | *Salmonella* phage wast | MT074451.1 | 92.30% (86%) |
| ILRI_K9 | ILRI_K26 | 99.99 (100%) | *Salmonella* phage wast | MT074451.1 | 92.70% (90%) |
| ILRI_K11 | None | -- | *Salmonella* phage SP6 | AY288927.2 | 89.67% (90%) |
| ILRI_K14 | ILRI_K1 & _K22 | 97.27% (96%) | *Salmonella* phage wast | MT074451.1 | 93.59% (92%) |
| ILRI_K22 | ILRI_K1 | 99.99 (100%) | *Salmonella* phage wast | MT074451.1 | 93.72% (90%) |
| ILRI_K24 | ILRI_K3 | 99.79% (100%) | *Salmonella* phage wast | MT074451.1 | 92.41% (90%) |
| ILRI_K26 | ILRI_K9 | 99.99 (93%) | *Salmonella* phage wast | MT074451.1 | 92.55% (90%) |
| ILRI_K47 | ILRI_K9 | 96.74% (93%) | *Salmonella* phage wast | MT074451.1 | 92.44% (91%) |

### 4.1.4. Evaluating the stability of phages in medium at different pH levels

The stability of the phages was evaluated in the low pH conditions present in the chicken gastrointestinal tract (cGIT) to find those that can survive there. All phages were comparatively stable between pH 4 and 9 after 12 hours of incubation in pH-adjusted TSB, with maximal stability near neutral pH. (Figure 4.2A). At pH 1 and 2, most phages were inactivated after 12 hours (Figure 4.2A). At pH 1 and pH 2, complete inactivation was seen after just 30 and 60 minutes, respectively. After 12 hours, phage titters at pH 3 drastically decreased (Figure 4.2A). A check was made on each phage's data for the specific pH values of 3 and 9, which are near to those of the chicken proventriculus (pH between 2 and 3) and gut (pH between 8 and 9). All phages were shown to behave uniformly with inactivation throughout time (Figures 4.2B and 4.2C, Appendix 1A). Phages ILRI K11 and ILRI K14 were slightly more quickly inactivated after two hours at pH 3 compared to the other phages (Figure 4.2B). Phage titres dropped for up to three hours at pH 9. (Figure 4.2C). Viral titres, however, were noticeably higher than those determined at pH 3. (Figure 4.2C). At pH 9, there were no discernible differences between the phages at any given time (Appendix 2A).

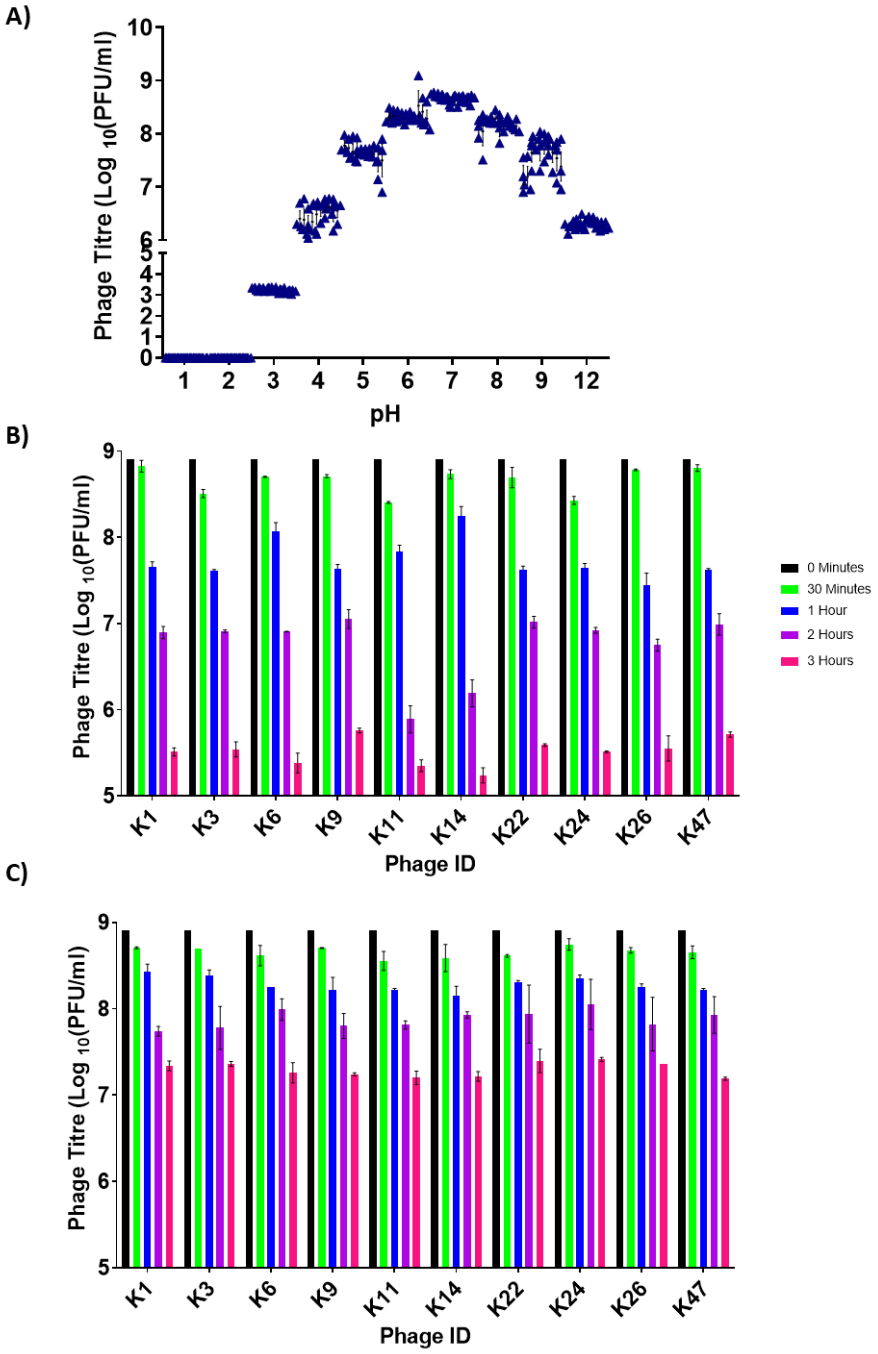



**Figure 4.2:** Phage stability in TSB medium with a modified pH

A) After 12 hours of incubation at 37 °C, stability of *S.* Enteritidis phages in TSB was adjusted to pH values of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 12. The black triangle on each bar graph represents a different phage. B) Individual *S.* Enteritidis phages can survive in TSB at pH 3 for up to three hours while being incubated at 37°C. Each bar represents the timing of phage titters. C) Individual *S.* Enteritidis phages can remain

viable in TSB at pH 9 for up to three hours of incubation at 37°C. Phage titres at times are shown by each bar. The standard error of the mean (SE) is shown by error bars. Black bar indicates 0 seconds, green bar 30 seconds, blue bar 1-hour, purple bar 2 hours, and magenta bar 3 hours. Each experiment was run twice, and the results were recorded in triplicate.

### 4.1.5. Evaluating the stability of phages in simulated gastric and intestinal fluids.

It was also evaluated how long the phage would remain infectious in commercially available simulated gastric (SGF) and intestinal (SIF) fluids. The product for SGF included distilled water, pepsin, sodium chloride, and hydrochloric acid. Along with sodium hydroxide, pancreatin and, potassium phosphate, the same components were employed in SIF. The mixes were designed to replicate the environment encountered in the cGIT. The pH of the proventriculus (real stomach) of chicken's ranges from 2 to 3. Due to the fact that the usual time for food to travel through this organ is 60 minutes, the 10 phages were put to SGF conditions at pH 2.5 for that duration (Ravindran, 2013). After 60 minutes of incubation, the phage titre significantly decreased by about 5 logs before stabilizing (Figure 4.3A). After the first two minutes, there was a decrease of about three logs. The most unstable phage, ILRI K29, had a final titre of 2 x 102 PFU/ml after 60 minutes of incubation in SGF (pH 2.5). (Figure 4.3A). The phages were entirely neutralized by longer incubation times. During the first 40 minutes, there were considerable variations among the phages (p values ranging from 0.001 to 0.0475, Appendix 3). Nevertheless, the changes in the last 20 minutes were not statistically significant (p values ranging from 0.0545 to > 0.9999, Appendix 3). The chicken cecum (intestine), which has a more basic environment with a pH of roughly 8, may be the possible hosts for these phages. For 120 minutes, the typical transit time for meals in this organ, the 10 phages were exposed to SIF with pH 8 adjustment. 30 minutes into the incubation period, a decrease in phage titre was seen (Figure 4.3B). Nevertheless, for up to 2 hours in SIF, all 13 phages were largely stable. While phage ILRI K6 and ILRI K47 had the highest final titre at 9.3 x 106 PFU/ml, phage ILRI K23 had the lowest phage titre at 120 minutes (2.2 x 106 PFU/ml). With time periods at 30, 60, and 90 minutes of

incubation showing the most noteworthy significant changes (p values ranging from 0.0001 to > 0.0472, Appendix 4), phage concentrations varied significantly among phages.

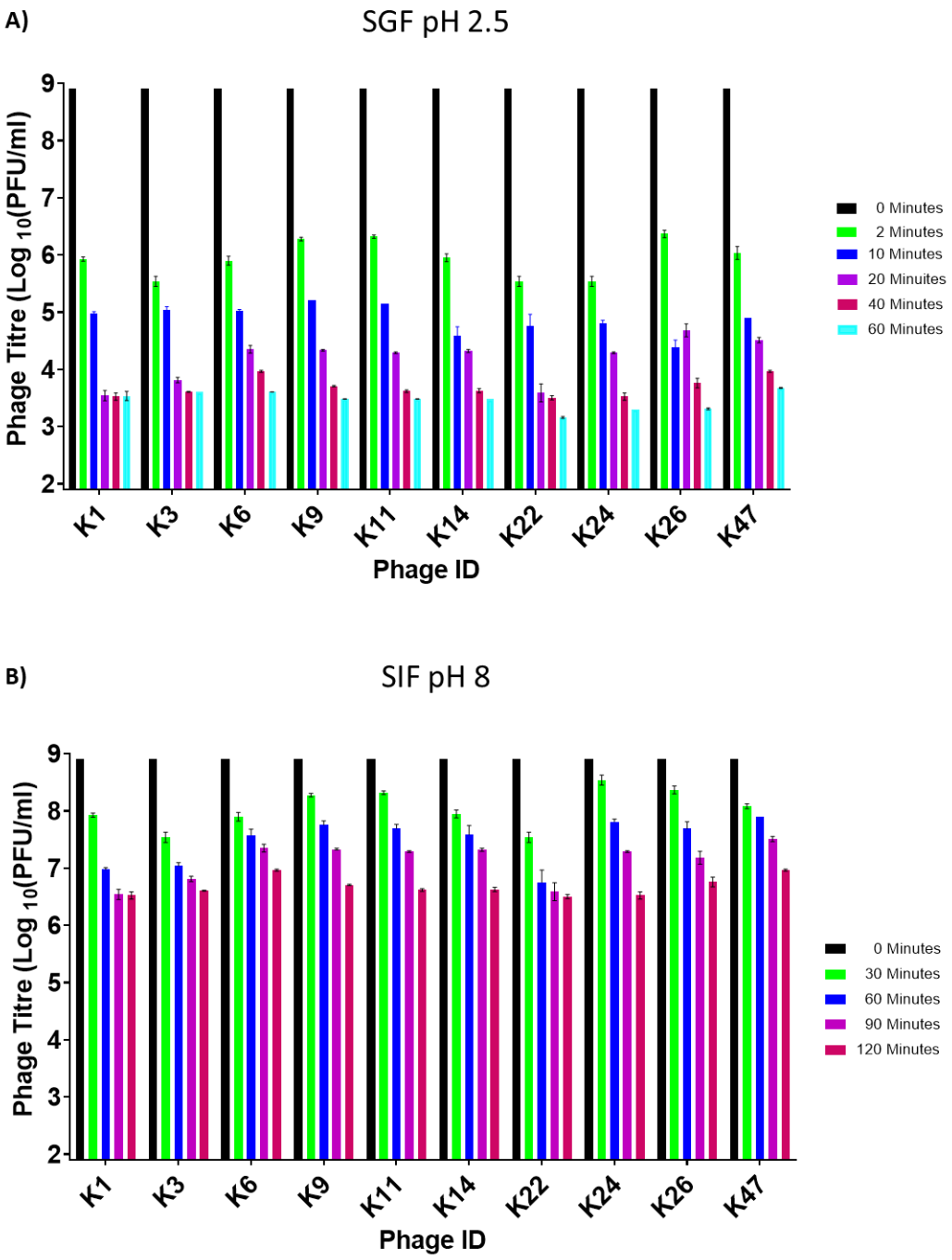



**Figure 4.3: Phage stability in simulated digestive environment**.

A) Individual phage stability during incubation in SGF for 60 minutes at 42 °C. Individual phage titres were calculated at each of the following time points: 0 (black bar), 2 (green), 10 (blue), 20 (purple), 40 (magenta), and 60 (turquoise). B) Individual phage stability throughout a 120-minute incubation period at 42 °C in SIF. Individual phage titres were measured at each of the following time points: 0 (black bar), 30 (green), 60 (blue), 90 (purple), and 120 (magenta). Each experiment was run twice, and the results were recorded in triplicate. Phage titres at times are shown by each bar. The standard error of the mean (SE) is shown by error bars.

### 4.1.6. Phage stability at different temperatures

The capacity of phages to maintain stability throughout a range of temperatures is another crucial factor to look at. The temperature range included 25 °C, which is the typical daily temperature for a large portion of Kenya for a significant portion of the year (KMD, 2022), 42 °C, which is the typical body temperature of chickens (Kentucky University, 2019), and 50–60 °C, which are temperatures that can be reached during phage production processes, such as spray–drying (Malik, 2021). After 12 hours, the phages were generally stable between 25 °C and 37 °C (Figure 4.4A). After 3 hours at 37 °C, a 1-log decrease in phage titre was seen (Figure 4.4B). For the first hour of incubation, there was a significant difference among the phages (p values ranging from 0.0001 to 0.0493, Appendix 5), but after that point, there was no longer a significant difference (p values ranging from 0.0582 to > 0.9999, Appendix 5). Phage titters were very equal at 42 °C after 3 hours of incubation, with phage ILRI K6 having the lowest concentration at 7.5 x $10^7$ PFU/ml and phage ILRI K47 having the highest concentration at 8 x $10^7$ PFU/ml (Figure 4.4C).

Phages were generally stable at 37 °C and 42 °C, but as soon as the incubation time at 50 °C began, there was a considerable decline in phage concentration (Figure 4.4D). The concentration of the ILRI K1, _K3, _K10, and _K11 phages was the lowest (Figure 4.4D). However, even after three hours at 50 °C, ILRI _K26, _K29, and _K47 phages were still present in rather high numbers. Only between 0 and 30 minutes of incubation, at this temperature, did phages differ significantly from one another (p values ranging from 0.0001 to 0.0243, Appendix 7). After that, no more

distinguishing characteristics amongst phages were found (p values ranging from 0.0518 to > 0.9999, Appendix 7).

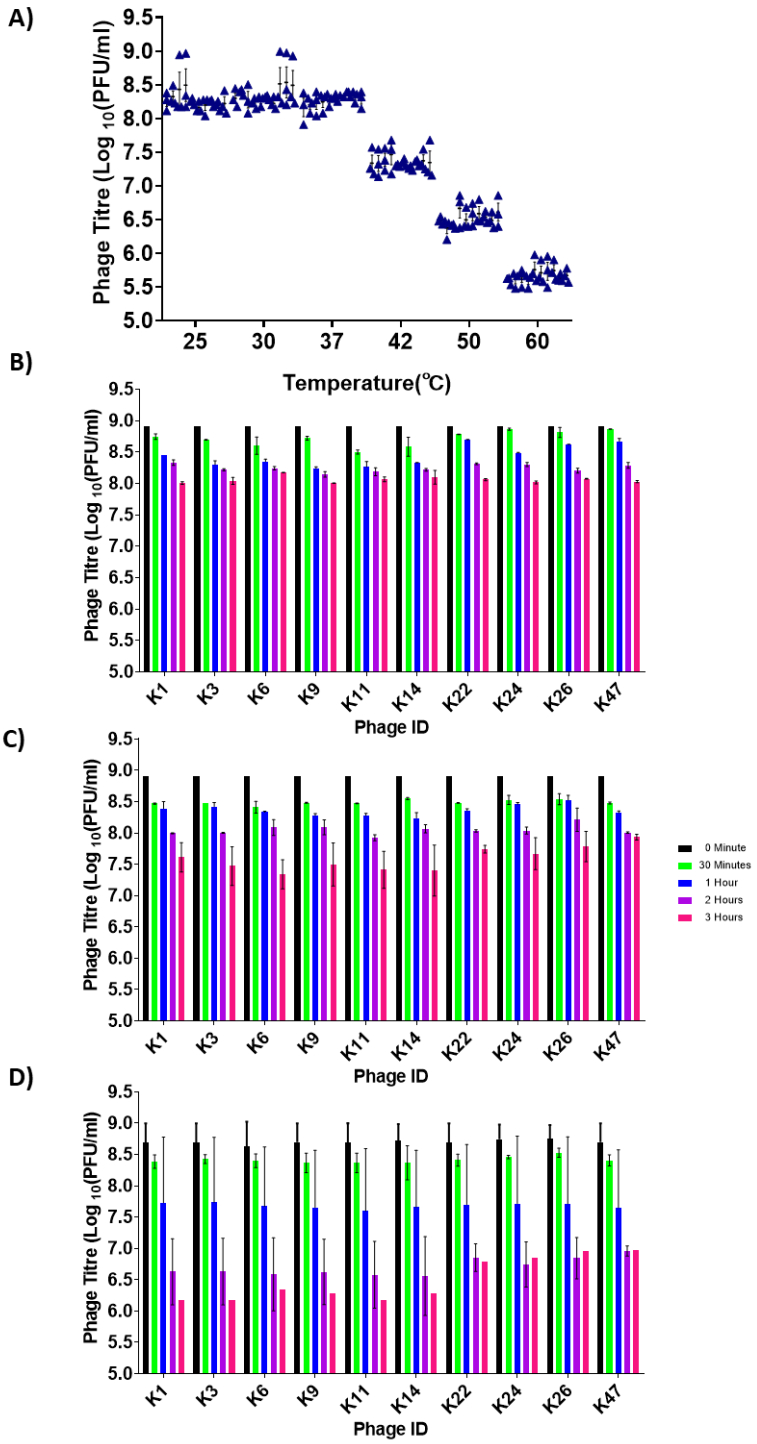



**Figure 4.4: Phage thermal stability assay**

**A)** *S.* Enteritidis phages were generally stable throughout a 12-hour period at 25°C, 30°C, 37°C, 42°C, 50°C, and 60°C. On each bar graph, the black triangle denotes a particular phage. After 12 hours of incubation, phage titres are shown in a bar graph. **B)** After incubation for three hours, phage stability at 37 °C. After 0, 0.5, 1, 2, and 3 hours, the specific phage titres were calculated. **C)** Phage stability following a 3-hour incubation at 42 C. After 0, 0.5, 1, 2, and 3 hours, the specific phage titres were calculated. **D)** After incubating for three hours, phage stability at 50 °C. After 0, 0.5, 1, 2, and 3 hours, the specific phage titres were calculated. Every experiment was performed three times.

### 4.1.7. Phage persistence in water from different sources

The viability and simplicity of administering phages through the water provided to hens in poultry farms have been established in earlier investigations. The persistence of a selection of these phages (ILRI_K1, ILRI_K6, ILRI_K14, ILRI_K24, and ILRI_K47) was examined in various water sources, including rivers, rain, boreholes, and tap water because the water source for hens in Kenya can range from one farm to another (Figure 4.5). Raw (unmodified) water samples from each of the four sources were analysed, as well as filtered and autoclaved samples. After 50 hours of incubation, phages from river water had the most unfavourable impact, with an average drop of 5 logs PFU/ml (Figure 4.5A). Only a 2-log PFU/ml reduction was visible in the rain (Figure 4.5B), borehole (Figure 4.5C), and tap water (Figure 4.5D). River water that has been autoclaved or filtered considerably decreased phage titters by 6 and 5 logs, respectively (Figure 4.5A). After 12 hours of incubation, the phage ILRI_K47 had, on average, the highest phage titre across all water sources (River: $3.8 \times 10^5$ PFU/ml, Borehole: $3.2 \times 10^8$ PFU/ml, Rain: $3.4 \times 10^8$ PFU/ml, Tap: $5.9 \times 10^8$ PFU/ml). On the other hand, after 12 hours of incubation in all water sources phage ILRI K14 had the average lowest phage concentration (River: $1.2 \times 10^4$ PFU/ml, Borehole: $1.1 \times 10^8$ PFU/ml, Rain: $1.2 \times 10^8$ PFU/ml, Tap: $1.2 \times 10^8$ PFU/ml).

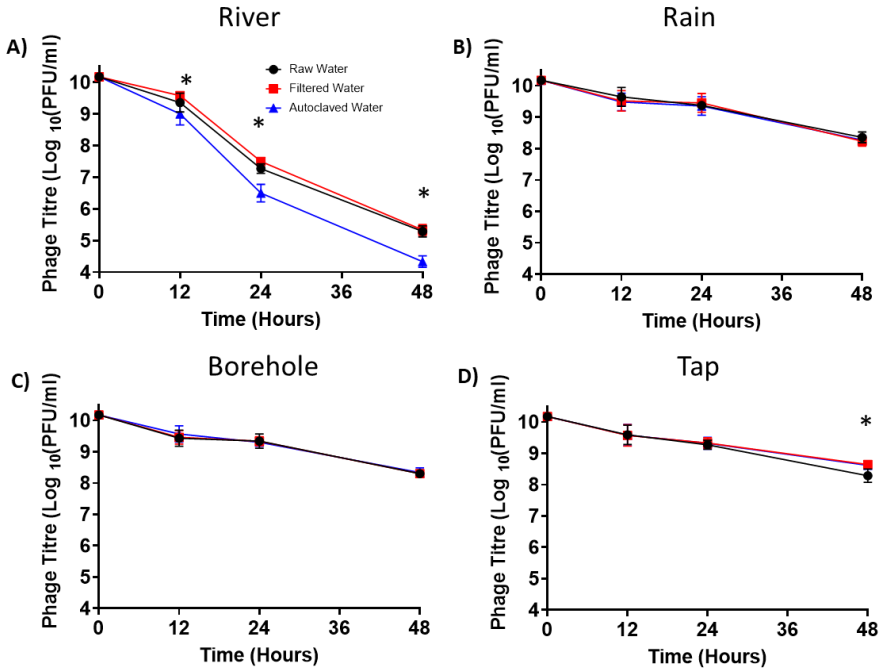



**Figure 4.5: Phage survival in various sources of water**

Significant difference between filtered and autoclaved water, from 12 to 40 hours of incubation (p values range from 0.0001 to 0.0396). Phage persistence in **A)** river water and B) rainwater. Significant differences between raw and filtered water at 48 hours of incubation (P = 0.0365). Phage persistence in **C)** borehole water and **D)** tap water. Blue triangle denotes autoclaved water, red square unfiltered water, and black circle raw water. Each experiment was run twice, and the results were recorded in triplicate. The standard error of the mean (SE) is shown by error bars.

### 4.2 Determination of the growth kinetics of *Salmonella* enterica serovar Enteritidis bacteriophages in rich media and simulated digestive system

#### 4.2.1 *Salmonella* eradication by phages in pH-adjusted medium

Phages can encounter their target bacteria in an animal host and reproduce, thereby lowering the number of the desired bacterium. Therefore, it was examined how phage presence in low and high pH-adjusted TSB media would affect a bacterial host. Because it is susceptible to all 10 phages, *S.* Enteritidis isolate 568 (Sal 568) was chosen. Optical density ($OD600_{nm}$) in TSB at pH 2, 3, and 8 and in the presence

of each phage were used to quantify bacterial growth at 42 °C. The OD in TSB at pH 2 was stable for up to 4 hours of incubation (Figure 4.6A). From 30 minutes to 4 hours at pH 2, most phages showed statistically significant alterations (p values ranging from 0.0001 to 0.0448, Appendix 8A). The $OD600_{nm}$ at pH 3 grew progressively for one hour before remaining stable for up to four hours of incubation (Figure 4.6B). From 30 minutes to 4 hours of incubation, most phages showed statistically significant changes (p values ranging from 0.0001 to 0.0497, Appendix 8B). At pH 8, Sal 568's OD600nm significantly dropped in less than an hour when phages were present before progressively rising from two to four hours of incubation (Figure 4.6C). From 30 minutes to 4 hours, most phages showed statistically significant changes at pH 8 (p values ranging from 0.0001 to 0.0494, Appendix 8C). At pH 2, ILRI K1, ILRI K9, and ILRI K11 were the phages that had the best control over Sal 568 growth at the end of the incubation.

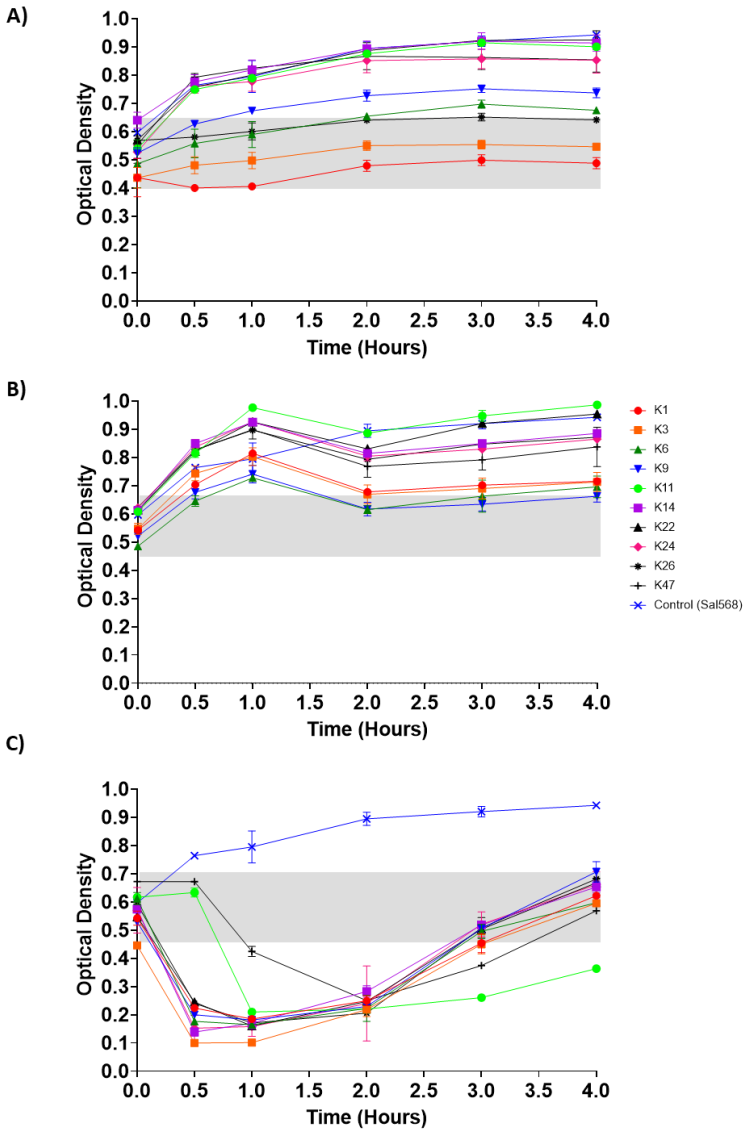



**Figure 4.6: Eradication of *S.* Enteritidis by phages in pH-adjusted media.**

TSB was adjusted to pH **A)** 2, **B)** 3, and **C)** 8. The optical density ($OD_{600nm}$) of the mixture of *S.* Enteritidis isolate 568 ($10^6$ CFU/ml) and the 10 phages (4.5 x $10^7$ PFU/ml) were measured after 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours. The grey shading indicates initial OD values at the start of the experiment. All experiments were repeated twice and measured in triplicate. Error bars represent the standard error of the mean (± SE).

### 4.2.2 Control of *Salmonella* by phages in Simulated Gastric Fluid and Simulated Intestinal Fluid

The same tests that were carried out in SGF and SIF were reported above. To simulate the transit time through the organs represented by these biorelevant dissolving medium, the incubation times for SGF and SIF were 60 minutes and 3 hours, respectively. For up to an hour of incubation, Sal568 ($10^6$ CFU/ml) and the 13 phages (4.5 x $10^7$ PFU/ml) in SGF adjusted to a pH of 2.5 maintained a steady OD600nm (Figure 4.7A). Phage ILRI K22 was more effective than phages ILRI K24 and _K26 at inhibiting the growth of the chosen Salmonella strain under these environmental conditions. In less than an hour and for up to three hours of incubation, the growth of Sal568 was dramatically reduced in the presence of each of the 13 phages in SIF adjusted to pH 8. (Figure 4.7B). In comparison to the other 12 phages, ILRI K1 was the most effective at inhibiting Salmonella development under

these environmental conditions.

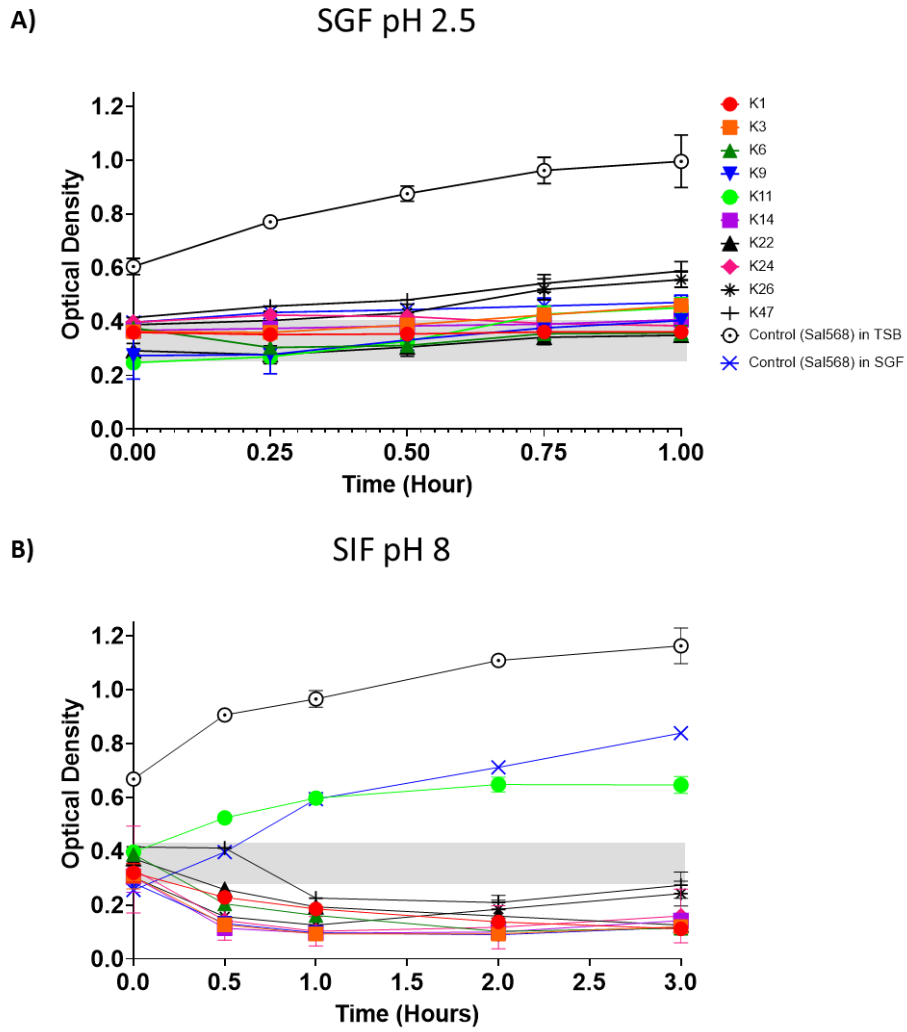



**Figure 4.7: Control of *S.* Enteritidis growth by phages in SGF and SIF.**

A) Effect of SGF on phage efficiency to control the growth of *S.* Enteritidis Sal568. B) Effect of SIF on phage efficiency to control the growth of *S.* Enteritidis Sal568. The optical density (OD600nm) of the mixture of *S.* Enteritidis Sal568 ($10^6$ CFU/ml) and the 10 phages (4.5 x $10^7$ PFU/ml) were measured at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours. The grey shading indicates initial OD values at the start of the experiment. All experiments were repeated twice and measured in triplicate. Error bars represent the standard error of the mean (± SE).

### 4.2.3 Phage replication in Simulated Gastric Fluid

All 10 phages were examined to see how their phage titres would be affected in SGF adjusted to pH 2.5 and in the presence of their bacterial host because they were all primarily affected by the conditions found in SGF at pH 2.5 (Figure 4.8). The same experimental procedure was used to regulate Salmonella growth in SGF, however viral titres rather than optical density brought on by bacterial growth were measured. It was noted that during the first 15 minutes of replication, the viral titres decreased by 0.5 log PFU/ml. The virus titres stayed steady after those 15 minutes for 45 minutes before gradually rising (Figure 4.8). At 15 minutes, there was a statistically significant difference between the phages ILRI K1 and ILRI K11 (P = 0.042) and ILRI K9 and ILRI K11 (P = 0.0471). At 30 minutes of incubation, significant differences were seen between ILRI K11 and ILRI K26 (P= 0.0356) as well as ILRI K14 and ILRI K26 (P= 0.0356). After 60 minutes of incubation, phage ILRI K47 had the highest titre while phage ILRI K9 had the lowest titre.

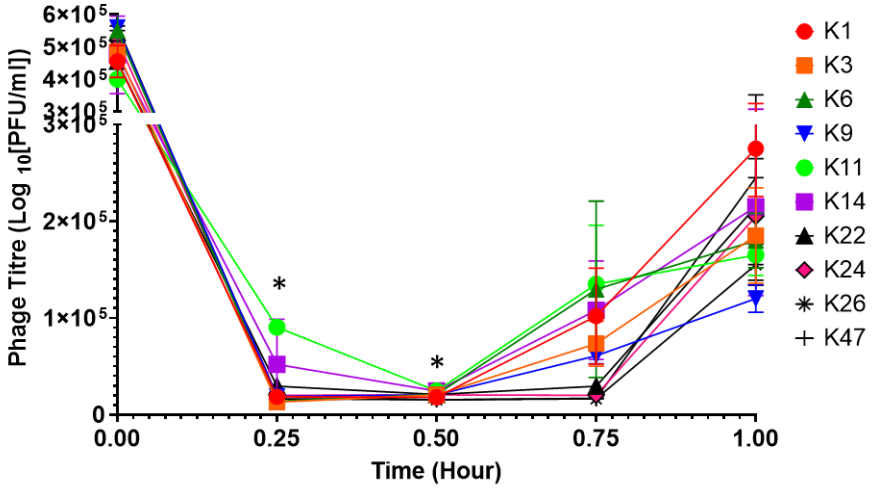



**Figure 4.8: Phage titres measured following infection of *S.* Enteritidis Sal 568 in SGF.**

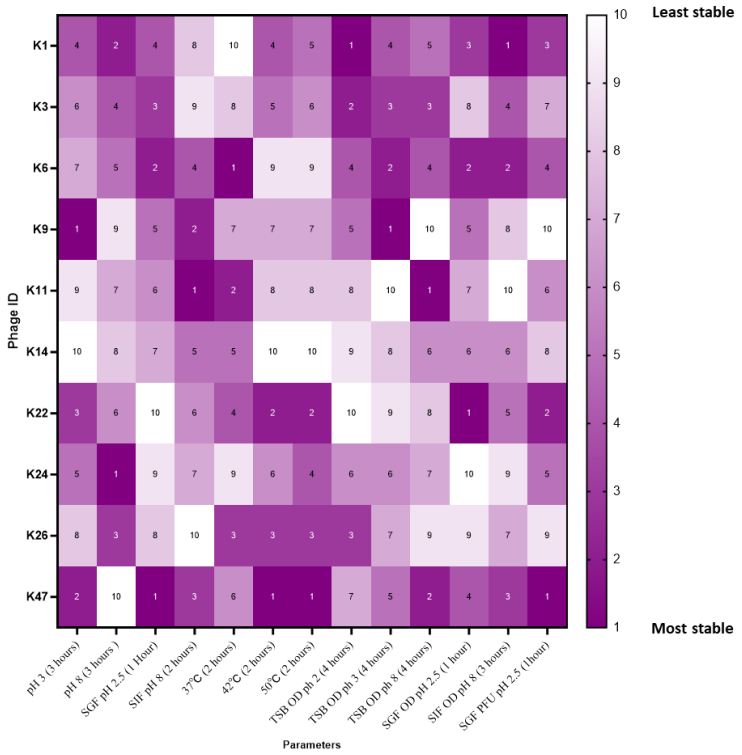
Viral titre was determined through spot assays following the infection of S. Enteritidis isolate 568 ($10^6$ CFU/ml) by the 10 phages (4.5 x $10^7$ PFU/ml). Error bars represent the standard error of the mean (± SE). All experiments were repeated twice and measured in triplicate. *Significant differences between phages ILRI_K1 and

ILRI_K11 (P = 0. 0.042), and between ILRI_K9 and ILRI_K11(P = 0.0471) at 15 minutes; as well as between ILRI_K11 and ILRI_K26 (P = 0.0356) and between phages ILRI_K14 and ILRI_K26 (P = 0.0356) at 30 minutes of incubation.

### 4.2.4 Phage stability scoring system.

To determine the top phages that are most likely to perform well in vivo, a scoring system (Figure 4.9A) was created considering all parameters aside from phage survival in various water sources. Stability at various temperatures (37, 42, and 50 °C), in TSB media with pH levels between 3 and 9, in SGF with a pH of 2.5, and in SIF were the characteristics that were used (pH 8). Under each of those circumstances, the phages were evaluated from best (score of 1) to worst (score of 13), and all the scores were summed. The phage with the lowest overall score was rated first, while the one with the greatest overall score came in last and was ranked at position 13. ILRI K47 was the most resilient of the 13 phages examined. ILRI K29, however, had the lowest final titters for most of the characteristics (Figure 4.9B).

A)




B)

| Phages | Total Score |
|---|---|
| ILRI_K47 | 46 |
| ILRI_K1 | 54 |
| ILRI_K6 | 55 |
| ILRI_K3 | 68 |
| ILRI_K22 | 68 |
| ILRI_K9 | 77 |
| ILRI_K26 | 82 |
| ILRI_K11 | 83 |
| ILRI_K24 | 84 |
| ILRI_K14 | 98 |

**Figure 4.9: Phage stability scoring system.**

**(A)** The heatmap showing the ranking of the 10 S. Enteritidis phages based on the stability of 13 parameters, excluding water and SVs. **(B)** A table showing the ranking and scoring system for the phages.

**4.3 Determination the phage binding capacity, rate of release and the phage protective capability of mesoporous silica vesicles in simulated digestive system, and in chicken model.**

### 4.3.1 Phage Binding to Silica Vesicles

With the goal to determine whether SVs can be used as a potential phage delivery tool in the gastrointestinal tract of chickens and other hosts, the ability of phages binding to the external and internal walls of the SVs using electrostatic interactions, was checked. To achieve this, the concentration of unbound phages before and after mixing with SVs had to be determined. All SVs led to the surface adsorption of active phages. SV 140-$C_{18}$ has the highest adsorption effect on phages with the phage concentration reduction in the supernatant by 5 logs PFU/ml. SV 100 and SV 140 resulted in the reduction by 4 logs PFU/ml (Figure 4.10). SV 100-$C_{18}$ had the highest average adsorption/encapsulation efficiency of 90.4% while SV 100 and SV 140 had 57.5% and 60% respectively (Figure 4.11). Phages K6 and K23 had the highest average adsorption/ encapsulation efficiencies of 96.8%.

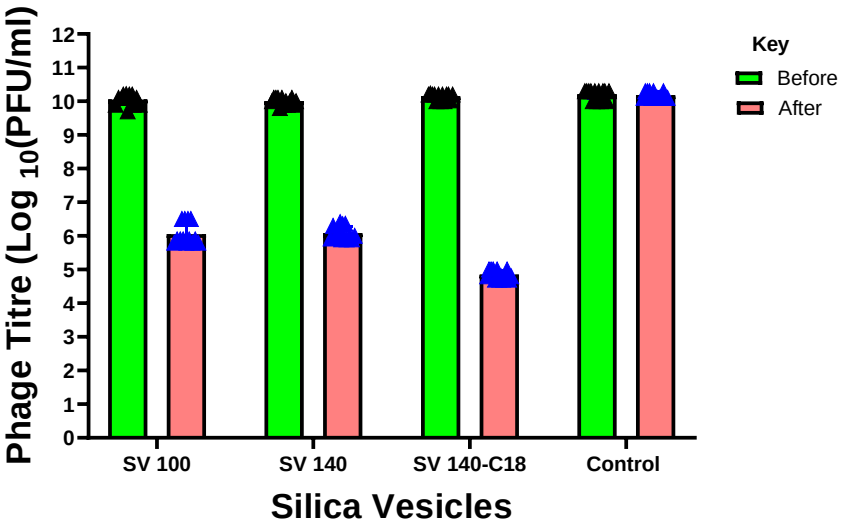



**Figure 4.10: Phage binding to Silica Vesicles.**

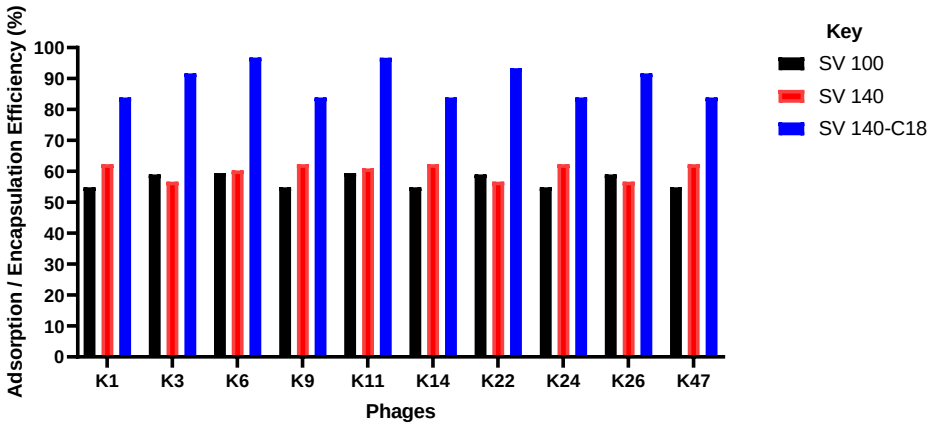



**Figure 4.11: Phage adsorption/ encapsulation efficiency.**

### 4.3.2 Release of Phages from Silica Vesicles

All three SVs showed that they could release for up to four days. With an average release concentration of 7.92 logs PFU/ml, SV 140-C18 had the highest concentration, followed by SV-100 (6.90 logs PFU/ml) and SV-140 (6.71 logs PFU/ml) (Figure 4.12).

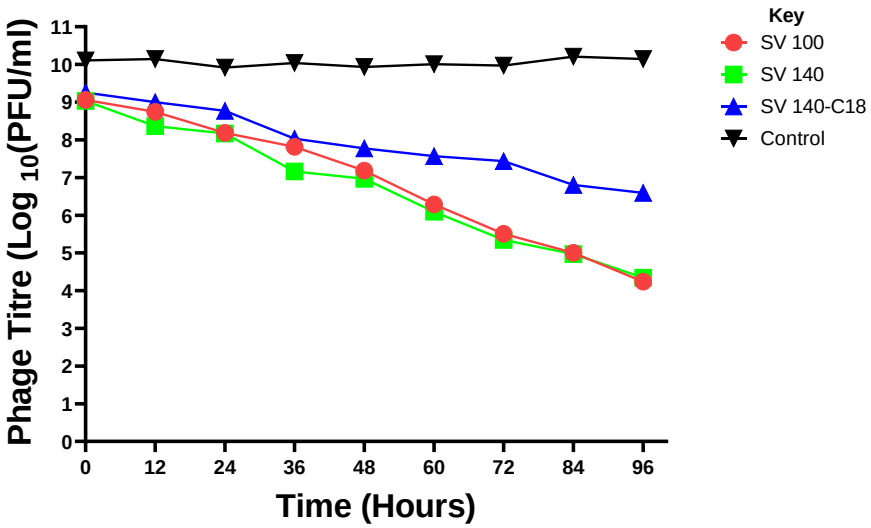



**Figure 4.12: Release of phages from SVs**.

### 4.3.3 Phage detection on functionalized particles in Simulated Gastric Fluid

With the aim to determine if the SVs protect phages *in vitro*, the stability of SV-encapsulated phages was determined using SGF. After 60 minutes of incubation, SVS 140-$C_{18}$ encapsulated phages reduced by 4 logs PFU/ml, SV 100 and SV140 both lost 6 logs PFU/ml. Phages without any encapsulation material reduced at a very fast rate, by almost 8 logs PFU/ml by 60 minutes of incubation. There was sudden drop in phage concentration in all the SVs at the first 15 minutes, afterwards the reduction in concentrations was relatively low (Figure 4.13).

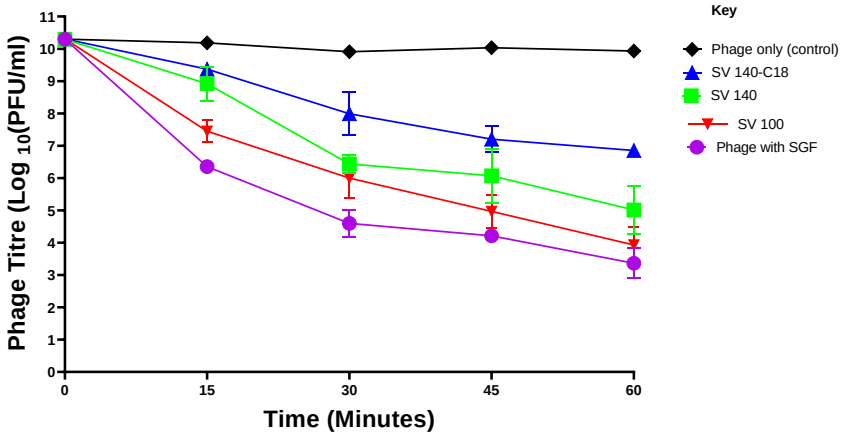



**Figure 4.13: SVs phage protection efficiency in SGF.**

### 4.3.4 In vivo Stability of SV-Encapsulated and Non-Encapsulated Phages

The difference between the SV-encapsulated phages and the non-encapsulated phages from day 1 to day 8 following phage enumeration was statistically significant (p values ranging from 0.0001 to 0.0396). (Figure 4.14). In the first 8 days, there were no statistically significant differences between SV-encapsulated phages (p values ranged from > 0.6194 to ->0.9999), and in the same way, there were no statistically significant differences between non-encapsulated phages (p values ranged from > 0.6194 ->0.9999). No statistically significant difference existed between SV-encapsulated and non-encapsulated phages from day 9 to day 27 (p values ranged from > 0.6194 to 0.9999). The highest titres on day 28 were found in

SV-encapsulated phages K47 and K11, whose difference from other phages was statistically significant (p values ranging from 0.0001 to 0.0002) (Figure 4.15).

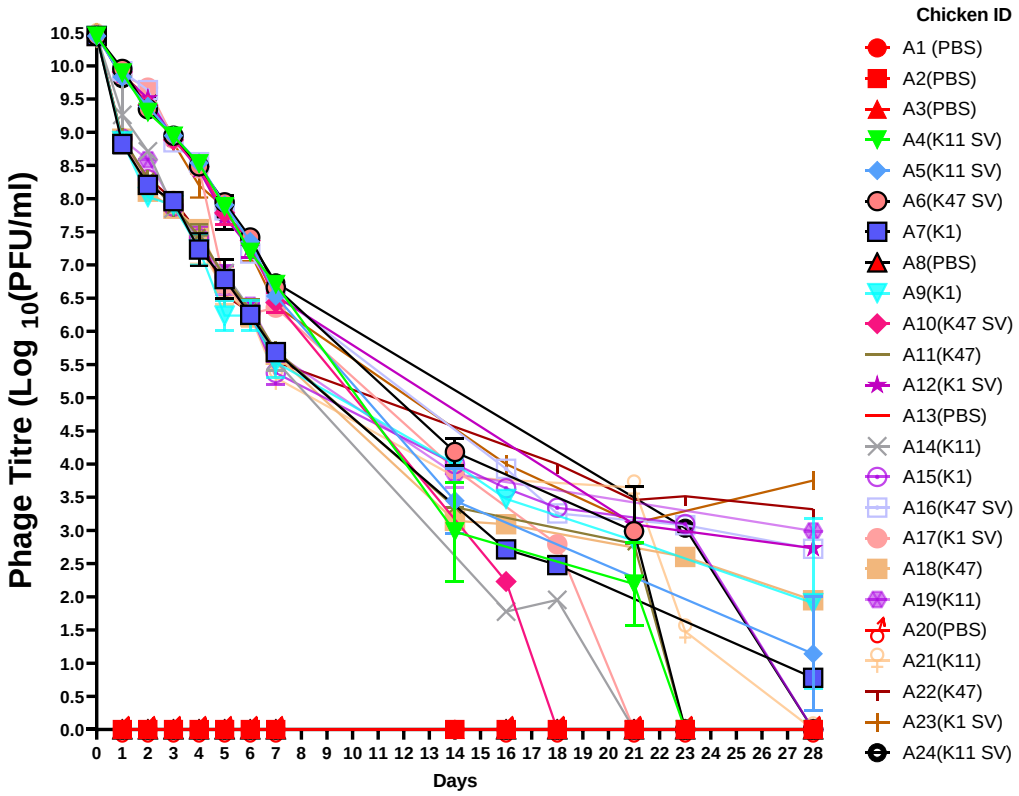



**Figure 4.14: Phage enumeration from individual birds from day 1 to day 28**

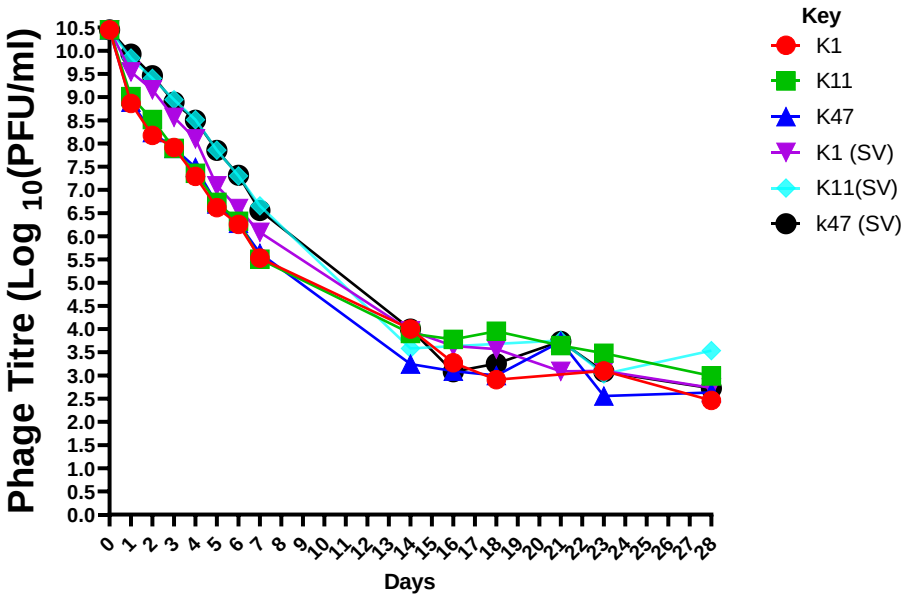



**Figure 4.15: Comparison of the SV-encapsulated phages and the non-encapsulated phages from day 1 to day 28**

# CHAPTER 5

# DISCUSSION, CONCLUSIONS & RECOMMENDATIONS

## 5.0.**DISCUSSION**

### 5.1 Characterisation of *Salmonella* Enteritidis Specific Bacteriophages

The *S.* Enteritidis phages in this study demonstrated a wider host range within and across serovars of *Salmonella.* This is attributed to the horizontal transfer of genes between different bacterial species and serovars, enabling them to express receptor binding proteins that recognize receptors from different hosts (de Jonge *et al.*, 2019). These findings are in line with what previous studies have shown. Islam *et al.*, (2019) reported that novel *Salmonella* phages LPSTLL, LPST94, and LPST153 from isolated from different biofilms were able to lyse *S. enterica* serovars Typhimurium, Enteritidis, Dublin, Choleraesuis, Paratyphi B, Pullorum, Javiana, Anatum and Kentucky, and subs. *Arizonae*. Santos *et al.*, (2010) isolated and characterized a multivalent *Salmonella* phage PVP-SE1, which was able to lyse 13 *Salmonella* serovars, *E. coli* (K5, N9), and *Enterobacter amnigenus* CECT 4078 (ATCC 33072). In cases of multiple infections wider host range phages are the most effective option because they can eliminate several serovars and species of bacteria at the same time. Tao *et al.,* (2021) demonstrated that *Salmonella* Phage SHWT1 had activity against muti-drug resistant *Salmonella* serovars Pullorum, Gallinarum, Enteritidis, and Typhimurium. Similarly Li *et al.*, (2020) found that phage STP4-a was able to eliminate were 95 strains, containing 91 *Salmonella* strains, 2 *E. coli* strains, and 2 *Klebsiella pneumonia* strains.

Phage DNA digestion assists in the identity of bacteriophages, as well as used for insertion of different sequences in plasmids. EcoRV restriction enzyme was able to efficiently digest all the 10 phage DNA used in this study, which exhibited different patterns. The efficiency of EcoRV is DNA digestion has been previously reported. Nikapitiya *et al.*, (2020) used SpeI, SacI, XhoI, BamHI, NdeI, PstI, EcoRV, HindIII and ClaI restriction enzymes for the digestion of *Edwardsiella tarda* phage (ETP-1), and demonstrated that only EcoRV was able to efficiently digest the phage DNA.

Shahin *et al.*, (2019) used the restriction enzymes; EcoRI, EcoRV, HindIII and BamHI for the digestion of *Shigella dysenteriae* phage vB-SdyS-ISF003, however only EcoRV was able to digest the software demonstrated that the size of vB-SdyS-ISF003 genome could be around 62 000 bp phage DNA. *Salmonella typhimurium* phage DT204c and *Salmonella* phage, LP7, also demonstrated efficient digestion by the restriction enzyme EcoRV (Baquar *et al*., 1993; Bernhard Petri & Schmieger, 1990).

Like the earlier findings, all 10 phages demonstrated thermal and pH stability at a range of 25 to 42 °C and 4 to 9. For instance, in SGF at pH 2.5, Vibrio vulnificus phage titters decreased by 3 logs in just 2 minutes (Koo *et al.,* 2000, 2001). At pH 2, *Salmonella* Phage Felix O1 lost its infectiousness after ten minutes, while at pH 2.5, it lost its infectiousness after one hour (Gomez-Garcia *et al.*, 2021). Coliphages JLA23, KP26, C119, and E142 were treated to SGF at pH 2.5, and their titters persisted after 2, 5, and 15 minutes but disappeared after 30 minutes (Ramirez *et al.*, 2018). In contrast, these coliphages in SIF were steady for 3 hours before decreasing 2 logs (Malik *et al*., 2017). Although the physiochemical circumstances of the GIT naturally promote digestion, they could be harmful to phages. *Salmonella* phages encounter several obstacles on their way to the small intestine, the site of *Salmonella* infection. The hydrochloric acid and a number of enzymes that are released by the gastric pits in the stomach can denature the phage structural proteins and render the virions inactive (Międzybrodzki *et al*., 2017; Vinner *et al*., 2019). The low pH can have an impact on the intricate structure of phage protein interactions by altering the protonation state of charged residues. The intensity and geometry of electrostatic interactions, which are crucial for protein interactions at low salt concentrations, are altered when the charge distribution varies (Zhou & Pang, 2018).

The preferred method for delivering phages that target gastrointestinal pathogens is frequently thought to be water (Kittler *et al*., 2020). Different water sources (river, rain, borehole, and tap water) that might be utilized in poultry husbandry were studied to see how they affected phage stability. According to findings from this and previous studies, river water is more harmful to viruses than groundwater and tap water (Pinon & Vialette, 2019). This is most likely because river water contains a lot

of organic substances. Additionally, the pH and temperature of river water are constantly changing, which could influence phage structure.

Compared to raw and filtered water, boiling river water causes the complex organic compounds to break down and releases ions that increase the acidity of the water. These conditions can be more harmful to phage infectivity. It should be noted that cations like calcium and magnesium ions may also encourage phage adsorption to the host bacteria, aiding in the viral production (Bhadauria *et al*., 2017; Pinon & Vialette, 2019; Wanhong *et al.*, 2020). If all parameters are considered, phage survival in water sources is influenced by their connection with solids, the presence of organic matter, ultraviolet radiation, temperature, pH, ion concentration, and type.

### 5.2 Growth Kinetics of *Salmonella* and Bacteriophages in Simulated Digestive Environments

Additionally, the impact of pH on phage replication was examined. It was discovered that acidic media (pH 2 and 3) have an impact on the phage's effectiveness in inhibiting Salmonella development (Figures 3, 4, and 5). In contrast, an alkaline environment (pH 8), as also noted by others (Śliwka *et al*., 2019; Verthé *et al*., 2004) did not significantly impact the phage replication process. Similar results were shown with SGF and SIF, with SGF decreasing phage efficacy whereas SIF did not (Figure 6). As phages are more likely to survive in the harsh gastrointestinal environment, which contains hydrochloric acid, enzymes, and other chemicals, their capacity to remain in an acidic environment is one of the key features used for phage selection.

Animal body temperature is a significant factor that influences how phages and bacteria interact since it is essential for phage adsorption, replication, burst size, and latent period length. Slower viral replication cycles are frequently the result of temperatures outside the bacterial host's ideal growth temperature (Parker *et al*., 2016). At temperatures ranging from 25°C to 42°C, every phage in our investigation displayed high titres. At 50 °C, phages began to lose their ability to infect, though. This is in line with earlier research on Salmonella phages, which showed that temperatures higher than 50 °C resulted in low phage titters (Huang, Virk, *et al.*,

2018; Karimi *et al*., 2016). Some phages have been reported to survive at greater temperatures, though. One such is the limited-spectrum phage LSE7621, which successfully lysed *Salmonella* Enteritidis and shown good thermal stability at temperatures up to 50 °C. Higher temperatures (often above 60 °C) can cause proteins to become inactive, which reduces the viability of viruses (Liu *et al.*, 2020).

**5.3 Mesoporous Silica Vesicles Efficiency in Phage Delivery**

Due to the proteinoid shell that shields the genetic material from deterioration, the net charge of most viruses is often negative. Bacteriophages are positively charged at the tail and negatively charged at the head. Through electrostatic forces, these charges allow them to "physio adsorb" to cationic or anionic surfaces (Duran-Meza *et al.*, 2021). Mesoporous SVs are designed using a two-step fabrication process to enable them to have the ability to adsorb different particles based on their electrostatic forces, have a constant rate of release from the adsorption status (Zhang *et al*., 2014; Zhou & Pang, 2018). All three SVs (SV 100, SV 140, and SV 140-$C_{18}$) demonstrated the ability to adsorb phages from the media facilitated by the electrostatic force that formed between cationic SVs and anionic phage capsids. SV 140-$C_{18}$ had the highest adsorption/ encapsulation efficiency because it contains the octadecyl groups (-$C_{18}$) which increases the cationic nature of the SV surface, at the same time strengthens the SVs (Bernardes et al., 2017). All the SVs in this study showed a modest rate of phage release, with SV 140-C18 showing the slowest rate of release. The alkyl chain order and six-fold siloxane rings increase as a result of the octadecyl content on the silica surface, which makes the pores of the vesicle hard without changing its form and causes the content of the interior chamber of the vesicle release more slowly and continuously (Bernardes *et al*., 2017; Duran-Meza *et al*., 2021). Longer retention of phages in the human gastrointestinal system is ensured by SVs' capacity to return phages for longer in the cavity.

When subjected to SGF, the SVs demonstrated the ability to protect phages from the harsh acidic as compared to phages alone on SIF. The hydrogen ions in the hydrochloric acid and the cations on the surface of the SVs repel each other, this to some extent protect the phages from being inactivated by the acid in the surrounding

(Nobrega *et al.*, 2016). Again, the silicon bonds (Si-O-Si) bonds on the surface of the SVs are hydrophobic, this reduces the interaction between the SGF and the vesicles, and further facilitate protection of phages in the SVs (Zhang *et al*., 2014). The ability of SVs to protect phages in SGF demonstrates that they can be able to protect them in the stomach in transit to small intestines.

In contrast to the simulated digestive system, the actual/real chicken digestive system has a number of complex factors that may affect how phages pass through the chicken gastrointestinal tract, including interactions between phages and the intestinal mucosa and the variety of bacteria and families that make up the gut microbiome (Costa *et al.*, 2017). Both encapsulated and non-encapsulated phages could survive through the GIT and were expelled in the cloacal swabs and feces, according to the in vivo data obtained following SV-encapsulated and non-encapsulated phages administration in 3-day-old chicks. These findings align with those of Lorenzo-Rebenaque et al (2021) who also observed that both encapsulated and non-encapsulated were able to pass through 1 day old chicks and were observed in faeces. The encapsulated phages demonstrated higher titres, in the first 8 days and on day 28, unlike non encapsulated phages, which had relatively lower concentrations. With the ability of SV 140-$C_{18}$ vesicles returning more phages for longer, they can be recommended for use in delivery of phages for therapy.

**5.4 The conclusions**

- The 10 Salmonella Enteritidis phages showed that they were stable at temperatures between 25 °C and 42 °C and pH levels between 5 and 9. River water had the most detrimental effects on phage titres, and they lost infectivity quickly in SGF but were more stable in SIF.
- The replication of the phage was greatly hindered in low pH media and in SGF, whereas it was unaffected in high pH media and in SIF.
- The SV 140-C18 has the highest adsorption/ encapsulation efficiency, highest retention of phages, highest protection efficiency in simulated digestive environment, and returned higher phage concentrations in vivo.

### 5.5 The recommendations

- The 10 *Salmonella* Enteritidis may be administered orally through drinking water and may survive gastrointestinal tract to prevent salmonellosis.
- SV 140-$C_{18}$ can be used for oral delivery of phages, as it has demonstrated the ability to protect and retain phages.

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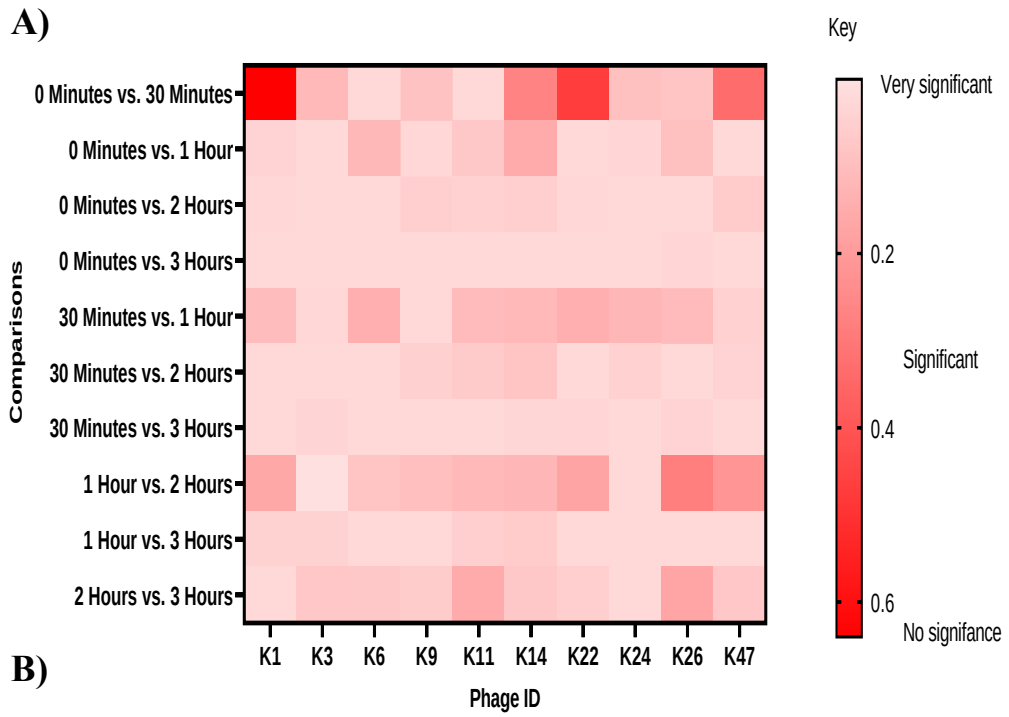
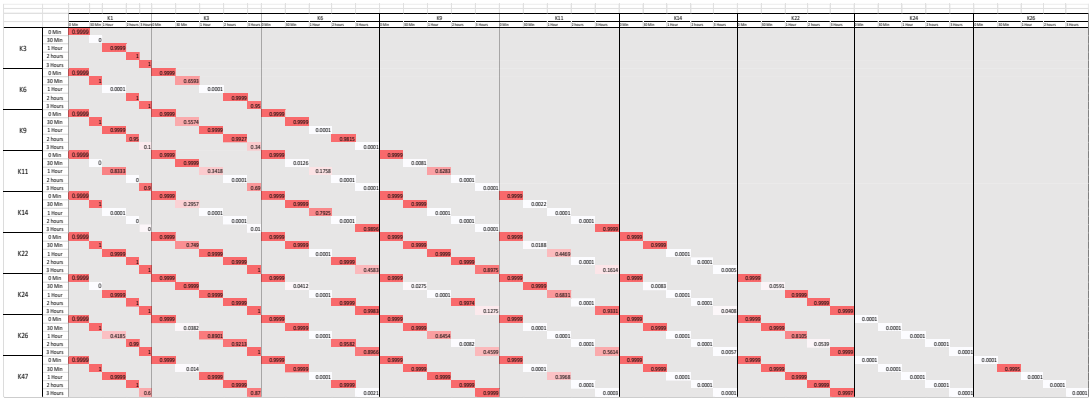
## APPENDICES

**Appendix I:** Phage stability P-values in pH 3-adjusted TSB assay.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

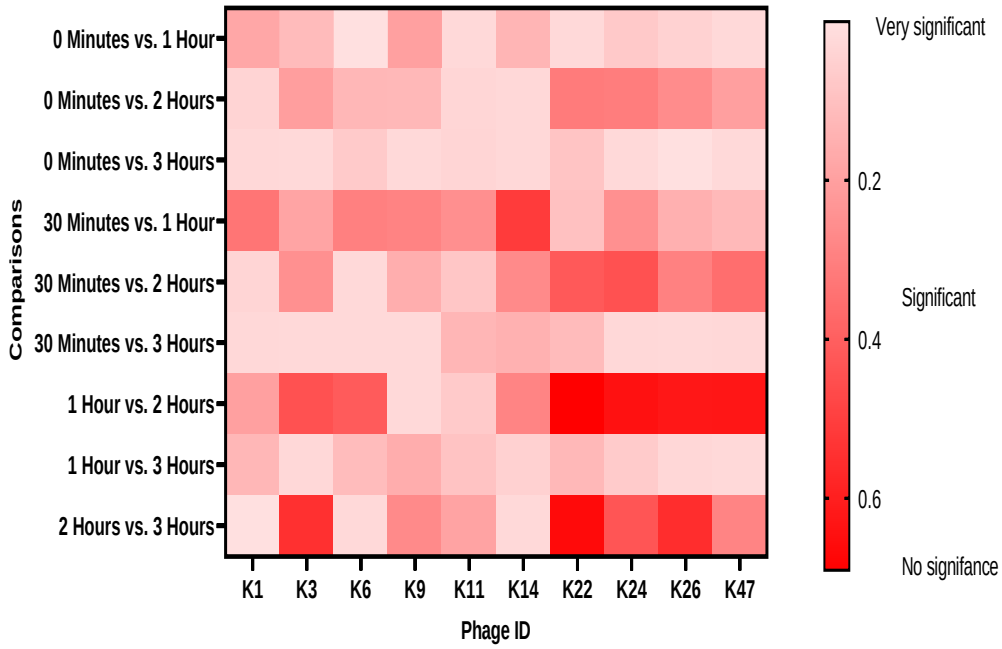


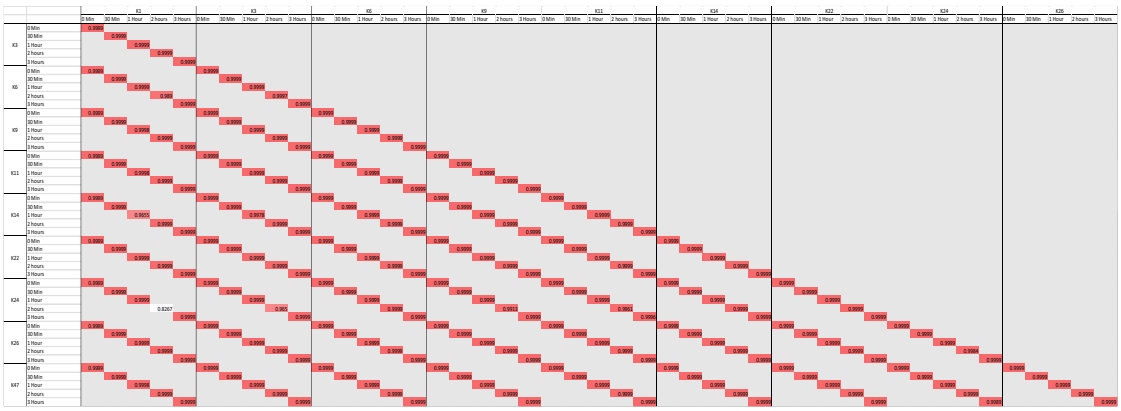
**Appendix II:** Phage stability P-values in pH 9-adjusted TSB assay.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**



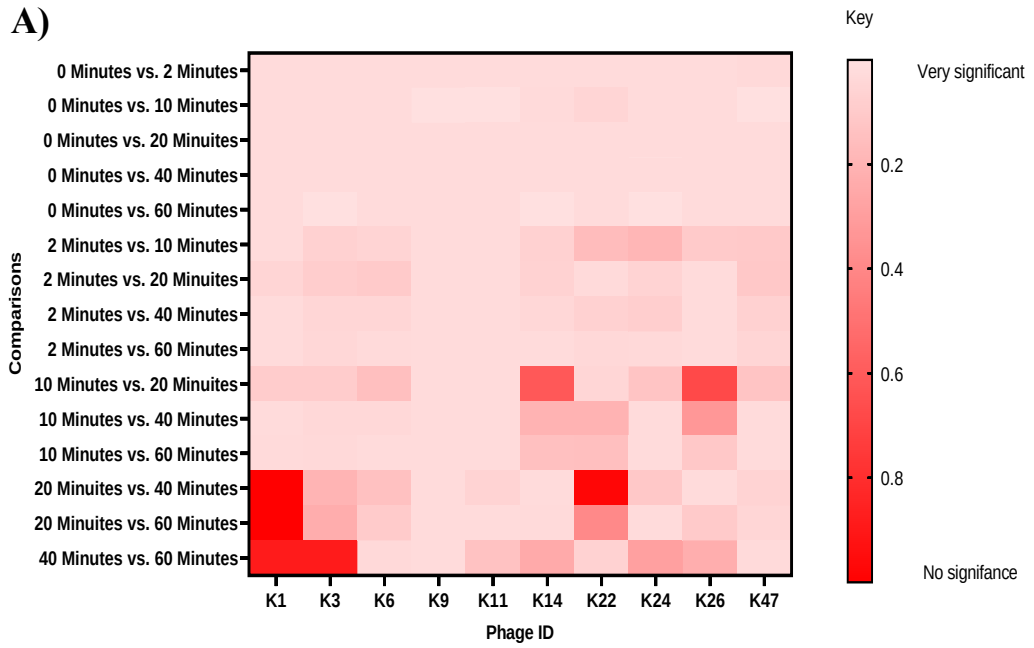

**B)**



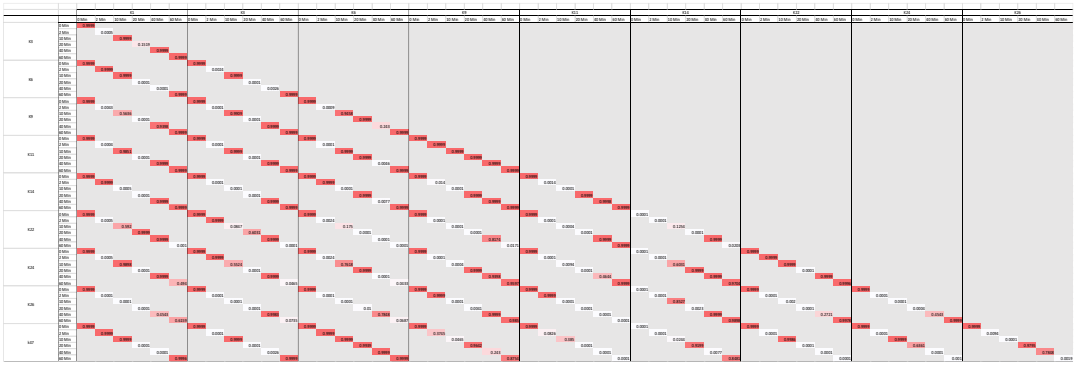
**Appendix III:** Phage stability P-values in SGF at 42 ℃.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**



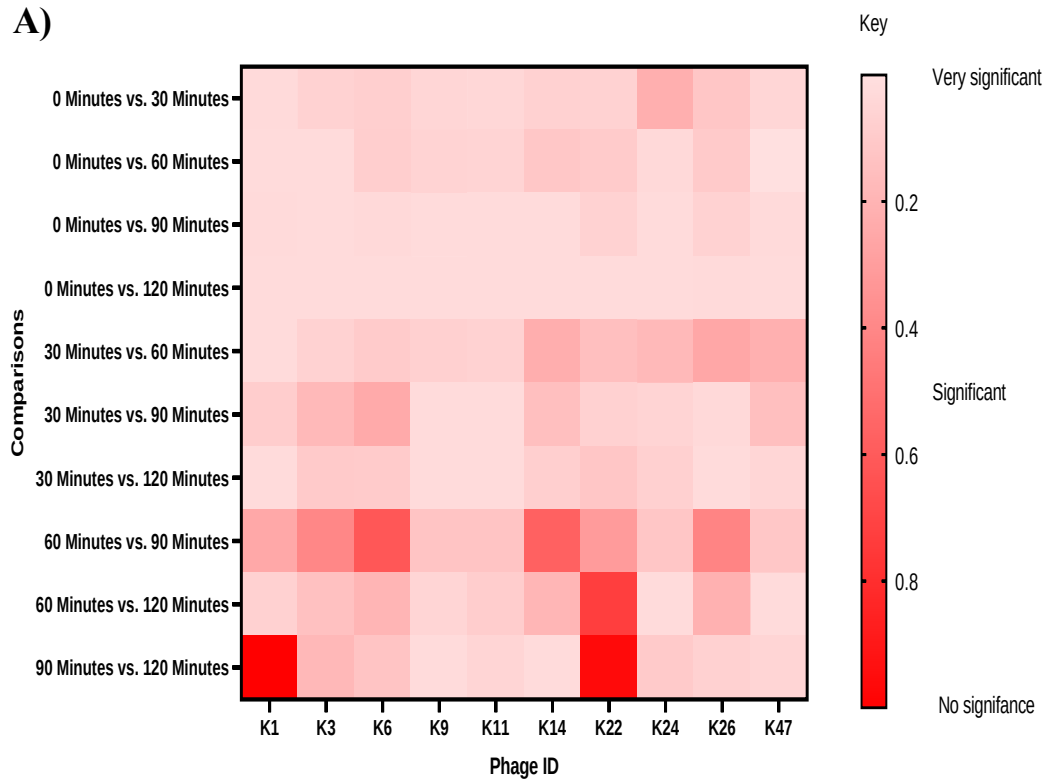

**B)**



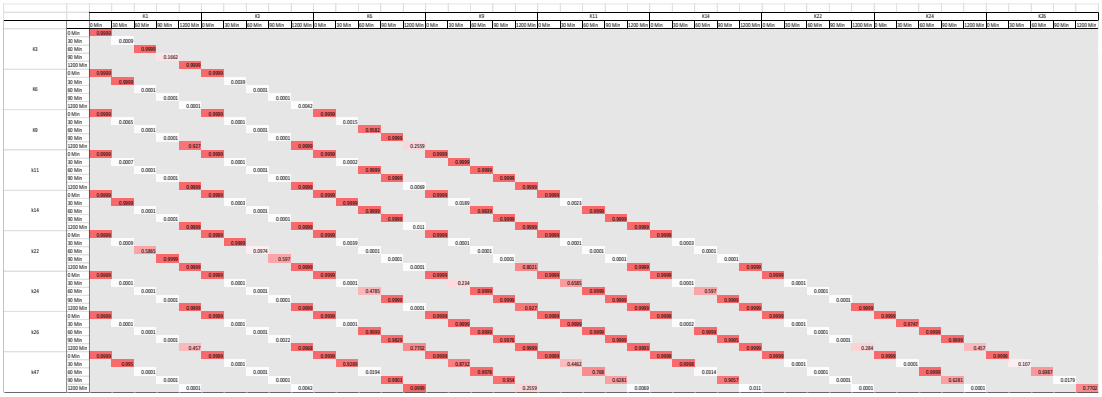
**Appendix IV**: Phage stability P-values in SIF at 42 ℃.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**



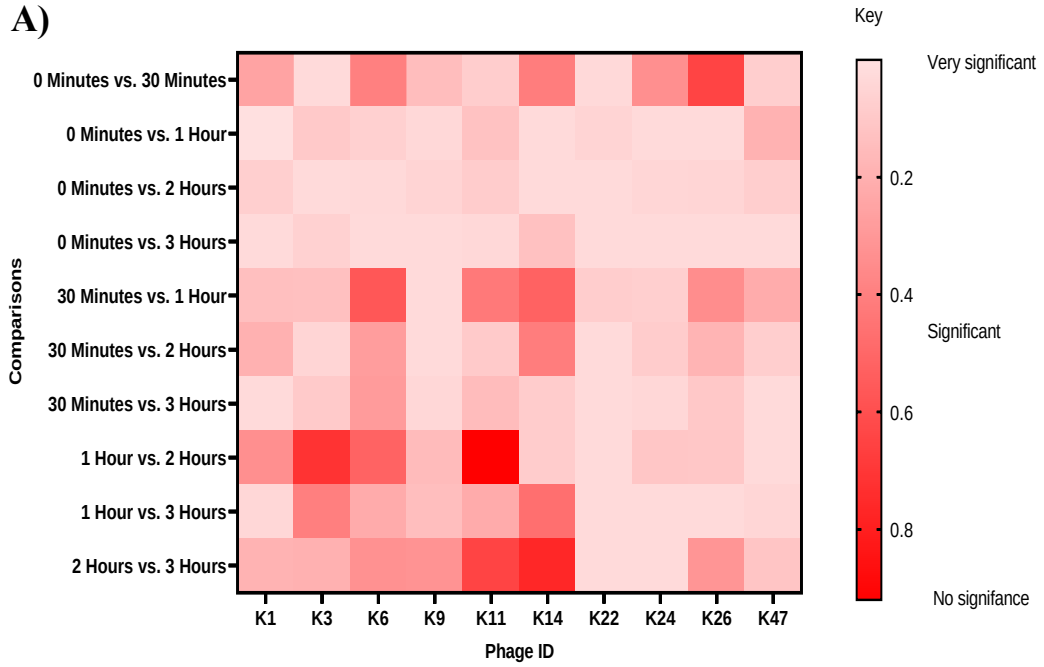

**B)**



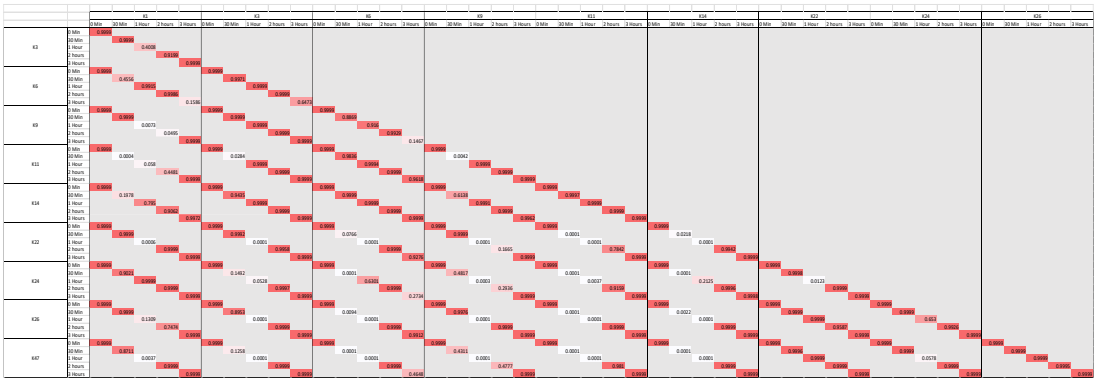
**Appendix V:** Phage stability P-values in TSB at 37 ℃.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**



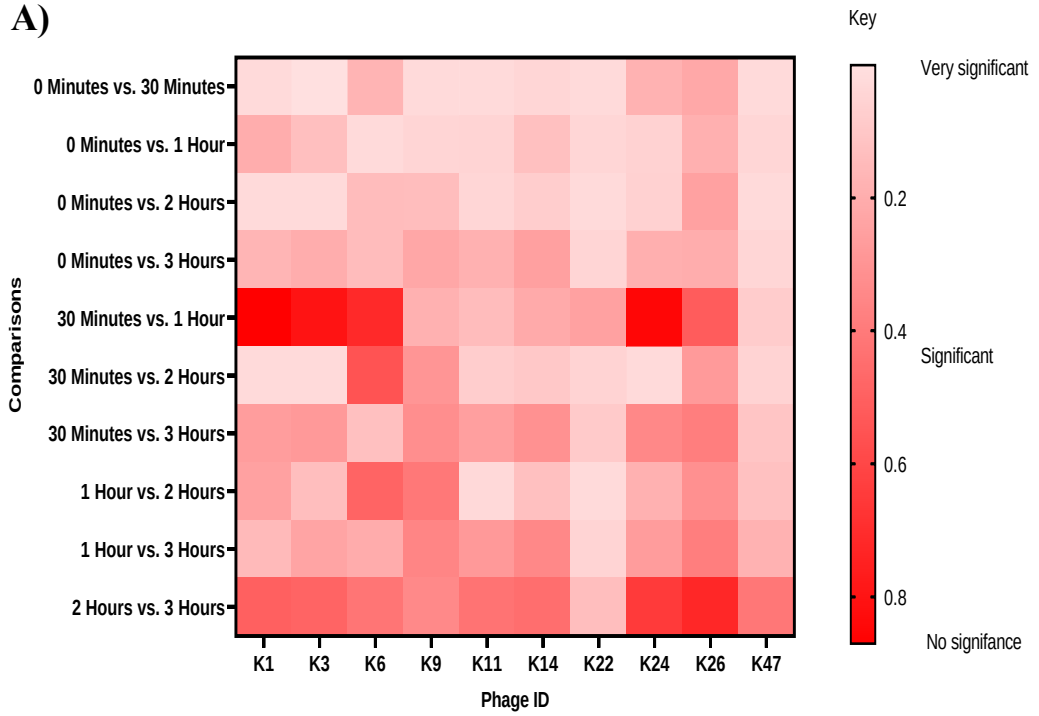

**B)**



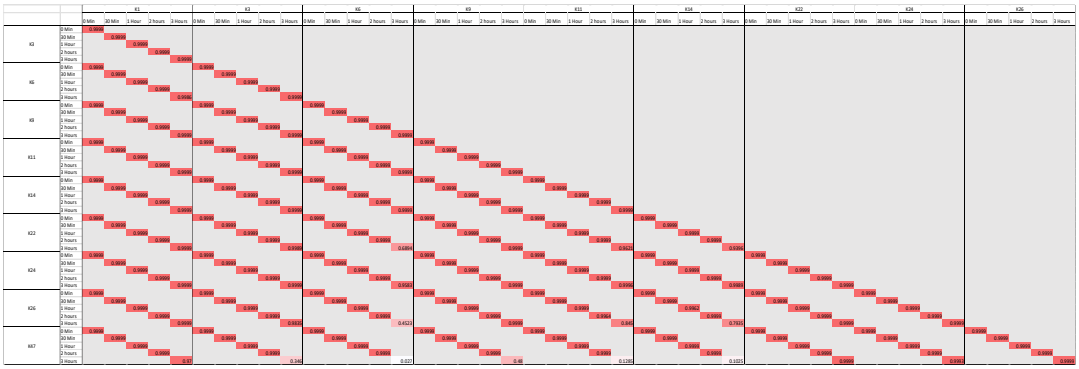
**Appendix VI:** Phage stability P-values in TSB at 42 °C.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**



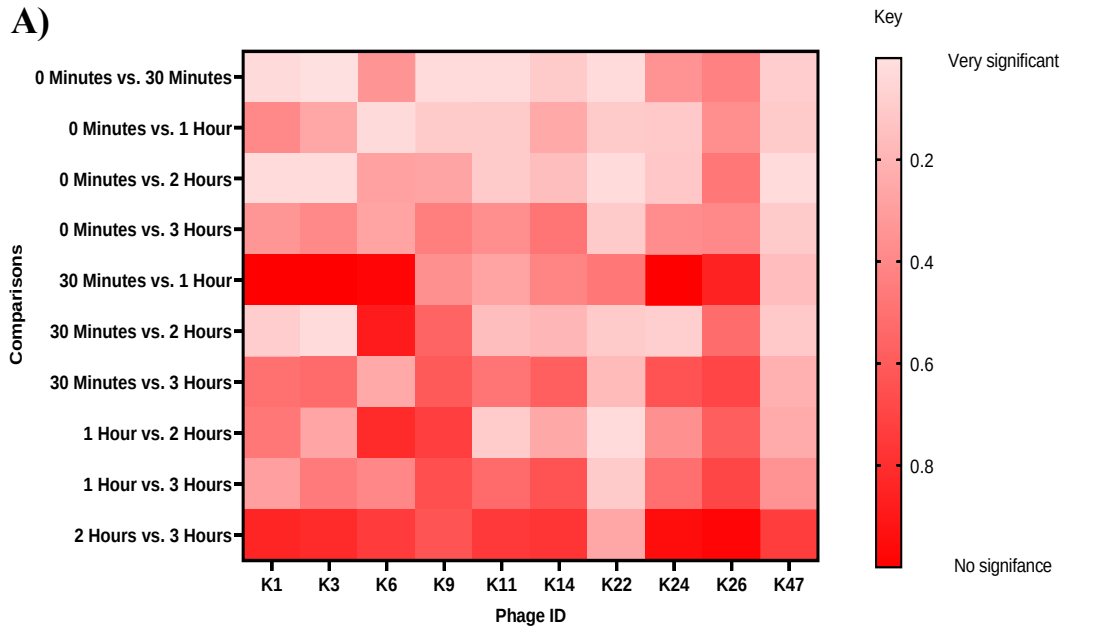

**B)**



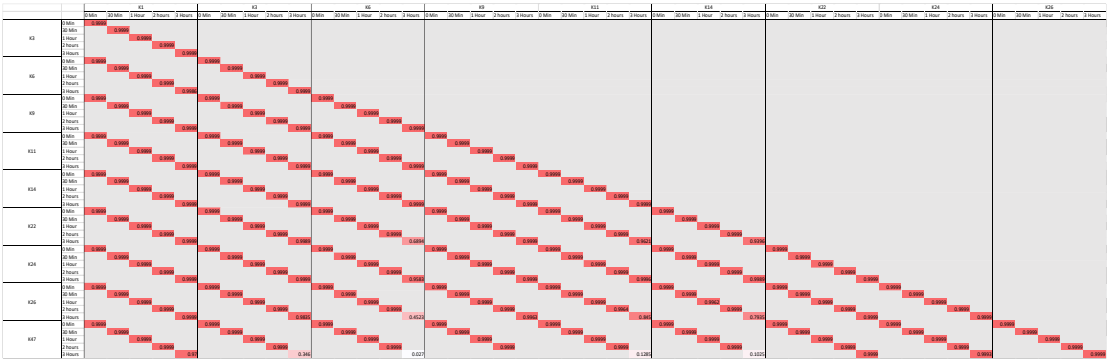
**Appendix VII:** Phage stability P-values in TSB at 50 °C.

**A)** P values of phage titres between time-points for a given phage. The colour intensity correlates with the P-value; deep red represents a high P-value, while light pink represents a low P-value. **B)** P-values of phage titres between different phages at a given time-point. The colour intensity correlates with the P-value; deep red represents a high P-value, while white represents a low P-value.

**A)**




**B)**



**Appendix VIII:** P-values of bacterial growth under phage control in pH-adjusted medium

**A).** P-values of phage titres between phages at a given time-point at pH 2. **B)** P-values of phage titres between phages at a given time-point at pH 3. **C).** P-values of phage titres between phages at a given time-point at pH 8. The colour intensity correlates with the P value; deep red represents a high P-value, while white represents a low P-value. Highlighted cells with a black border indicate significant P-values.

**A)**

| TSB | Time Hours | | | | | |
|---|---|---|---|---|---|---|
| Correlation | 0 | 0.5 | 1 | 2 | 3 | 4 |
| K1 vs. K3 | 0.9999 | 0.4757 | 0.4057 | 0.3307 | 0.4389 | 0.4321 |
| K1 vs. K6 | 0.9993 | 0.414 | 0.328 | 0.1312 | 0.0521 | 0.1217 |
| K1 vs. K9 | 0.8148 | 0.0395 | 0.0304 | 0.0382 | 0.0341 | 0.035 |
| K1 vs. K11 | 0.7256 | 0.0211 | 0.0191 | 0.0635 | 0.0228 | 0.0567 |
| K1 vs. K14 | 0.3997 | 0.0951 | 0.1111 | 0.0261 | 0.0326 | 0.0287 |
| K1 vs. K22 | 0.8091 | 0.0524 | 0.0669 | 0.0611 | 0.0184 | 0.0409 |
| K1 vs. K24 | 0.7973 | 0.0958 | 0.124 | 0.1005 | 0.0665 | 0.1207 |
| K1 vs. K26 | 0.614 | 0.022 | 0.2089 | 0.0889 | 0.084 | 0.1519 |
| K1 vs. K47 | 0.6718 | 0.0311 | 0.0222 | 0.118 | 0.1063 | 0.1064 |
| K1 vs. Control (sal 568) | 0.5253 | 0.0211 | 0.1951 | 0.0172 | 0.0131 | 0.0473 |
| K3 vs. K6 | 0.9947 | 0.7834 | 0.6313 | 0.1883 | 0.0645 | 0.0392 |
| K3 vs. K9 | 0.5135 | 0.2496 | 0.1869 | 0.0667 | 0.0344 | 0.1177 |
| K3 vs. K11 | 0.439 | 0.1522 | 0.1173 | 0.063 | 0.0153 | 0.018 |
| K3 vs. K14 | 0.1561 | 0.0569 | 0.0622 | 0.0436 | 0.0525 | 0.1051 |
| K3 vs. K22 | 0.7182 | 0.0892 | 0.0579 | 0.0607 | 0.0131 | 0.1165 |
| K3 vs. K24 | 0.4931 | 0.0629 | 0.0802 | 0.1451 | 0.1002 | 0.2042 |
| K3 vs. K26 | 0.3188 | 0.3859 | 0.3833 | 0.1671 | 0.1289 | 0.0735 |
| K3 vs. K47 | 0.3797 | 0.0817 | 0.0747 | 0.1634 | 0.1468 | 0.1916 |
| K3 vs. Control (sal 568) | 0.2992 | 0.1439 | 0.1878 | 0.0257 | 0.0151 | 0.018 |
| K6 vs. K9 | 0.9984 | 0.7784 | 0.6396 | 0.3386 | 0.3138 | 0.3368 |
| K6 vs. K11 | 0.9812 | 0.3482 | 0.2967 | 0.0005 | 0.023 | 0.0008 |
| K6 vs. K14 | 0.6481 | 0.2504 | 0.1869 | 0.1443 | 0.1187 | 0.1538 |
| K6 vs. K22 | 0.9822 | 0.31 | 0.2436 | 0.0008 | 0.0211 | 0.1714 |
| K6 vs. K24 | 0.9967 | 0.2776 | 0.2577 | 0.2851 | 0.2392 | 0.3405 |
| K6 vs. K26 | 0.9077 | 0.9979 | 0.9999 | 0.8696 | 0.4123 | 0.1197 |
| K6 vs. K47 | 0.963 | 0.2645 | 0.2368 | 0.2952 | 0.3119 | 0.3186 |
| K6 vs. Control (sal 568) | 0.7913 | 0.3246 | 0.3169 | 0.1154 | 0.0385 | 0.0001 |
| K9 vs. K11 | 0.9587 | 0.0872 | 0.0381 | 0.1623 | 0.0374 | 0.113 |
| K9 vs. K14 | 0.2525 | 0.192 | 0.2927 | 0.1356 | 0.1782 | 0.1434 |
| K9 vs. K22 | 0.9918 | 0.0828 | 0.1302 | 0.1506 | 0.0352 | 0.1704 |
| K9 vs. K24 | 0.9999 | 0.2095 | 0.4116 | 0.4135 | 0.3921 | 0.4889 |
| K9 vs. K26 | 0.6008 | 0.1683 | 0.5075 | 0.2333 | 0.1031 | 0.2083 |
| K9 vs. K47 | 0.7922 | 0.0433 | 0.036 | 0.4121 | 0.4747 | 0.4539 |
| K9 vs. Control (sal 568) | 0.3317 | 0.0817 | 0.5633 | 0.1001 | 0.0654 | 0.0846 |
| K11 vs. K14 | 0.3446 | 0.8707 | 0.943 | 0.9825 | 0.9999 | 0.9979 |
| K11 vs. K22 | 0.9995 | 0.9892 | 0.994 | 0.5655 | 0.999 | 0.9676 |
| K11 vs. K24 | 0.9979 | 0.9969 | 0.9997 | 0.9897 | 0.6936 | 0.8861 |
| K11 vs. K26 | 0.7194 | 0.0138 | 0.1994 | 0.0497 | 0.0153 | 0.0001 |
| K11 vs. K47 | 0.9508 | 0.3956 | 0.4075 | 0.9999 | 0.832 | 0.8727 |
| K11 vs. Control (sal 568) | 0.1501 | 0.0525 | 0.9999 | 0.9538 | 0.9999 | 0.0805 |
| K14 vs. K22 | 0.9542 | 0.9893 | 0.9983 | 0.9999 | 0.9999 | 0.9999 |
| K14 vs. K24 | 0.2804 | 0.9995 | 0.9583 | 0.9575 | 0.7561 | 0.8661 |
| K14 vs. K26 | 0.4823 | 0.1616 | 0.1288 | 0.0962 | 0.0955 | 0.1335 |
| K14 vs. K47 | 0.3848 | 0.9934 | 0.9999 | 0.9983 | 0.8689 | 0.8549 |
| K14 vs. Control (sal 568) | 0.7194 | 0.9965 | 0.9999 | 0.9999 | 0.9999 | 0.8952 |
| K22 vs. K23 | 0.9999 | 0.1954 | 0.1302 | 0.017 | 0.2754 | 0.2457 |
| K22 vs. K24 | 0.9965 | 0.9999 | 0.9934 | 0.9428 | 0.629 | 0.8082 |
| K22 vs. K26 | 0.9999 | 0.0803 | 0.1257 | 0.047 | 0.0143 | 0.1505 |
| K22 vs. K47 | 0.9999 | 0.5983 | 0.8613 | 0.9986 | 0.7751 | 0.7842 |
| K22 vs. Control (sal 568) | 0.9998 | 0.998 | 0.9999 | 0.9999 | 0.9999 | 0.9927 |
| K24 vs. K26 | 0.7569 | 0.1703 | 0.1867 | 0.2456 | 0.1788 | 0.2879 |
| K24 vs. K47 | 0.9265 | 0.8507 | 0.7948 | 0.9999 | 0.9999 | 0.9999 |
| K24 vs. Control (sal 568) | 0.4328 | 0.9999 | 0.9999 | 0.9396 | 0.6743 | 0.6162 |
| K26 vs. K47 | 0.9816 | 0.0562 | 0.1355 | 0.2604 | 0.2402 | 0.2692 |
| K26 vs. Control (sal 568) | 0.6807 | 0.0121 | 0.3304 | 0.0655 | 0.0271 | 0.0001 |
| K29 vs. K47 | 0.9779 | 0.9843 | 0.9999 | 0.9075 | 0.6614 | 0.5727 |
| K29 vs. Control (sal 568) | 0.0661 | 0.1253 | 0.9919 | 0.9573 | 0.9048 | 0.9999 |
| K47 vs. Control (sal 568) | 0.3599 | 0.5699 | 0.9949 | 0.9996 | 0.8149 | 0.5924 |

**B)**

| TSB | Time Hours | | | | | |
|---|---|---|---|---|---|---|
| Correlation | 0 | 0.5 | 1 | 2 | 3 | 4 |
| K1 vs. K3 | 0.9999 | 0.7246 | 0.9995 | 0.9999 | 0.9998 | 0.9999 |
| K1 vs. K6 | 0.0422 | 0.4073 | 0.1169 | 0.2685 | 0.9714 | 0.9944 |
| K1 vs. K9 | 0.5458 | 0.8474 | 0.5187 | 0.4884 | 0.4486 | 0.4807 |
| K1 vs. K11 | 0.2053 | 0.1539 | 0.0122 | 0.0025 | 0.102 | 0.0154 |
| K1 vs. K14 | 0.0375 | 0.0143 | 0.0338 | 0.0072 | 0.0201 | 0.0004 |
| K1 vs. K22 | 0.0261 | 0.0125 | 0.0269 | 0.056 | 0.0166 | 0.0059 |
| K1 vs. K24 | 0.0207 | 0.2563 | 0.0217 | 0.1496 | 0.0076 | 0.0738 |
| K1 vs. K26 | 0.2211 | 0.0478 | 0.0572 | 0.0251 | 0.0029 | 0.0154 |
| K1 vs. K47 | 0.0158 | 0.0444 | 0.4893 | 0.5286 | 0.5022 | 0.8604 |
| K1 vs. Control (sal 568) | 0.0335 | 0.0845 | 0.9997 | 0.1339 | 0.1097 | 0.0008 |
| K3 vs. K6 | 0.3994 | 0.2936 | 0.5148 | 0.7109 | 0.9997 | 0.9999 |
| K3 vs. K9 | 0.8371 | 0.5392 | 0.7505 | 0.7948 | 0.7967 | 0.797 |
| K3 vs. K11 | 0.3845 | 0.4424 | 0.2349 | 0.195 | 0.1242 | 0.1674 |
| K3 vs. K14 | 0.3741 | 0.306 | 0.3194 | 0.2979 | 0.298 | 0.2577 |
| K3 vs. K22 | 0.3696 | 0.4261 | 0.321 | 0.2415 | 0.2025 | 0.1887 |
| K3 vs. K24 | 0.3293 | 0.3736 | 0.3225 | 0.2697 | 0.3419 | 0.2616 |
| K3 vs. K26 | 0.3485 | 0.4038 | 0.4286 | 0.351 | 0.3068 | 0.2836 |
| K3 vs. K47 | 0.329 | 0.3804 | 0.4809 | 0.5323 | 0.5176 | 0.675 |
| K3 vs. Control (sal 568) | 0.4947 | 0.9516 | 0.9999 | 0.1123 | 0.1489 | 0.1905 |
| K6 vs. K9 | 0.2779 | 0.6978 | 0.9993 | 0.9999 | 0.9979 | 0.9853 |
| K6 vs. K11 | 0.0874 | 0.0673 | 0.0401 | 0.0451 | 0.2283 | 0.177 |
| K6 vs. K14 | 0.0132 | 0.093 | 0.0229 | 0.0713 | 0.39 | 0.2646 |
| K6 vs. K22 | 0.0113 | 0.125 | 0.0257 | 0.0245 | 0.2862 | 0.1982 |
| K6 vs. K24 | 0.0104 | 0.1025 | 0.0297 | 0.0407 | 0.433 | 0.271 |
| K6 vs. K26 | 0.1032 | 0.1418 | 0.0801 | 0.1023 | 0.3963 | 0.288 |
| K6 vs. K47 | 0.0105 | 0.0853 | 0.2181 | 0.297 | 0.5647 | 0.6102 |
| K6 vs. Control (sal 568) | 0.0164 | 0.2137 | 0.8354 | 0.0475 | 0.2587 | 0.2013 |
| K9 vs. K11 | 0.1001 | 0.1419 | 0.1685 | 0.1013 | 0.0328 | 0.0914 |
| K9 vs. K14 | 0.0653 | 0.1556 | 0.2002 | 0.1534 | 0.1359 | 0.1125 |
| K9 vs. K22 | 0.0716 | 0.2005 | 0.2034 | 0.0996 | 0.09 | 0.0954 |
| K9 vs. K24 | 0.0629 | 0.1409 | 0.2063 | 0.0897 | 0.158 | 0.0715 |
| K9 vs. K26 | 0.1131 | 0.2079 | 0.2621 | 0.1825 | 0.1474 | 0.1311 |
| K9 vs. K47 | 0.0809 | 0.1611 | 0.2207 | 0.2752 | 0.2309 | 0.489 |
| K9 vs. Control (sal 568) | 0.121 | 0.3478 | 0.9508 | 0.0435 | 0.04 | 0.0813 |
| K11 vs. K14 | 0.9999 | 0.5465 | 0.1443 | 0.0354 | 0.2361 | 0.0275 |
| K11 vs. K22 | 0.9992 | 0.9998 | 0.1229 | 0.1863 | 0.7736 | 0.0288 |
| K11 vs. K24 | 0.8696 | 0.9785 | 0.1026 | 0.2275 | 0.2039 | 0.1228 |
| K11 vs. K26 | 0.9999 | 0.9641 | 0.032 | 0.0711 | 0.2449 | 0.0001 |
| K11 vs. K47 | 0.797 | 0.9884 | 0.5236 | 0.419 | 0.2435 | 0.5691 |
| K11 vs. Control (sal 568) | 0.9308 | 0.3919 | 0.3998 | 0.9997 | 0.8956 | 0.1446 |
| K14 vs. K22 | 0.9999 | 0.2177 | 0.9999 | 0.735 | 0.0448 | 0.0446 |
| K14 vs. K24 | 0.8023 | 0.9978 | 0.9999 | 0.961 | 0.2607 | 0.8477 |
| K14 vs. K26 | 0.9999 | 0.368 | 0.3666 | 0.2333 | 0.9991 | 0.3188 |
| K14 vs. K47 | 0.6206 | 0.5053 | 0.9571 | 0.8366 | 0.699 | 0.9729 |
| K14 vs. Control (sal 568) | 0.4433 | 0.0849 | 0.5329 | 0.3671 | 0.3204 | 0.0443 |
| K22 vs. K23 | 0.7733 | 0.8795 | 0.3544 | 0.0828 | 0.048 | 0.1073 |
| K22 vs. K24 | 0.8541 | 0.986 | 0.9999 | 0.77 | 0.0385 | 0.1656 |
| K22 vs. K26 | 0.9999 | 0.7874 | 0.3231 | 0.3627 | 0.0681 | 0.0029 |
| K22 vs. K47 | 0.6448 | 0.9934 | 0.9522 | 0.7096 | 0.3549 | 0.6816 |
| K22 vs. Control (sal 568) | 0.3329 | 0.092 | 0.5316 | 0.4382 | 0.9999 | 0.5237 |
| K24 vs. K26 | 0.9856 | 0.9994 | 0.2786 | 0.9688 | 0.1908 | 0.9886 |
| K24 vs. K47 | 0.9999 | 0.9996 | 0.9472 | 0.9416 | 0.8743 | 0.9992 |
| K24 vs. Control (sal 568) | 0.1825 | 0.4847 | 0.5302 | 0.2793 | 0.2567 | 0.1411 |
| K26 vs. K47 | 0.9528 | 0.9999 | 0.9999 | 0.9814 | 0.7161 | 0.9957 |
| K26 vs. Control (sal 568) | 0.8409 | 0.0211 | 0.6447 | 0.3022 | 0.3229 | 0.0896 |
| K29 vs. K47 | 0.982 | 0.2115 | 0.716 | 0.5802 | 0.3549 | 0.6699 |
| K29 vs. Control (sal 568) | 0.4412 | 0.1044 | 0.4607 | 0.61 | 0.9999 | 0.4198 |
| K47 vs. Control (sal 568) | 0.0982 | 0.1885 | 0.8768 | 0.3558 | 0.3173 | 0.7296 |

**C)**

| TSB | Time Hours | | | | | |
|---|---|---|---|---|---|---|
| Correlation | 0 | 0.5 | 1 | 2 | 3 | 4 |
| K1 vs. K3 | 0.3128 | 0.0192 | 0.1468 | 0.889 | 0.9999 | 0.523 |
| K1 vs. K6 | 0.4702 | 0.1409 | 0.5918 | 0.9697 | 0.9104 | 0.352 |
| K1 vs. K9 | 0.9945 | 0.0378 | 0.9996 | 0.9728 | 0.7472 | 0.5227 |
| K1 vs. K11 | 0.419 | 0.0454 | 0.6051 | 0.8055 | 0.1965 | 0.0204 |
| K1 vs. K14 | 0.841 | 0.2286 | 0.99 | 0.8216 | 0.6204 | 0.7578 |
| K1 vs. K22 | 0.9999 | 0.0286 | 0.0745 | 0.9999 | 0.7613 | 0.4293 |
| K1 vs. K24 | 0.9999 | 0.7126 | 0.9586 | 0.9999 | 0.8254 | 0.9417 |
| K1 vs. K26 | 0.7624 | 0.7144 | 0.6972 | 0.4952 | 0.8796 | 0.1555 |
| K1 vs. K47 | 0.249 | 0.0029 | 0.0914 | 0.9999 | 0.5232 | 0.0928 |
| K1 vs. Control (sal 568) | 0.5565 | 0.0001 | 0.1227 | 0.0103 | 0.0423 | 0.0006 |
| K3 vs. K6 | 0.1364 | 0.0308 | 0.1456 | 0.9999 | 0.8755 | 0.9999 |
| K3 vs. K9 | 0.0433 | 0.0494 | 0.0919 | 0.9999 | 0.7095 | 0.3948 |
| K3 vs. K11 | 0.0162 | 0.0183 | 0.0726 | 0.9999 | 0.2035 | 0.0718 |
| K3 vs. K14 | 0.0611 | 0.4728 | 0.3278 | 0.515 | 0.593 | 0.4394 |
| K3 vs. K22 | 0.1486 | 0.0115 | 0.2173 | 0.9925 | 0.7226 | 0.2233 |
| K3 vs. K24 | 0.7206 | 0.8451 | 0.6964 | 0.9999 | 0.7947 | 0.7844 |
| K3 vs. K26 | 0.2214 | 0.0613 | 0.117 | 0.996 | 0.8472 | 0.0931 |
| K3 vs. K47 | 0.0083 | 0.0006 | 0.0264 | 0.8005 | 0.5467 | 0.5262 |
| K3 vs. Control (sal 568) | 0.0304 | 0.0087 | 0.0968 | 0.0101 | 0.0417 | 0.012 |
| K6 vs. K9 | 0.3044 | 0.3116 | 0.8208 | 0.9999 | 0.9999 | 0.423 |
| K6 vs. K11 | 0.9998 | 0.0215 | 0.3254 | 0.9999 | 0.0889 | 0.0087 |
| K6 vs. K14 | 0.6788 | 0.4679 | 0.9999 | 0.8279 | 0.9115 | 0.4787 |
| K6 vs. K22 | 0.394 | 0.0911 | 0.9987 | 0.9998 | 0.9999 | 0.3006 |
| K6 vs. K24 | 0.9961 | 0.993 | 0.9999 | 0.9999 | 0.9974 | 0.7725 |
| K6 vs. K26 | 0.9948 | 0.1535 | 0.9992 | 0.9996 | 0.9999 | 0.1558 |
| K6 vs. K47 | 0.4402 | 0.0029 | 0.0367 | 0.9856 | 0.2492 | 0.2564 |
| K6 vs. Control (sal 568) | 0.9666 | 0.0129 | 0.1092 | 0.042 | 0.0203 | 0.0049 |
| K9 vs. K11 | 0.0392 | 0.0397 | 0.581 | 0.9999 | 0.0167 | 0.1416 |
| K9 vs. K14 | 0.2668 | 0.3146 | 0.9968 | 0.5462 | 0.9002 | 0.7949 |
| K9 vs. K22 | 0.8792 | 0.0091 | 0.5199 | 0.9996 | 0.9999 | 0.9024 |
| K9 vs. K24 | 0.9944 | 0.8754 | 0.982 | 0.9999 | 0.9998 | 0.9709 |
| K9 vs. K26 | 0.5201 | 0.3454 | 0.9575 | 0.8311 | 0.9999 | 0.979 |
| K9 vs. K47 | 0.0322 | 0.0129 | 0.0443 | 0.8659 | 0.0831 | 0.3324 |
| K9 vs. Control (sal 568) | 0.0819 | 0.0029 | 0.1139 | 0.0091 | 0.0158 | 0.1978 |
| K11 vs. K14 | 0.3043 | 0.0076 | 0.6281 | 0.4 | 0.059 | 0.0991 |
| K11 vs. K22 | 0.2847 | 0.052 | 0.3186 | 0.988 | 0.0143 | 0.0809 |
| K11 vs. K24 | 0.8696 | 0.0926 | 0.7537 | 0.9999 | 0.202 | 0.2102 |
| K11 vs. K26 | 0.9227 | 0.0167 | 0.3866 | 0.9234 | 0.1678 | 0.0285 |
| K11 vs. K47 | 0.0459 | 0.5189 | 0.0399 | 0.6002 | 0.0666 | 0.0234 |
| K11 vs. Control (sal 568) | 0.2607 | 0.1653 | 0.113 | 0.0103 | 0.0034 | 0.018 |
| K14 vs. K22 | 0.7423 | 0.184 | 0.9962 | 0.964 | 0.8093 | 0.9987 |
| K14 vs. K24 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9999 |
| K14 vs. K26 | 0.9879 | 0.109 | 0.9999 | 0.3288 | 0.9999 | 0.8248 |
| K14 vs. K47 | 0.1238 | 0.0172 | 0.0337 | 0.704 | 0.058 | 0.3034 |
| K14 vs. Control (sal 568) | 0.6003 | 0.0197 | 0.0865 | 0.0108 | 0.0648 | 0.0824 |
| K22 vs. K23 | 0.0556 | 0.132 | 0.4126 | 0.9992 | 0.0882 | 0.2811 |
| K22 vs. K24 | 0.9999 | 0.5943 | 0.9999 | 0.9999 | 0.9994 | 0.9999 |
| K22 vs. K26 | 0.7266 | 0.9985 | 0.8135 | 0.8934 | 0.9999 | 0.9779 |
| K22 vs. K47 | 0.149 | 0.0029 | 0.0859 | 0.9999 | 0.0653 | 0.173 |
| K22 vs. Control (sal 568) | 0.3823 | 0.0001 | 0.1174 | 0.0328 | 0.0185 | 0.0626 |
| K24 vs. K26 | 0.9999 | 0.6175 | 0.9997 | 0.9999 | 0.9999 | 0.9985 |
| K24 vs. K47 | 0.8069 | 0.1161 | 0.11 | 0.9999 | 0.3816 | 0.6008 |
| K24 vs. Control (sal 568) | 0.9998 | 0.1009 | 0.0528 | 0.2587 | 0.0999 | 0.2175 |
| K26 vs. K47 | 0.4231 | 0.0123 | 0.045 | 0.1734 | 0.3502 | 0.0688 |
| K26 vs. Control (sal 568) | 0.9999 | 0.0183 | 0.113 | 0.023 | 0.0643 | 0.025 |
| K29 vs. K47 | 0.0877 | 0.0029 | 0.0652 | 0.2342 | 0.2732 | 0.0697 |
| K29 vs. Control (sal 568) | 0.3013 | 0.0129 | 0.1176 | 0.0123 | 0.0578 | 0.0474 |
| K47 vs. Control (sal 568) | 0.0136 | 0.0077 | 0.172 | 0.0361 | 0.0178 | 0.0002 |

**Appendix IX:** P-values for bacterial growth under phage control in SGF and SIF

**A)** P-values of phage titres between phages at a given time-point in SGF. **B)** P-values of phage titres between phages at a given time-point in SIF. The colour intensity correlates with the P value; deep red represents a high P-value, while white represents a low P-value. Highlighted cells with a black border indicate significant P-values.

**A)**

| SGF | Time Hours | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Correlation | 0 | 0.25 | 0.5 | 0.75 | 1 | 2 | 3 | 4 |
| K1 vs. K3 | 0.9999 | 0.9999 | 0.4715 | 0.2571 | 0.0989 | 0.017 | 0.0239 | 0.0949 |
| K1 vs. K6 | 0.8508 | 0.3669 | 0.4205 | 0.9999 | 0.9999 | 0.3578 | 0.2351 | 0.4045 |
| K1 vs. K9 | 0.9025 | 0.894 | 0.9521 | 0.9999 | 0.8428 | 0.2071 | 0.1126 | 0.173 |
| K1 vs. K11 | 0.0749 | 0.1839 | 0.6017 | 0.5737 | 0.5187 | 0.0686 | 0.0701 | 0.069 |
| K1 vs. K14 | 0.9999 | 0.8146 | 0.6906 | 0.8406 | 0.4327 | 0.0908 | 0.4767 | 0.6855 |
| K1 vs. K22 | 0.4888 | 0.5492 | 0.7738 | 0.9894 | 0.9969 | 0.78 | 0.6355 | 0.7014 |
| K1 vs. K24 | 0.4106 | 0.2501 | 0.3102 | 0.667 | 0.5173 | 0.6486 | 0.6244 | 0.5347 |
| K1 vs. K26 | 0.8984 | 0.7684 | 0.5023 | 0.2909 | 0.1639 | 0.1128 | 0.2841 | 0.3975 |
| K1 vs. K47 | 0.2447 | 0.1088 | 0.1095 | 0.1874 | 0.1846 | 0.0859 | 0.0318 | 0.1074 |
| K1 vs. Control (sal 568) with acid | 0.5406 | 0.1635 | 0.1081 | 0.3656 | 0.2968 | 0.2443 | 0.3733 | 0.0928 |
| K1 vs. Control 568 | 0.1202 | 0.0254 | 0.0476 | 0.0829 | 0.207 | 0.1516 | 0.262 | 0.258 |
| K3 vs. K6 | 0.8636 | 0.318 | 0.1679 | 0.4026 | 0.3545 | 0.1742 | 0.1198 | 0.0978 |
| K3 vs. K9 | 0.8924 | 0.8569 | 0.7519 | 0.8676 | 0.7009 | 0.3269 | 0.2296 | 0.1181 |
| K3 vs. K11 | 0.048 | 0.1751 | 0.1709 | 0.9999 | 0.9999 | 0.2801 | 0.9999 | 0.4903 |
| K3 vs. K14 | 0.9999 | 0.9736 | 0.9999 | 0.6753 | 0.399 | 0.022 | 0.2165 | 0.2002 |
| K3 vs. K22 | 0.4711 | 0.4925 | 0.5107 | 0.4169 | 0.27 | 0.1357 | 0.0991 | 0.0185 |
| K3 vs. K24 | 0.3666 | 0.303 | 0.7167 | 0.5112 | 0.0672 | 0.2086 | 0.2642 | 0.2463 |
| K3 vs. K26 | 0.9146 | 0.8549 | 0.7836 | 0.5127 | 0.3859 | 0.7792 | 0.9786 | 0.8881 |
| K3 vs. K47 | 0.2109 | 0.1372 | 0.1429 | 0.3514 | 0.359 | 0.237 | 0.6233 | 0.7784 |
| K3 vs. Control (sal 568) with acid | 0.5454 | 0.2111 | 0.2391 | 0.87 | 0.9991 | 0.4403 | 0.9378 | 0.9345 |
| K3 vs. Control 568 | 0.134 | 0.0326 | 0.0531 | 0.1146 | 0.2476 | 0.2192 | 0.3968 | 0.3998 |
| K6 vs. K9 | 0.839 | 0.9997 | 0.9968 | 0.9993 | 0.9025 | 0.4718 | 0.0033 | 0.4086 |
| K6 vs. K11 | 0.0251 | 0.2499 | 0.9516 | 0.5687 | 0.5246 | 0.1963 | 0.0003 | 0.1249 |
| K6 vs. K14 | 0.994 | 0.1168 | 0.2273 | 0.8412 | 0.6949 | 0.8743 | 0.9995 | 0.9995 |
| K6 vs. K22 | 0.4034 | 0.9569 | 0.9999 | 0.9999 | 0.9999 | 0.6719 | 0.9304 | 0.6764 |
| K6 vs. K24 | 0.5213 | 0.0362 | 0.1379 | 0.7334 | 0.9132 | 0.3513 | 0.4612 | 0.381 |
| K6 vs. K26 | 0.9981 | 0.4391 | 0.3044 | 0.253 | 0.1163 | 0.0839 | 0.4521 | 0.6961 |
| K6 vs. K47 | 0.2829 | 0.0236 | 0.1048 | 0.1525 | 0.118 | 0.042 | 0.0161 | 0.1088 |
| K6 vs. Control (sal 568) with acid | 0.7894 | 0.0553 | 0.0636 | 0.3423 | 0.2963 | 0.4943 | 0.5775 | 0.178 |
| K6 vs. Control 568 | 0.1669 | 0.002 | 0.0336 | 0.06 | 0.1814 | 0.1452 | 0.2952 | 0.2964 |
| K9 vs. K11 | 0.9999 | 0.9999 | 0.9999 | 0.9248 | 0.9362 | 0.5453 | 0.0097 | 0.2081 |
| K9 vs. K14 | 0.8796 | 0.7858 | 0.7999 | 0.9999 | 0.9999 | 0.6332 | 0.6 | 0.5042 |
| K9 vs. K22 | 0.9999 | 0.9999 | 0.9983 | 0.986 | 0.7909 | 0.203 | 0.3347 | 0.1261 |
| K9 vs. K24 | 0.7606 | 0.6945 | 0.5351 | 0.9997 | 0.9938 | 0.2339 | 0.3551 | 0.3159 |
| K9 vs. K26 | 0.8094 | 0.6911 | 0.5065 | 0.3932 | 0.2407 | 0.1965 | 0.8261 | 0.9862 |
| K9 vs. K47 | 0.7026 | 0.5025 | 0.3351 | 0.2931 | 0.1968 | 0.0794 | 0.0683 | 0.1068 |
| K9 vs. Control (sal 568) with acid | 0.7668 | 0.5646 | 0.4127 | 0.6932 | 0.6977 | 0.9999 | 0.9645 | 0.3173 |
| K9 vs. Control 568 | 0.3183 | 0.1967 | 0.0278 | 0.04 | 0.1845 | 0.1619 | 0.3359 | 0.3311 |
| K11 vs. K14 | 0.1072 | 0.0621 | 0.3054 | 0.9012 | 0.8788 | 0.0565 | 0.2773 | 0.0985 |
| K11 vs. K22 | 0.6985 | 0.9999 | 0.9895 | 0.4917 | 0.4532 | 0.1327 | 0.1782 | 0.1471 |
| K11 vs. K24 | 0.0185 | 0.0229 | 0.1853 | 0.8888 | 0.6693 | 0.2256 | 0.2783 | 0.151 |
| K11 vs. K26 | 0.1923 | 0.3309 | 0.3704 | 0.5733 | 0.463 | 0.4 | 0.9756 | 0.477 |
| K11 vs. K47 | 0.0191 | 0.0196 | 0.0838 | 0.3676 | 0.3485 | 0.1241 | 0.211 | 0.6001 |
| K11 vs. Control (sal 568) with acid | 0.061 | 0.0396 | 0.0647 | 0.9844 | 0.9997 | 0.7323 | 0.9346 | 0.8586 |
| K11 vs. Control 568 | 0.0914 | 0.0012 | 0.0488 | 0.056 | 0.199 | 0.2006 | 0.3987 | 0.4806 |
| K14 vs. K22 | 0.4404 | 0.4222 | 0.533 | 0.7316 | 0.5021 | 0.3748 | 0.9602 | 0.9887 |
| K14 vs. K24 | 0.6305 | 0.2439 | 0.7277 | 0.9999 | 0.7917 | 0.3372 | 0.4166 | 0.3918 |
| K14 vs. K26 | 0.9791 | 0.9566 | 0.7711 | 0.357 | 0.1853 | 0.1574 | 0.4913 | 0.637 |
| K14 vs. K47 | 0.3907 | 0.0929 | 0.2128 | 0.2197 | 0.2023 | 0.0729 | 0.2183 | 0.1971 |
| K14 vs. Control (sal 568) with acid | 0.744 | 0.207 | 0.298 | 0.5675 | 0.5377 | 0.6618 | 0.6604 | 0.1504 |
| K14 vs. Control 568 | 0.1099 | 0.0072 | 0.0321 | 0.0759 | 0.216 | 0.1734 | 0.2816 | 0.279 |
| K22 vs. K24 | 0.3106 | 0.2975 | 0.3517 | 0.6557 | 0.7282 | 0.5091 | 0.4929 | 0.4492 |
| K22 vs. K26 | 0.3501 | 0.3516 | 0.3334 | 0.2206 | 0.0991 | 0.055 | 0.3528 | 0.5387 |
| K22 vs. K47 | 0.2549 | 0.2203 | 0.2607 | 0.1364 | 0.1156 | 0.0315 | 0.1326 | 0.0154 |
| K22 vs. Control (sal 568) with acid | 0.2869 | 0.2467 | 0.2892 | 0.3095 | 0.2313 | 0.2347 | 0.4888 | 0.1845 |
| K22 vs. Control 568 | 0.0535 | 0.0901 | 0.0196 | 0.0473 | 0.187 | 0.1325 | 0.2728 | 0.2856 |
| K24 vs. K26 | 0.9996 | 0.9892 | 0.9998 | 0.3806 | 0.2065 | 0.1386 | 0.2352 | 0.2218 |
| K24 vs. K47 | 0.7972 | 0.3599 | 0.3731 | 0.2509 | 0.2199 | 0.1239 | 0.2559 | 0.2399 |
| K24 vs. Control (sal 568) with acid | 0.9999 | 0.9696 | 0.8511 | 0.5649 | 0.3943 | 0.2105 | 0.2471 | 0.1792 |
| K24 vs. Control 568 | 0.1743 | 0.0001 | 0.0283 | 0.095 | 0.2165 | 0.062 | 0.1371 | 0.1404 |
| K26 vs. K47 | 0.8914 | 0.7363 | 0.7364 | 0.9996 | 0.9854 | 0.4444 | 0.7776 | 0.781 |
| K26 vs. Control (sal 568) with acid | 0.9999 | 0.9537 | 0.9999 | 0.8165 | 0.4658 | 0.2768 | 0.9999 | 0.7638 |
| K26 vs. Control 568 | 0.1041 | 0.1232 | 0.0297 | 0.0671 | 0.2728 | 0.2081 | 0.3436 | 0.3171 |
| K47 vs. Control (sal 568) with acid | 0.899 | 0.7096 | 0.4468 | 0.5532 | 0.3602 | 0.1292 | 0.7525 | 0.9993 |
| K47 vs. Control 568 | 0.1838 | 0.0113 | 0.0972 | 0.0791 | 0.2858 | 0.2531 | 0.4272 | 0.4162 |
| Control (sal 568) with acid vs. Control 568 | 0.1407 | 0.0225 | 0.0474 | 0.063 | 0.2269 | 0.1525 | 0.3226 | 0.416 |

**B)**

| SIF | Time Hours | | | | | |
|---|---|---|---|---|---|---|
| Correlation | 0 | 0.5 | 1 | 2 | 3 | 4 |
| K1 vs. K3 | 0.9999 | 0.2348 | 0.1269 | 0.2978 | 0.8691 | 0.0819 |
| K1 vs. K6 | 0.6328 | 0.907 | 0.5263 | 0.1543 | 0.9997 | 0.0389 |
| K1 vs. K9 | 0.9409 | 0.285 | 0.0647 | 0.2797 | 0.9973 | 0.1054 |
| K1 vs. K11 | 0.5624 | 0.0303 | 0.004 | 0.0841 | 0.0809 | 0.0942 |
| K1 vs. K14 | 0.9999 | 0.2145 | 0.3073 | 0.6631 | 0.7937 | 0.8498 |
| K1 vs. K22 | 0.7455 | 0.7647 | 0.9735 | 0.8699 | 0.6198 | 0.9847 |
| K1 vs. K24 | 0.9999 | 0.8606 | 0.7347 | 0.9999 | 0.9968 | 0.9565 |
| K1 vs. K26 | 0.9975 | 0.3321 | 0.207 | 0.7646 | 0.4515 | 0.3933 |
| K1 vs. K47 | 0.4534 | 0.071 | 0.2893 | 0.4814 | 0.3937 | 0.1436 |
| K1 vs. Control (sal 568) with acid | 0.6509 | 0.1041 | 0.0139 | 0.0207 | 0.0095 | 0.017 |
| K1 vs. Control 568 | 0.0852 | 0.0054 | 0.0367 | 0.021 | 0.0905 | 0.0971 |
| K3 vs. K6 | 0.6917 | 0.3232 | 0.2113 | 0.9522 | 0.9822 | 0.1532 |
| K3 vs. K9 | 0.9986 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.7363 |
| K3 vs. K11 | 0.6375 | 0.0239 | 0.0122 | 0.049 | 0.0793 | 0.095 |
| K3 vs. K14 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9168 | 0.9999 |
| K3 vs. K22 | 0.7817 | 0.2277 | 0.163 | 0.3543 | 0.9919 | 0.0727 |
| K3 vs. K24 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9992 | 0.9999 |
| K3 vs. K26 | 0.9999 | 0.865 | 0.5436 | 0.4538 | 0.4783 | 0.519 |
| K3 vs. K47 | 0.5401 | 0.0379 | 0.066 | 0.273 | 0.4128 | 0.1866 |
| K3 vs. Control (sal 568) with acid | 0.8848 | 0.0439 | 0.0194 | 0.0005 | 0.0074 | 0.0029 |
| K3 vs. Control 568 | 0.1445 | 0.0035 | 0.0179 | 0.0052 | 0.0903 | 0.0994 |
| K6 vs. K9 | 0.2245 | 0.2232 | 0.1063 | 0.8656 | 0.9999 | 0.2495 |
| K6 vs. K11 | 0.9998 | 0.0137 | 0.002 | 0.0722 | 0.0811 | 0.0971 |
| K6 vs. K14 | 0.7759 | 0.3038 | 0.4383 | 0.9999 | 0.8373 | 0.9787 |
| K6 vs. K22 | 0.9709 | 0.3039 | 0.2718 | 0.4409 | 0.7712 | 0.2763 |
| K6 vs. K24 | 0.9998 | 0.9472 | 0.8745 | 0.9999 | 0.9979 | 0.9967 |
| K6 vs. K26 | 0.2619 | 0.3874 | 0.2884 | 0.5083 | 0.4608 | 0.4427 |
| K6 vs. K47 | 0.7883 | 0.0294 | 0.1219 | 0.3276 | 0.4005 | 0.1646 |
| K6 vs. Control (sal 568) with acid | 0.1203 | 0.1015 | 0.0117 | 0.0096 | 0.0095 | 0.0143 |
| K6 vs. Control 568 | 0.024 | 0.0003 | 0.0364 | 0.0163 | 0.0906 | 0.0987 |
| K9 vs. K11 | 0.1968 | 0.0367 | 0.0039 | 0.0487 | 0.0583 | 0.0921 |
| K9 vs. K14 | 0.9997 | 0.9678 | 0.9999 | 0.9993 | 0.9124 | 0.9999 |
| K9 vs. K22 | 0.3012 | 0.0001 | 0.0784 | 0.3351 | 0.9895 | 0.0976 |
| K9 vs. K24 | 0.9999 | 0.9999 | 0.9999 | 0.9998 | 0.9989 | 0.9999 |
| K9 vs. K26 | 0.9305 | 0.6858 | 0.4136 | 0.4397 | 0.4638 | 0.4923 |
| K9 vs. K47 | 0.1899 | 0.0822 | 0.0344 | 0.2649 | 0.3988 | 0.1729 |
| K9 vs. Control (sal 568) with acid | 0.9026 | 0.1186 | 0.0139 | 0.0005 | 0.0007 | 0.0029 |
| K9 vs. Control 568 | 0.0249 | 0.0207 | 0.026 | 0.0052 | 0.0841 | 0.0985 |
| K11 vs. K14 | 0.7274 | 0.0293 | 0.062 | 0.0169 | 0.0231 | 0.1838 |
| K11 vs. K22 | 0.833 | 0.0822 | 0.0001 | 0.0207 | 0.0771 | 0.0711 |
| K11 vs. K24 | 0.9992 | 0.2484 | 0.1495 | 0.1673 | 0.2459 | 0.3689 |
| K11 vs. K26 | 0.2232 | 0.0121 | 0.017 | 0.0293 | 0.0757 | 0.4933 |
| K11 vs. K47 | 0.9227 | 0.099 | 0.0062 | 0.0232 | 0.0988 | 0.1049 |
| K11 vs. Control (sal 568) with acid | 0.1007 | 0.1715 | 0.975 | 0.5362 | 0.1558 | 0.081 |
| K11 vs. Control 568 | 0.0237 | 0.0116 | 0.1076 | 0.0197 | 0.1149 | 0.1185 |
| K14 vs. K22 | 0.8531 | 0.228 | 0.31 | 0.5105 | 0.9757 | 0.8256 |
| K14 vs. K24 | 0.9999 | 0.9997 | 0.9999 | 0.9999 | 0.9999 | 0.9999 |
| K14 vs. K26 | 0.9999 | 0.7161 | 0.8054 | 0.4988 | 0.5699 | 0.5989 |
| K14 vs. K47 | 0.6287 | 0.0454 | 0.1909 | 0.2718 | 0.4563 | 0.5153 |
| K14 vs. Control (sal 568) with acid | 0.9454 | 0.0436 | 0.0685 | 0.0112 | 0.0079 | 0.1161 |
| K14 vs. Control 568 | 0.1927 | 0.0061 | 0.0106 | 0.001 | 0.061 | 0.0332 |
| K22 vs. K24 | 0.9999 | 0.7215 | 0.692 | 0.9963 | 0.9998 | 0.945 |
| K22 vs. K26 | 0.1214 | 0.2002 | 0.0834 | 0.8681 | 0.4971 | 0.3825 |
| K22 vs. K47 | 0.2573 | 0.1308 | 0.3599 | 0.7155 | 0.4258 | 0.1072 |
| K22 vs. Control (sal 568) with acid | 0.0448 | 0.2284 | 0.0029 | 0.0117 | 0.0056 | 0.0004 |
| K22 vs. Control 568 | 0.0236 | 0.0207 | 0.0475 | 0.0014 | 0.0898 | 0.092 |
| K24 vs. K26 | 0.9999 | 0.9999 | 0.9987 | 0.9657 | 0.9706 | 0.7943 |
| K24 vs. K47 | 0.9948 | 0.3519 | 0.5469 | 0.8734 | 0.8978 | 0.776 |
| K24 vs. Control (sal 568) with acid | 0.9972 | 0.359 | 0.1523 | 0.1739 | 0.1917 | 0.2044 |
| K24 vs. Control 568 | 0.5871 | 0.1181 | 0.033 | 0.0837 | 0.0614 | 0.078 |
| K26 vs. K47 | 0.0465 | 0.02 | 0.133 | 0.9963 | 0.9994 | 0.9999 |
| K26 vs. Control (sal 568) with acid | 0.2151 | 0.0609 | 0.0087 | 0.0517 | 0.073 | 0.1891 |
| K26 vs. Control 568 | 0.0377 | 0.0003 | 0.0402 | 0.0114 | 0.0318 | 0.0578 |
| K47 vs. Control (sal 568) with acid | 0.0217 | 0.997 | 0.0157 | 0.0354 | 0.0876 | 0.032 |
| K47 vs. Control 568 | 0.0305 | 0.0067 | 0.0403 | 0.0054 | 0.0326 | 0.0879 |
| Control (sal 568) with acid vs. Control 568 | 0.0147 | 0.021 | 0.1112 | 0.0304 | 0.2549 | 0.3914 |