Characterization of *Salmonella* Bacteriophages Isolated from Farm Environments for Use in Decontamination of Liquid Whole Egg

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Yue Yi, B.S.

Graduate Program in Food Science and Technology

The Ohio State University

2019

Master's Examination Committee:

Dr. Ahmed Yousef, Advisor

Dr. Abigail Snyder

Dr. Jiyoung Lee

Abstract

Salmonella enterica is the most prevalent bacterial pathogen causing foodborne illnesses. Globally, contamination of eggs has been identified as a major vehicle for transmitting foodborne salmonellosis. Two *S. enterica* serovars, Typhimurium and Enteritidis, are efficient colonizers of the reproductive organs of hens, hence, these two serovars are closely associated with salmonellosis transmitted by eggs. Current decontamination procedures for the production of microbiologically safe eggs rely on thermal treatments, but these adversely affect egg quality. Hence, research is needed to explore effective decontamination methods that do not damage egg quality and functionality.

The objectives of this research were (1) to isolate and select *Salmonella* bacteriophages from environmental samples; (2) to characterize selected phage isolates and assess their lytic activities; and (3) to evaluate the effectiveness of promising phage isolates against *Salmonella* in liquid whole eggs.

To achieve these objectives, the following procedure was followed. Environmental samples (water, animal feces, feather, etc.) were collected from animal farms in Ohio. Samples were inoculated with a cocktail of *S.* Typhimurium, *S.* Enteritidis and *S.* Heidelberg and incubated to allow for the amplification of potential phages. Incubated samples were micro-filtered to remove bacterial cells and large particles. The presence of bacteriophages was determined by spotting 10 μl of filtrates onto *Salmonella*-

embedded soft agar, followed by isolation and purification of bacteriophages from the filtrates by double agar overlay technique. The most promising phage candidates were selected based on their host range which was determined against 37 *Salmonella* strains.

Two phage isolates, OSY-STA and OSY-SHC, were characterized by determining replication kinetics, measuring pH and thermal stability, examining phage morphology, estimating phage genome size and evaluating phage lytic ability against *Salmonella* in Tryptic Soy Broth (TSB). For determination of replication kinetics, titer of phages in the presence of their host at each treatment were enumerated during incubation at 37 °C. The phages had similar latent periods and generation times, while burst size of OSY-STA and OSY-SHC were 176 and 243 PFU/cell, respectively. Both OSY-STA and OSY-SHC were stable while holding in media at pH 4 – 12 and during storage at temperatures of 4, 25, 37 and 55 °C. Phage morphology was examined by Transmission Electron Microscope (TEM), and results showed that OSY-STA and OSY-SHC are members of family *Siphoviridae* of tailed bacteriophages. Phage OSY-STA was subjected to DNA sequencing considering its broad host range. Sequencing results revealed a 111,373 bp dsDNA genome containing 169 ORFs organized into four major categories: DNA replication and nucleotides metabolism-related proteins, structural proteins, regulator proteins and cell lysis proteins.

Lytic activity of phage was determined at 4 °C or 25 °C by enumerating viable *Salmonella* after phage treatments. At 25 °C, application of the mixture of two isolated phages significantly reduced *Salmonella* population after 24 h (Tukey's test, $P<0.05$). Furthermore, the growth of *Salmonella* Enteritidis was significantly (Tukey's test,$P <$

0.05) inhibited after 3-day incubation at 4 °C. Lysis results deliver valuable information to assess the potential of the phage cocktail as a biocontrol agent against diverse *Salmonella* serovars in contaminated food products.

Liquid egg samples were inoculated with 100 µl of 10^5 CFU/ml *Salmonella* Typhimurium or Enteritids in 10 ml liquid whole egg sample, and treated with 100 µl of the phage cocktail (OSY-STA + OSY-SHC) at a concentration of 10^7 PFU/ml. Therefore, the final concentration of *Salmonella* and phage in liquid egg sample were 10^3 CFU/ml and 10^5 PFU/ml, respectively (i.e., MOI of 100). Efficiency of the phage cocktail was determined by counting viable *Salmonella* population after 24 h incubation at 4 °C. Significant reduction (Tukey's test, $P<0.05$) in bacterial counts was only observed in *S.* Enteritidis-inoculated samples. Overnight phage cocktail treatment (at 4°C) following heating at 55 °C showed higher efficiency in reducing bacterial counts than when the phage or thermal treatment was applied individually. There was at least 2.8 $\log_{10}$ CFU/ml reduction in the population of *S.* Enteritidis when contaminated liquid egg was treated with a combination of phage cocktail, followed by 13-min of heating.

This study illustrated the characteristics of isolated phages and provided a basis for phage application in food processing. The study specifically demonstrated the applicability of phage in conjunction with thermal treatment to pasteurize liquid eggs.

Acknowledgments

I would like to first express my sincere gratitude to my advisor Dr. Ahmed Yousef for his instructive advice and useful suggestions all the time through these two years at The Ohio State University. He has walked me through all the stages of my research and inspired me to set my mind to work on my current project. Without his consistent encouragement and illuminating instructions, this research could not have reached its present form. To my committee member: Dr. Jiyoung Lee, thank you for your teaching in Global Health and Environmental Microbiology class. I really appreciate all the projects and researches you have done in the developing countries; I learned how important it is to apply our knowledge in the real world. To my other committee member: Dr. Abigail Snyder, one of your papers spiked my interest in using bacteriophages to control foodborne pathogens.

Second, I would like to thank all of the members in Yousef lab: Dr. Ahmed Abdelhamid, Dr. Pengkang Ren, David Kasler, Emily Campbell, Walaa Hussein, and Yumin Xu and former lab member, Mustafa Yesil and Xiaochang Liu, for their insightful criticism. Special thanks go to David Kasler for always helping me solving technique problems when I was working in the Howlett lab. Thank you Yumin for your encouragements, suggestions and helps during my experiments.

Last my thanks would go to my beloved family for their loving considerations and great confidence in me. You have been always supporting my education and dreams.

Especially, to my grandmother, the life experience you taught me is invaluable. I also owe my gratitude to my friends who gave me their help and time in listening to me and helping me work out my problems through these years.

Vita

August 11, 1995 .....Born, Nanjing, Jiangsu, China

2017.....B.S. Food quality and Safety, Yangzhou University

Fields of Study

Major Field: Food Science and Technology

Table of Contents

Abstract ii

Acknowledgments v

Vita vii

Fields of Study vii

Table of Contents viii

List of Tables xiii

List of Figures xv

Chapter 1: Literature review 1

1.1 *Salmonella* sp. and foodborne disease 1

1.2 *Salmonella* serovars and eggs 2

1.3 Decontamination of liquid egg products 4

1.4 Bacteriophage 8

1.4.1 History 8

1.4.2 Diversity and abundance of bacteriophage 8

1.4.3 Phage taxonomy and structure 9

1.4.4 Phage infection processes and life cycles 12

1.5 Biocontrol of *Salmonella* in foods using bacteriophages 15

1.5.1 Control of *Salmonella enterica* in primary production 18

1.5.2 Postharvest control of *Salmonella* 19

1.6 Commercial *Salmonella* phage products and their applications 26

1.7 Phage as a part of hurdle technologies in the food industry 28

Reference 32

Chapter 2: isolation and characterization of *Salmonella* bacteriophage from farm environments in Ohio 39

2.1 Abstract 39

2.2 Introduction 40

2.3 Materials and methods 42

2.3.1 Sample collecting 42

2.3.3 Buffer preparation 44

2.3.4 Bacteriophage screening 45

2.3.5 Bacteriophage isolation, purification and propagation 46

2.3.6 Host range determination 48

2.3.7 Phage replication kinetics 50

2.3.8 pH stability 51

2.3.9 Thermal stability 51

2.3.10 Phage morphology 52

2.3.12 Efficiency of phage and phage cocktail against *Salmonella* in TSB 53

2.3.13 Statistical analysis 53

2.4 Results 54

2.4.1 Screening, isolation and purification of bacteriophages from environmental samples 54

2.4.2 Host range determination 56

2.4.3 Phage replication kinetics 58

2.4.4 pH stability 61

2.4.5 Thermal stability 63

2.4.6 Phage morphology 65

2.4.7 Inactivation of *Salmonella* serotypes in TSB by phages and a phage cocktail 67

2.5 Discussion 73

Reference 77

Additional tables 82

Chapter 3: Bioinformatic analyses of *Salmonella* phage OSY-STA 94

3.1 Abstract 94

3.2 Introduction 94

3.3 Materials and methods..... 96

3.3.1 DNA extraction, library preparation and whole genome sequencing 96

3.3.2 Bioinformatic analyses of phage genome..... 97

3.4 Results 98

3.4.1 General features of the phage genome..... 98

3.4.2 Regulatory sequences 112

3.4.3 DNA replication and nucleotide metabolism 112

3.4.4 Structure and cell wall lysis related sequence..... 112

3.5 Discussion..... 115

Reference..... 118

Chapter 4: Use of phage cocktail alone or in combination with heat against *Salmonella* Typhimurium and Enteritidis in liquid whole egg..... 120

4.1 Abstract..... 120

4.2 Introduction 121

4.3 Materials and methods..... 124

4.3.1 Recovery test 124

4.3.2 Egg inoculation 124

4.3.3 Phage cocktail treatment..... 125

4.3.4 Phage treatment combined with mild heating 125

4.3.5 Statistical analysis..... 126

4.4 Results 127

4.4.1 Recovery test 127

4.4.2 Application of the phage cocktail as a biocontrol agent against *Salmonella* in liquid whole egg 128

4.4.3 Efficiency of phage cocktail combined with thermal treatment at 55 °C against *Salmonella* in liquid whole egg 129

4.5 Discussion..... 132

Reference..... 135

List of reference…….....136

List of Tables

Chapter 1.

Table 1.1 Overview of double-strand DNA (dsDNA) *Salmonella* bacteriophages in recent literatures. 11

Table 1.2 Some commercial *Salmonella* phage products. 26

Chapter 2.

Table 2.1 Locations, operation types and sampling information of farms. 43

Table 2.2 *Salmonella enterica* and *Escherichia coli* strains used in host range determination analysis for phage isolates. 48

Table 2.3 Number of positive samples with potential phage candidates and ID of each isolated phage. 54

Table 2.4 Host range of phage OSY-STA and OSY-SHC determined against 39 strains of *Salmonella enterica* and *Escherichia coli*. 57

Table 2.5 Host range of 31 phage isolates determined against 39 bacterial strains (From A-DW-SE to D-CW-SH). 82

Table 2.6 Host range of 31 phage isolates determined by against 39 bacterial strains (From D-Body-SH to E-Drain-Q-SH). 85

Table 2.7 Host range of 31 phage isolates determined by against 39 bacterial strains (From E-Cage-C-SH to G-Gost-Hay-SH). 88

Table 2.8 Host range of 31 phage isolates determined by 39 bacterial strains (From H-DW-C-SH to I-Wall-SH). 91

Chapter 3.

Table 3.1 Features of the open reading frames of bacteriophage OSY-STA and homology to protein databases. 100

Table 3.2 tRNA coding regions in phage genome. 114

Chapter 4.

Table 4.1 Phage titer in liquid whole egg after 24 h incubation at 4°C 127

List of Figures

Chapter 1.

Figure 1.1 Flow chart of liquid egg pasteurization (Clark 2014; SPX Process Bulletin, 2008). 5

Figure 1.2 Schematic diagram of Escherichia coli phage. 9

Figure 1.3 Schematic diagram of phage lytic life cycle (Bertani, 1973). 14

Figure 1.4 The one-step growth curve for a bacteriophage (Lenski, 1988). 15

Figure 1.5 Phage applications along the food production processes (Moye et al., 2018). 17

Chapter 2.

Figure 2.1 Examples of phage lysis reactions on specific *Salmonella* serovars. 55

Figure 2.2 An example of phage-forming plaques on TSA. 56

Figure 2.3 Replication curves (changes in phage titer) for two phages determined against selected host bacteria. 60

Figure 2.4 Stability of selected phage isolates at different pH values. 62

Figure 2.5 Stability of phage isolates upon holding at different temperatures. 64

Figure 2.6 Morhplogy of bacteriophages examined by TEM. 66

Figure 2.7 Lytic effects of single phage isolate OSY-STA, OSY-SHC and the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes of liquid cultures (TSB) *in vitro* at 25 °C. 68

Figure 2.8 Lytic effects of single phage isolate OSY-STA, OSY-SHC and the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes in tryptic soy broth held at 4 °C. 71

Chapter 3.

Figure 3.1 Schematic diagram of the OSY-STA genome and its open reading frames, colored-coded by protein functional categories. 110

Figure 3.2 Mauve genome alignment of *Salmonella* phage OSY-STA, 3-29 and BSP22A. 111

Chapter 4.

Figure 4.1 Flow chart of phage cocktail and phage cocktail-thermal treatments in liquid whole eggs. 126

Figure 4.2 Lytic effects of the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes in liquid whole eggs at 4 °C. 129

Figure 4.3 Effects of phage cocktail and thermal treatment at 55 °C on *Salmonella* serotypes with different treatment time. 130

Figure 4.4 Morphology of *Salmonella* Typhimurium colonies. 133

Chapter 1: Literature review

1.1 *Salmonella* sp. and foodborne disease

Salmonella enterica is one of the most important human pathogens and the leading causes of foodborne diseases (CDC,2018). In 2011, it was estimated that in the United states, 31 pathogens caused 37.2 million illnesses, and nontyphoidal *Salmonella* spp. was the second most common pathogens after norovirus, causing about 1.0 million illnesses (11 %), 80,000 hospitalizations, and 730 deaths annually (Scallan et al., 2011). European Food Safety Authority (EFSA) also confirmed that over 100,000 salmonellosis cases in humans are reported each year in the European Union (EU), which cost approximately €3 billion a year. The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2017 suggests that though a statistically significant decreasing trend of confirmed salmonellosis cases has been observed in EU/EEA between 2008 and 2017, however, over the most recent 5 years (2013 – 2017), the overall trend of salmonellosis cases has not shown any significant change (EFSA, 2018).

In 2018, CDC reported 15 salmonellosis outbreaks linked to food; this was considered the most frequent outbreaks/year within the most recent five years. This number also accounts for over 60 % of foodborne disease outbreaks in that year (CDC, 2018). Foods involved in these outbreaks include fresh produce, raw meat, poultry products, and even highly processed food. As detection technologies improved through

years, researchers are more capable of effectively monitoring and controlling *Salmonella* contamination within food commodities.

Salmonellosis is characterized by gut inflammation. In most cases, stomach cramps, diarrhea, fever and vomiting are the common symptoms with *Salmonella* infection, but they usually do not last more than one week, and no special treatments needed. For patients with complications such as reactive arthritis, dehydration or ongoing high fever for more than a couple of days, further treatments should be considered. People with compromised immune system may also need doctor's prescription (Eng et al.,2015)

SopE is one of the virulence factor of certain *Salmonella* strains which is capable of triggering the diarrhea (Lara-Tejero et al., 2006). When the protein is injected into an intestinal epithelium cell, it tampers with two specific GTPases (Cdc42 and Rac1) which play essential role in cell's early warning system via activating Caspase-1, resulting in inflammatory responses in the cell. Activated Caspase-1, in turn, triggers phagocytes to eliminate bacterial pathogens (Muller et al., 2009). In addition to SopE, other virulence factors induce SipA which is engaged in a completely different proinflammatory signal cascade than that is affected by SopE.

1.2 *Salmonella* serovars and eggs

Over 2,500 *Salmonella* serovars have been identified; among these, Enteritidis, Typhimurium, Newport and Heidelberg are the most common serotypes associated with foodborne disease outbreaks. Overall, egg is the most common commodity associated with salmonellosis outbreaks, followed by poultry, raw meat and fresh produce.

Enteritidis and Heidelberg are the most prevalent serotypes within egg, whereas Typhimurium is more likely to contaminate chicken (CDC,2017).

The most notable egg-associated *Salmonella* serovar is *S.* Enteritidis. Although egg shell can be penetrated by various bacterial species, *S.* Enteritidis is the only serovar can be isolated from egg contents, both in yolk and albumen (Humphrey et al., 1994). It has been reported that *S.* Enteritidis has high ability to colonize in ovary and the preovulatory follicules than five other serotypes (*S.* Typhimurium, *S.* Infantis, *S.* Heidelberg, *S.* Hadar, *S.* Montevideo) and shows higher affinity to reproductive organs in hens. Molecular analysis has revealed the few possible mechanisms of the interaction between *S.* Enteritidis and hen's reproductive tract and how bacteria adapt to this particular ecological niche. Stress-induced protective and reparative responses is one of the factors contributing to the persistence of bacteria in hostile environment and to overcoming host defense reactions. Few genes encode cell wall integrity, regulation of fimbrial operons and stress response would be highly induced during *S.* Enteritidis colonization of the reproductive tract (Gantois et al., 2008). For example, when *S.* Enteritidis resides extracellularly in the oviduct lumen, in the presence of albumen during incubation at 42℃, *S.* Enteritidis *fimZ* will be highly induced, further activates the transcription of type-1 fimbriae, resulting in the attachment of bacteria to the reproductive cells (Gantois at al., 2008). High-molecular-weight lipopolysaccharide in *S.* Enteritidis also may be considered a major factor related to *S.* Enteritidis virulence in reproductive tracts. Different compositions and structures of lipopolysaccharide affect the levels of attachment of different *Salmonella* serotypes (Guard-Petter et al., 2001). Type

III secretion systems (T3SSs) of *Salmonella* also plays important role in invasion (Gu et al., 2005). In general, many factors contribute to the ability of *Salmonella* Enteritidis to reside in reproductive organs of chicken, resulting in contamination inside eggs.

1.3 Decontamination of liquid egg products

Eggs has been considered one of the most nutritious and economic foods as it is rich in vitamins, essential fatty acids and amino acids. Foods contains contaminated raw eggs such as mayonnaise and ice cream may present potential risk to consumers if those eggs have not undergone pasteurization or other decontamination processes. Pasteurization is the most widely used process for production of microbiologically safe eggs. Commercial liquid egg pasteurization process is presented in **Figure 1.1** (Clark 2014; SPX Process Bulletin, 2008).

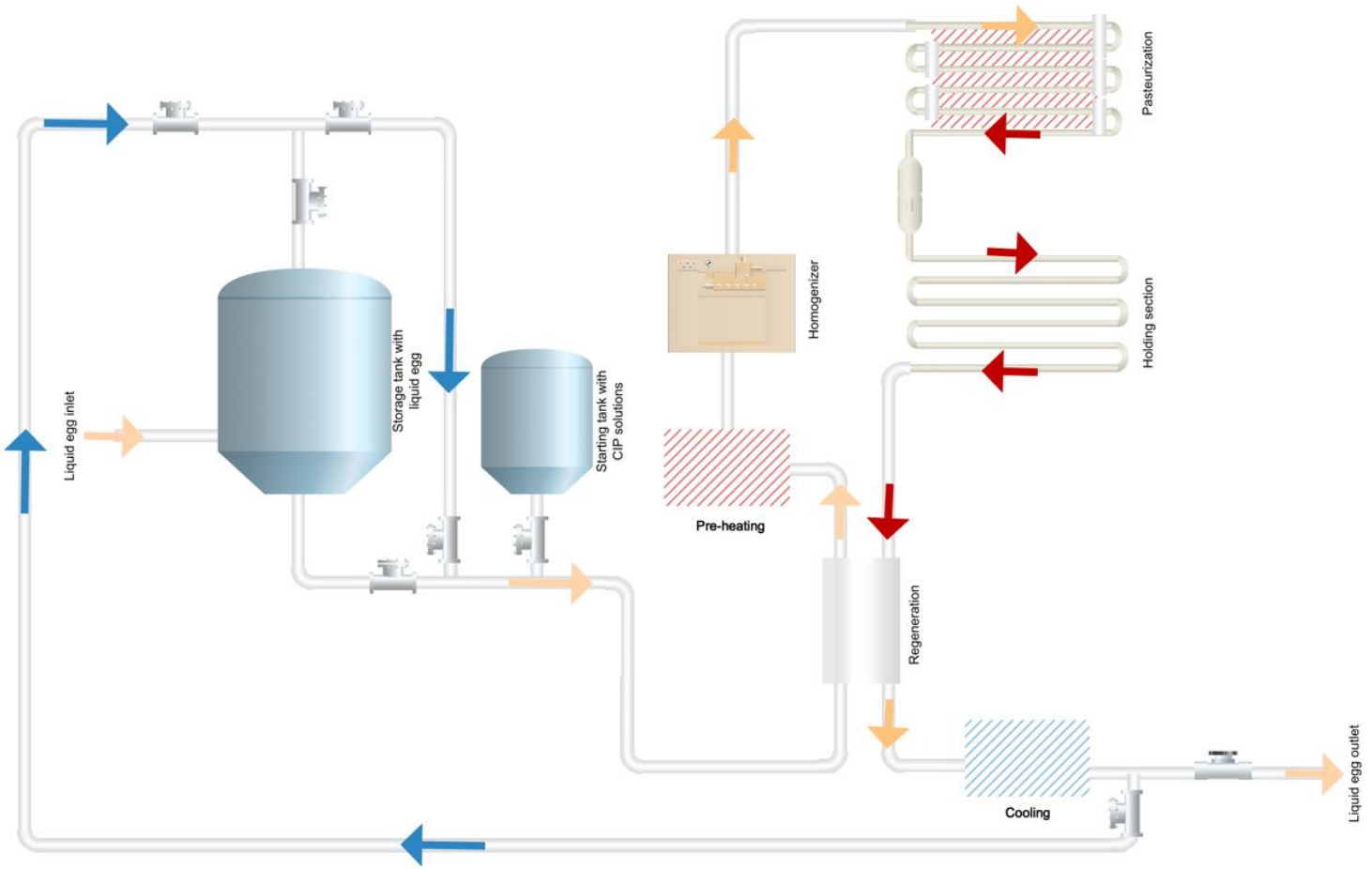


Figure 1.1 Flow chart of liquid egg pasteurization (Clark 2014; SPX Process Bulletin, 2008).

Refrigerated unpasteurized liquid egg is first received in a closed storage tank (balance tank) to ensure a continuous supply of the product. From the storage tank, the liquid egg product is pumped into a heat exchanger for pre-heating. The product is then transferred to a homogenizer in order to (a) protects the functional properties of the liquid egg in pasteurization; (b) prevents separation between egg yolk and white; (c) improves the foaming power of product; (d) reduces protein denaturation. Following homogenization, product is heated to the desired pasteurization temperature in the pasteurizer through heat exchange between hot water and liquid egg product. Heated product is kept in holding tubes at a temperature close to pasteurization temperature for a certain amount of time, followed by cooling process in another heat exchanger and final product is filled in appropriate packages (Clark, 2014).

Protein denaturation happens during the heating process, resulting in protein aggregation and egg gelatinization. Moreover, the changes of egg properties including coagulation, foaming and emulsifying depend heavily on temperature and heating time. In most cases, pasteurization temperatures used in egg industry are limited by the sensitivity of egg protein. It has been reported that pasteurization is accomplished in 2-10 min at 60 to 68 °C but these conditions decrease ovotranferrin, livetin, apovitellenin, and lysozyme, thus altering the egg proteins electrophoretic pattern. Furthermore, egg viscosity increases as pasteurization temperature reaches 56 °C or above (Lechevalier et al., 2016), yet some studies considered that foaming and emulsifying properties remain stable in liquid egg heated at temperatures up to 60 °C (Montfort et al., 2012).

In addition to thermal pasteurization, some non-thermal treatments have been explored as alternatives for liquid egg decontamination. According to Badr (2016), gamma irradiation at 3 kGy dose did not show significant effects on fatty acids and amino acids composition of liquid egg. Furthermore, after irradiation at 3 kGy, significant reduction of total plate count was observed and no *Salmonella* or other members of *Enterobacteriaceae* could be detected during storage at 4 °C. According to another study by Serrano et al. (1997), 1.5 kGy irradiation dose is enough to cause 4 $\log_{10}$ CFU/ml reduction of *Salmonella* counts without damaging color and thermal characteristics of both shell and liquid whole egg. Despite the proposed advantages of irradiation, formation of free radicals due to lipid oxidation caused by irradiation is inevitable, even at small dose (Pinto et al., 2004). Another non-thermal treatment for production of microbiologically safe eggs has been introduced recently is high pressure carbon dioxide processing (HPCD). Related study stated that HPDC processing at 13.0 MPa, 45 °C, 50% working volume ratio and 400 min^{-1} stirring speed during 10 min were the optimum conditions for inactivating native microorganisms in liquid whole egg (LWE). Shelf life of HPCD treated LWG under 4 °C was extended up to 5 weeks, which is comparable to that of thermally-pasteurized LWG products (Garcia-Gonzalez et al., 2009).

Despite these efforts, raw egg and egg products are still a major cause of salmonellosis outbreaks, therefore, optimization of available procedures and development of new intervention strategies are required. Research is needed for identifying the optimum conditions such as the precise pasteurization temperature that could be

commercialized to obtain *Salmonella*-free eggs. Also, combination of pasteurization and other decontamination methods should be further explored for the possibility to produce safe eggs and maintain egg properties at the same time.

1.4 Bacteriophage

1.4.1 History

Bacteriophages were first discovered in 1915 by William Twort. In 1917, Félix d'Herelle recognized that phages are small virus with the ability to infect host bacteria without affecting cells of other organisms (Wittebole et al., 2014). Afterwards, the significance of phages and phage applications were disregarded due to the prevalence of antibiotics usage. During this time, research on using phages to treat a number of diseases did continue in few countries such as Georgia. Very little detailed information of phages could be obtained for several decades. However, interest in phage research has increased in recent years. This renaissance was triggered by the development of electron microscopy and whole genome sequencing technologies. Additionally, the current threat of antibiotic-resistant bacteria has risen the interests of the scientific community in exploring bacteriophages as an alternative biocontrol agent (Matsuzaki et al., 2005).

1.4.2 Diversity and abundance of bacteriophage

Bacteriophages (phages) are everywhere on earth, and their abundance and distribution are highly dependent on the host organisms. They are most frequently isolated from aquatic environments with an estimated population range of 10^4 to 10^8 virions per ml. In addition to the aquatic systems, most of the earth's bacteria and archaea can be found in soil environments where there are an estimated 10^9 virions per gram

(Wittebole et al., 2014). Only a tiny fraction of phages has been characterized and elucidated clearly, compared to the estimated total number of 10^{32} bacteriophages on the planet (Hanlon, 2007).

1.4.3 Phage taxonomy and structure

Phages have diverse structures with a variety of morphological types, but many share some common characteristics as shown in **Figure 1.2 (**Ackermann, 2009).

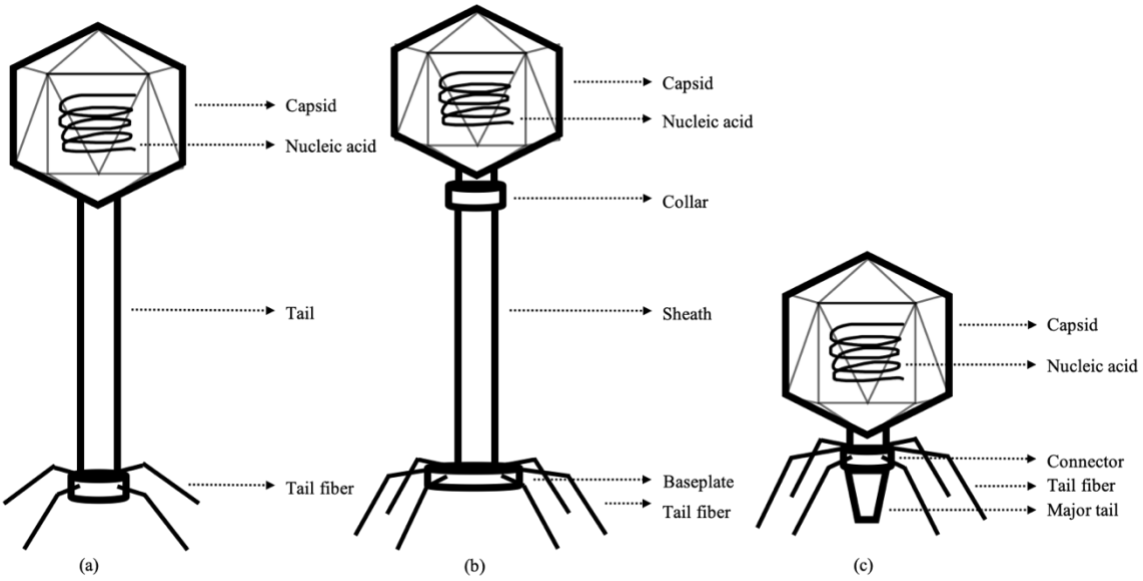


Figure 1.2 Schematic diagram of *Escherichia coli* phage **(**Ackermann, 2009). (a) T1 (*Siphoviridae*); (b) T4 (*Myoviridae*); (c) T7 (*Podoviridae*), representing typical phage virion structures.

Many phages have an icosahedral head structure comprised of repeat protein subunits. Usually the head contains double-strand DNA representing viral genome. There are few tail fibers connected to the body and phage utilize the receptors inside the tails to recognize the attachment sites on bacterial surface. However, not all the phage has a tail and the tail may or may not be a contractile structure. In this case, other attachment mechanisms are present.

The review will focus on taxonomy of *Salmonella* bacteriophages available in recent publications (2013-2019). Related information is illustrated in **Table 1.1.**

Table 1.1 Overview of double-strand DNA (dsDNA) *Salmonella* bacteriophages in recent literatures.

Phage	Family	Morphology		Reference
		Head diameter (nm)	**Tail length (nm)**	
wkls3	*Siphoviridae*[a]	63	121±7.9	Kang et al., 2013
Φ st1		67.43 ± 1.73	172.69 ± 30.03	Wong et al., 2014
fmb-p1		57.2 ± 2.0	171.2 ± 9.0	Wang et al., 2017
SLMP1		62	110	Xu et al., 2018
LPST10		83.26 ± 7.12	144.89	Huang et al., 2018
vB_SenS_CSP01		63.5±1.6	154.9±3.2	Chen et al., 2018
vB_SenS_PHB06		57.4±2.8	154.4±1.4	
vB_SenS_PHB07		55.4±0.9	146.3±1.7	
CGG3-1		76.03 ± 1	211.54 ± 2	El-Dougdoug et al., 2019
CGG3-2		66.47 ± 1	203.44 ± 2	
PM10	*Ackermannviridae*[b]	94 ± 4.0	106 ± 7.0	Newase et al., 2018
SE07	*Podoviridae*[c]	58.04 ± 1.23	11 ± 0.53	Thung et al., 2017
STP4-a	*Myoviridae*[d]	78.3 ± 4.9	112.7 ± 4.2	Li et al., 2015
PA13076		66	90	Bao et al., 2015
PC2184		65	106	
ST02		71.68 ± 1.53	181.42 ± 10.23	Thung et al., 2019
CGG4-1		104.07 ± 0.5	110.21 ± 3	El-Dougdoug et al., 2019
CGG4-2		88.02 ± 2	94.44 ± 2	

[a] *Siphoviridae*: icosohedral head; long, non-contractile tail.
[b] *Ackermannviridae*: icosohedral head; long, contractile tail.
[c] *Podoviridae*: icosohedral head; short, non-contractile tail.
[d] *Myoviridae*: icosohedral head; long, contractile tail.

The majority of known *Salmonella* phages belong to three family: *Siphoviridae*, *Podoviridae* and *Moviridae*. Those virions contain double-strand DNA (dsDNA) and have icosahedral or elongated heads without envelope. They also have long or short tails with fixation structure such as spikes, fibers and baseplates. Among the three families, Siphoviruses are the most numerous of tailed phages, accounting for 61 % out of 3,200 tailed phages (Ackermann, 2009).

Ackermannviridae is a new bacteriophage family created in 2017 which contains two new subfamilies including four genera. The morphology of phages within this family is very similar to Myoviruses and it used to be considered as a new genus of Myoviruses, which was accepted by the International Committee on Taxonomy of Viruses (ICTV) and renamed Vilvirus. After detailed BLAST reassessment of this genus and its related phages, this genus was replaced by two subfamilies and classified into a new family '*Ackermannviridae*' within the order *Caudoviridae* (Kropinski et al, 2017).

1.4.4 Phage infection processes and life cycles

Bacteriophages exhibit one or two types of life cycles, lytic and lysogenic. Phages only use the lytic cycle are called virulent phages, during the infection, virulent phage particles rapidly invade host and lyse bacterial cell in a short time, whereas temperate phages (phages use both lytic and lysogenic cycles for replication) integrate their genomic information into host genome and maintain 'silence' state inside the host. In the lysogenic cycle, propagated bacterial cell inherits the viral DNA. For the purpose of phage application in foods, this thesis will mainly focus on virulent phages.

Phage and bacterial cell encounter each other during random motion; adsorption process begins after phage attach to some highly specific binding sites on the surface of bacterial host.

The types of bacterial receptor sites depend on host and vary from phage to phage as well. Attachment sites include lipopolysaccharide in Gram-negative bacteria, peptidoglycan and teichoic acid in Gram-positive bacteria. Some protein receptors are essential in nutrients transportation. In other cases, receptors would be present on bacterial flagella and conjugative pili. The attachment is initially reversible and eventually phage genomic material is ready to be transferred to host and association between bacteria and phage becomes irreversible. The densities of both bacteria and phage should be considered carefully during adsorption process. When phage concentration is lower or slightly higher than bacteria, the chance for multiple phages attach to a single bacterium could be ignored. If the multiplicity of phage is extremely high, a large number of phage particles compete for limited receptor sites on a single bacterium, causing cell rupturing almost immediately. This phenomenon is termed as lysis-from-without and in this case, phage infection becomes nonproductive since phages are not able to infect more host cells (Lenski, 1988).

After attachment, phage injects its genome into the cytoplasm usually depends on the morphological characteristics such as the contraction of sheath and tail. The viral genome is subsequent transcribed by host cell RNA polymerase without degraded, producing early mRNA to take over bacterial metabolic systems. Eventually, the bacterium is converted into a factory for production of phage components and these components are then assembled into complete phage particles (Hanlon, 2007).

In order to disperse its progeny to find new target, phage has to escape from host cell and overcome peptidoglycan followed by construction and assembly of new particles. All dsDNA phages are capable of producing endolysin, which requires a second lysis factor to be activated to lyse cell wall. The second enzyme needs to be present here is holin. It disrupts the cytoplasmic

membrane and allows endolysin to reach its substrate. In this way, holin controls the timing of cell lysis (Young et al., 2000). A typical phage lytic life cycle is presented in **Figure 1.3** (Bertani, 1973).

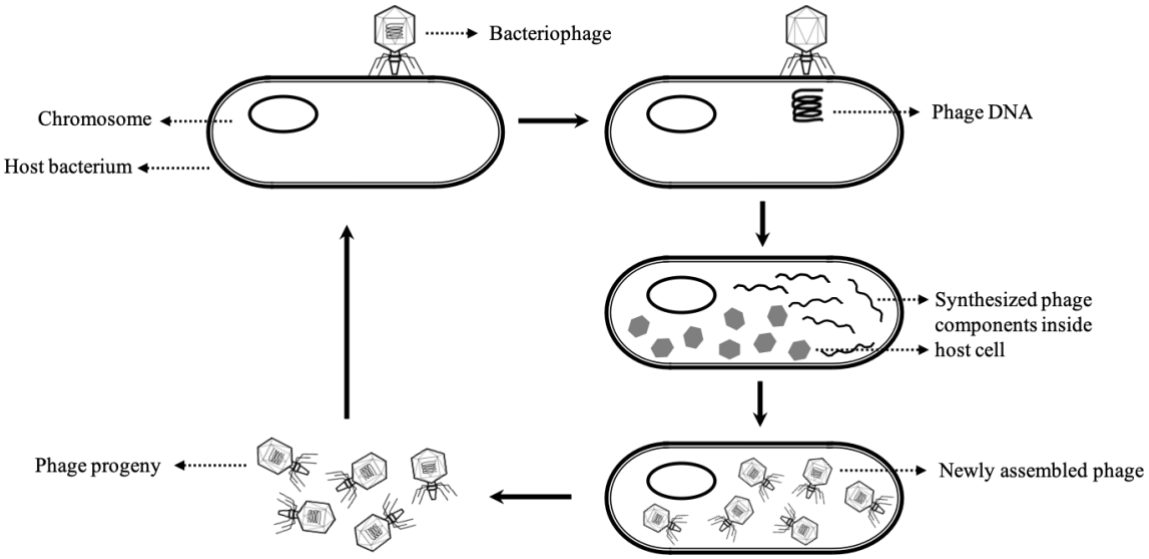


Figure 1.3 Schematic diagram of phage lytic life cycle (Bertani, 1973).

Ellis and Delbruck (1939) were first to describe these parameters by phage one-step growth curve (**Figure 1.4**). During latent period, the concentration of phage plaque-forming units should remain unchanged due to the intracellular dynamics of phage growth before the first cell lysis begins. Subsequently, an increasing phage titer could be observed, and all host cells will eventually burst. Phage titer gradually remains constant due to the 'dilution' of bacterial cells has stopped further phage adsorption and infection (Lenski, 1988), According to the definition, latent period is the duration extending from phage adsorption to the lysis of the first bacterial cell, and the number of phage progeny released upon lysis is referred as burst size.

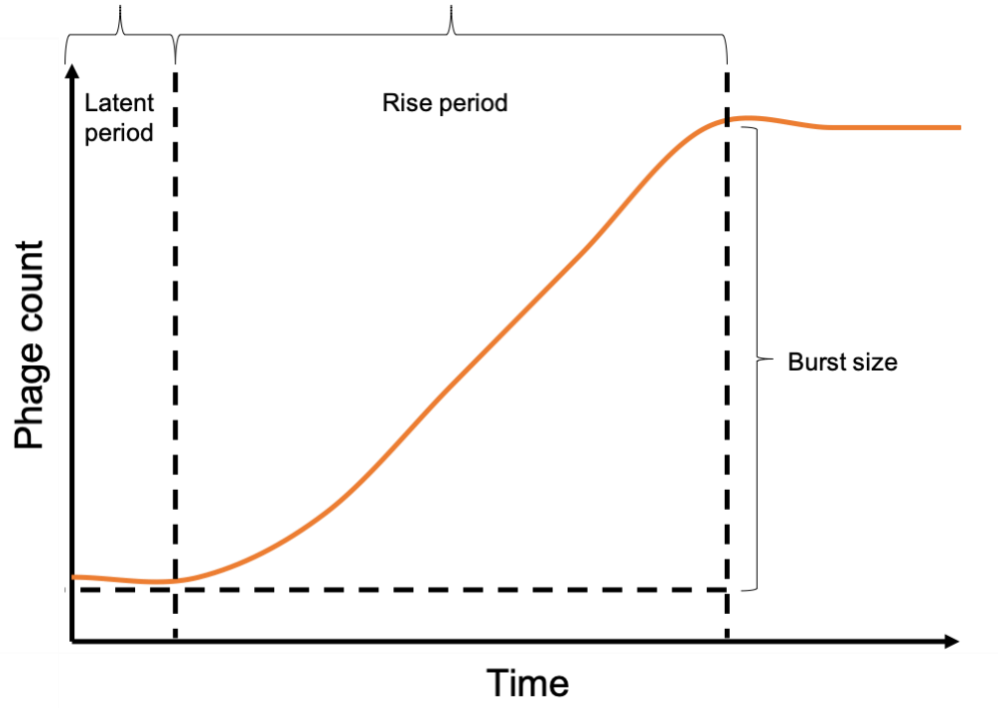


Figure 1.4 The one-step growth curve for a bacteriophage (Lenski, 1988).

1.5 Biocontrol of *Salmonella* in foods using bacteriophages

Foodborne illnesses caused by various pathogens such as *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and others have been a great concern to the food industries. CDC estimated that 1 out of 6 Americans get sick every year because of contaminated foods or beverages, whereas a report released by USDA in 2014 showed that the yearly cost of foodborne diseases in the U.S. reaches $ 15.6 billion (CDC, 2018).

Some pathogens are frequently transmitted by infected persons when they are handling food products. People with signs and symptoms such as diarrhea, vomiting, fever, dark urine or jaundice are possible carryiers of contaminates. On the other hand, the failure of food-handlers to

properly wash or sanitize their hands, wear clean outfits or use of clean and snitized utensils is one of the reasons for the transmission of foodborne pathogens. Intrinsically contaminated or cross-contaminated foods during production, processing, transportation or storage is another route for foodborne diseases transmission. Those contaminating bacteria have to multiply in food and reach an infectious dose to cause diseases (CDC, 2017). Over the last few decades, many strategies have been explored to minimize the microbial load of raw products. Physical treatments such as dry heat, UV light and steam not only affect food organoleptic properties, but also cannot be applied on fresh produce and ready-to-eat products. The other major food decontamination strategy is to use chemical sanitizers. However, low consumers acceptability of food chemical preservatives and increasing problems related to the development of bacterial resistance are the two major concerns in food industries. Furthermore, a common shortcoming shared by all of these techniques is that both pathogens and potentially advantageous normal microbiota are killed indiscriminately. All these issues lead to the urgent requirements of novel decontamination methods which limit the use of chemicals as well as have minimal effects on various food types, especially fresh produce. One of the emerging techniques is to utilize lytic bacteriophages as natural antimicrobials to target specific foodborne pathogen in foods. Therefore, “bacteriophage biocontrol” has been proposed to term this approach.

Phage-based biocontrol has been accepted in the food industry as a green technology to enhance microbiologically food safety. The majority of existing commercial phage products contain natural phages which are not genetically modified, instead, isolated directly from environment. A 2015-report stated that phage treatment alone without considering it as a part of hurdle approaches will only cost 1-4 cents per pound of food treated, whereas physical treatments such as irradiation typically cost 10-30 cents per pound (Viator et al., 2015).

Bacteriophages are usable at all stages of the food supply chain in the classic "farm to fork" concept. This includes preventing pathogens colonization and illnesses in livestock, decontaminating raw products such as meat and fresh vegetables, sanitizing food contact surfaces or equipment, and serving as natural preservatives in order to extend product shelf life (**Figure 1.5**). Many studies have been conducted at both pre- and postharvest stages of production by using phage or phage derivatives to control food pathogens and spoilage organisms, and, achieving promising results (Abuladze et al., 2008; Lone et al., 2015). In this review, studies of bacteriophages biocontrol which was used to combat *Salmonella* through food production process is discussed and its limitations and drawbacks are addressed.

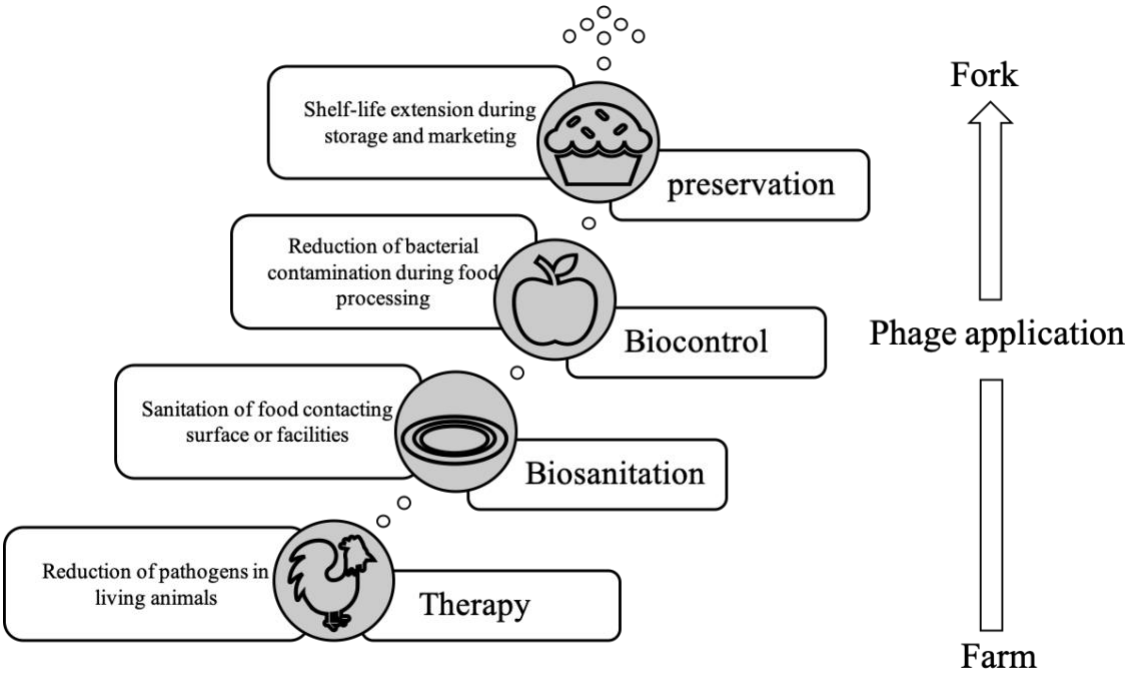


Figure 1.5 Phage applications along the food production processes (Moye et al., 2018).

1.5.1 Control of *Salmonella enterica* in primary production

Controlling pathogens on farm has become increasingly difficult due to the antibiotics overuse. Many researchers have evaluated the potential benefits of bacteriophages to control *Salmonella* in food-producing animals on the farm. As poultry is one of the major reservoirs of *Salmonella*, plenty of works have been published on the applications of phage to control *Salmonella* contamination in poultry. Atterbury et al. (2007) assessed three lytic bacteriophages exhibiting broad host ranges against *Salmonella* Enteritidis, Hadar and Typhimurium. All three of them were able to reduce the numbers of their respective *Salmonella* hosts with MOI of 10^0, 10^3, and 10^6 in nutrient broth. However, when phages were administrated at MOI of 10^6 by oral gavage, after six days treatment, no significant reductions in the cecal carriage of *Salmonella* in the broiler chickens could be observed. The researchers assumed that the viscosity of the gut matrix, complicated intestinal environments and host defense may have affected the ability of phages to locate a suitable host. A second experiment was conducted in order to assess the efficiency of phage treatments at a higher MOI (10^8). As a result, phage Φ151 caused 4.2 $\log_{10}$ CFU reduction in *Salmonella* Enteritidis population, whereas *Salmonella* Typhimurium population was reduced by 2.9 $\log_{10}$ CFU with phage Φ10. However, no significant reduction in *Salmonella* Hadar population was recorded even with higher MOI. This study suggested that cecal colonization of *Salmonella* Enteritidis and Typhimurium in commercial broiler chickens can be effectively reduced by certain phage treatments. Nabil et al. (2018) found that phage treatment is efficacious in reducing *S.* Typhimurium and *S.* Enteritidis colonization in the cecum of broiler chicken. In their study, chickens were administrated orally with high titer (10^{12} PFU/ml) phage suspension at 5 different days. Using quantitative real time PCR (RT-PCR) to determine *Salmonella* loads in each sample, they found that bacterial loads decreased after four

times of phage treatments and they were able to detect bacterial host after the fifth dose. In other words, *Salmonella* were successfully cleared from infected chickens within a short period. Those findings further confirmed the feasibility of using bacteriophages as antibiotics replacement to treat chickens on farms.

Bardina et al. (2012) evaluated the efficacy of a three-phage cocktail (UAB_Phi20, UAB_Phi78, and UAB_Phi87) in infected chicken models. They observed 4.4 $\log_{10}$ CFU and 3.2 $\log_{10}$ CFU reduction in *Salmonella* Typhimurium in chicken cecum respectively by days 2 and 6 post-infection. However, they also found that phage titer decreased dramatically if only two doses of phage cocktail were administrated, and treatment became less effective. On contrary, frequent treatment of the chicken with bacteriophage on scheduled dates effectively maintain phage concentration at around 10^4 to 10^5 PFU/ml throughout the whole experiment. Their results indicated the effectiveness of phage cocktail, also proposed current challenge of phage application is to further minimize the number of required doses. To conclude, all those studies demonstrated the potential of phage or phage cocktail for the biocontrol of *Salmonella* in primary production settings.

1.5.2 Postharvest control of *Salmonella*

Phage biocontrol is usually more effective at the postharvest stage of the food supply chain than in living animals with complex ever-changing microenviroments. Therefore, direct food application of phage biocntrol has been demonstrated by many studies and yielded promising results. Such foods include meat, fresh fruit and vegetables, ready-to-eat food, and pasteurized milk.

The value of phage biocontrol for post-harvest food applications was assessed by Leverentz et al. (2001). The authors found that approximately 3.5 $\log_{10}$ CFU reduction in

Salmonella Enteritidis population was achieved on honeydew melon slices at 5 and 10 °C by using a phage mixture. Moreover, with the storage temperature increased to 20 °C, 2.5 $\log_{10}$ CFU reduction could still be observed. However, phage was not effective on apple slices with low pH. Similar studies have been done on other fresh produce. Huang et al. (2018) applied single phage LPST10 treatment on lettuce with MOI from 1 to 10. At MOI of 1, *Salmonella* Typhimurium counts on lettuce decreased 0.7 to 1.7 $\log_{10}$ CFU/cm^2 during 3 to 5 hours following phage treatment. When the MOI was 100, bacterial counts decreased from 1.9 to 2.7 $\log_{10}$ CFU/cm^2. These results are consistent with previous reports that phage tend to be effective in a short period of time with a higher MOI (Andreatti Filho et al., 2007, Lopez-Cuevas et al., 2011). El-Dougdoug et al., (2019) observed that a phage cocktail composed of 4 isolates (vB_SnwM_CGG4-1, vB_SnwM_CGG4-2, vB_SnwM_CGG3-1, vB_SnwM_CGG3-2) reduced 4.5 $\log_{10}$ CFU *S.* Newport population on cherry potatoes at 22 °C for 3 days. The researchers investigated the genome of phage B_SnwM_CGG4-1 which revealed no homology to virulence or lysogenic genes. These findings confirmed the safety of their phage isolate at the genomic level and suggested the phage potential use to mitigate *Salmonella* risk on ready-to-eat produce.

Researches on phage biocontrol in the area of meat includes experiments by Kang et al. (2013), where phage wksl3 suspension was sprayed on chicken skins. A single-dose application resulted in 3.0 $\log_{10}$ CFU reduction in viable *Salmonella* Enteritidis after 24 h incubation at 8 °C, yet growth of bacteria resumed after 2 days incubation. Nonetheless, no significant growth was observed from day 2 to day 7 and *Salmonella* counts was successfully reduced below their detection limit. The investigators conducted oral toxicity studies by feeding 8 weeks male mice with phage stock solutions. Experimental mice were weighed before and after 1-week period, any abnormal behavior and toxicological effects were recorded as well within the first 6 h after

the test solution was administrated. Complete gross pathological examination showed no clear harmful effects. This encouraging work demonstrated the optimal application conditions for phage biocontrol on artificially contaminated chicken skin. Similar study conducted by Thung et al. (2017) which demonstrated the effectiveness of phage SE07 on beef and chicken meat in which a high titer phage suspension (10^{12} PFU/ml) was sprayed on the entire surface of the *S.* Enteritidis contaminated meat samples to obtain MOI of 10^7, followed by incubation at 4 °C up to 48 h. Within 48 h, bacterial population was reduced by 2.1 and 2.0 $\log_{10}$ CFU on phage-treated beef and chicken meat samples, respectively. The authors further applied a single phage strain ST02 on beef and chicken meat to inhibit *S.* Typhimurium and achieved approximately 2.0 and 2.3 $\log_{10}$ CFU reduction, respectively (Thung et al., 2019).

Viability of *Salmonella* after phage treatment in fruit juice was reported by the same authors. Phage was directly added to juices to obtain MOI of 10^5. Phage SE07 successfully reduced *S.* Enteritidis population by 2 $\log_{10}$ CFU/ml. Similarly, phage ST02 resulted in 2.2 $\log_{10}$ CFU/ml reduction in *S.* Typhimurium in kiwi juice. Since pH plays an important role in phage stability and fruit juice usually has low pH values, Huang et al. (2018) assessed efficacy of phage LPST10 and LPSE1 against S*almonella* Typhimurium strains in milk, where pH is approximately neutral. Phage efficiency was assessed at both 4 °C, lower than average refrigeration temperature, and 28 °C, average room temperature. A remarkable decrease in bacterial count was observed in milk following phage LPST10 application at MOI of 1 and 100. Especially, 4.1 $\log_{10}$ CFU/ml reduction was achieved at MOI of 100 and at 28°C. At the same time, phage titer increased significantly after 6 h treatment. However, when phage LPSE1 was applied at 4 °C, no appreciable change in *Salmonella* counts could be observed. Even at 28 °C, reduction in bacterial population resulted from phage treatment was only 2.37 $\log_{10}$ CFU/ml at

MOI of 100, which suggested that phage LPSE1 may be less effective than phage LPST10 under the same conditions in the same food matrices. On the other hand, at lower temperature, growth of bacterial host in milk was hindered, whereas replication of phage LPSE1 completely depends on multiplication of its host.

The potential of phage biocontrol has been investigated on seafood. In 2012 and 2019, two outbreaks were reported by CDC respectively due to tuna sushi consumption in the U.S., and the consumption of smoked salmon resulted in salmonellosis outbreaks in Netherland in 2012 (Friesema et al., 2012). Seafood is a common *Salmonella* vehicle since contaminants may become concentrated in tissues by filter feeding or transferred from environments. It was reported that the application of a phage cocktail against *S.* Enteritidis on salmon result in a largest reduction of 3.2 $\log_{10}$ CFU/g at 18 °C and 2.8 $\log_{10}$ CFU/g at 4 °C in raw salmon, compared to 2.0 $\log_{10}$ CFU/g at 18 °C and 1.2 $\log_{10}$ CFU/g at 4 °C in smoked salmon (Galarce et al., 2014). Raw salmon has higher water content compared to smoked salmon, which favors phage mobilization to achieve greater *Salmonella* reduction. Regardless the effect of different textures, temperature could also be a major factor. Cooling temperature limit not only the growth of bacterial host, but also some bacterial enzymatic machineries required for phage replication (Wu, 2008). Xu et al. (2018) analyzed the effectiveness of phage as a biocontrol agent in raw salmon and scallop adductors as a function of host inoculum level, phage dose, incubation time and temperature. The authors chose 10^2 and 10^4 CFU/g of *Salmonella* Typhimuriun for food inoculation, representing low and high inoculum level. Phage suspension was added accordingly to yield a final dose of 10^8, 10^7, 10^6, 10^5, and 10^4 PFU/g in food matrices. Reduction of *Salmonella* count was highly related to the phage concentration. At the inoculum level of 10^2 CFU/g, *Salmonella* was undetectable when treated with phage at a dose of 10^8 PFU/ml. With the

same phage concentration, *Salmonella* counts were reduced by 1.5 to 2.5 log CFU/g at the host inoculum level of 10^4 CFU/g. In terms of different incubation temperature and time, food samples were stored at 4 °C for 7 days, 15 °C for 4 days and 25 °C for 2 days, respectively. With lower inoculum level (10^2 CFU/g), under all the time and temperature combinations, no bacteria could be detected after up to 8 h phage treatment. Whereas with the increased inoculum level of bacteria, population of *Salmonella* decreased initially, then increased during extended incubation. The higher the temperature, the faster the recovery of *Salmonella*. Overall, significant reduction in bacterial counts was still achieved in each application compared with control groups without any phage treatment. In this study, phage effectiveness difference on raw salmon and scallop adductors could be observed, especially at the inoculum level of 10^2 CFU/g. Bacterial counts were under detection limit in all treatments on raw salmon, however, *Salmonella* population was first decreased below detection limit, subsequently increased after a certain time period. In this case, food type highly influenced phage effectiveness. The hardness and springiness of flesh of scallop adductors are higher than those of raw salmon, which restrict the phage diffusion to the receptors on the bacterial surface.

Based on this literature search, phage application has yielded promising results in various food matrices including meat, fresh produce, seafood and liquid products regardless of different effectiveness level. However, searching deeper into phage applications, we found that phage was rarely used to decontaminate two types of foods: dried foods and eggs. Consistency of dry food matrices would prevent the phage mobilization. Drying process limits or prevents the growth of pathogens, which makes the situation even harder for phage to locate bacteria. A study conducted by Heyse et al. (2015) investigated phage potential application in dried pet food. Pet food was first inoculated with a mixture of equal proportions of several overnight *Salmonella*

cultures (Enteritidis, Montevideo, Senftenberg and Typhimurium) with a concentration of approximately 10^6 CFU/ml. After mixing the sample to ensure even distribution of *Salmonella*, phage cocktail was surface sprayed onto each sample to attain a final concentration of 10^5, 10^6, 10^7 PFU/g, respectively, while control groups were treated with phosphate-buffered saline (PBS). All samples were incubated at room temperature for 1 hour to allow phage-host interactions; thereafter, *Salmonella* population was quantified by most-probable-number (MPN) assay. Their results showed that at a phage treatment concentration of 10^5, 10^6, and 10^7 PFU/g, significant decreases of 0.8, 1.4 and 2.0 log MPN/g were observed in contrast to control groups treated with PBS. This study suggested that bacteriophage biocontrol is technically feasible to reduce *Salmonella* contamination risk in commercial pet food by simply surface spray. It is obvious that testing phage as a biocontrol agent in dried foods needs to be explored further.

Unlike dried foods, water content is high in egg or egg products. Egg yolk contains approximately 60 % moisture, whereas egg white contains 90 % moisture. However, high viscosity and complex compositions of the eggs may affect phage mobilization and distribution. Recently, phage application in eggs was studied, targeting *Salmonella* serotypes on egg shell or in egg products. Spricigo et al. (2013) determined effectiveness of a phage cocktail composed of three lytic phages (UAB_Phi 20, UAB_Phi78, and UAB_Phi87) in experimentally *Salmonella* contaminated fresh eggs. Shell eggs were sanitized by ethanol to get rid of natural contaminants. Eggs were soaked into bacterial suspension containing 10^7 CFU/ml of *Salmonella* Enteritidis or Typhimurium. Phage cocktail was sprayed on the surface of egg shell to maintain MOI of 10^3 PFU/CFU and the remaining eggs were treated with $MgSO_4$ as control. After incubation at room temperature for 2 h, bacteriophages and bacteria were subsequently recovered by removing the egg contents. Shells were then homogenized with Buffered Peptone Water (BPW), followed by

determination of the concentration of both bacteria and bacteriophage. The results demonstrated that phage cocktail significantly decreased *Salmonella* population by 0.9 $\log_{10}$ CFU/ml and phage concentration remained stable in eggs after the treatment. The authors noticed that the effectiveness of the phage cocktail on *Salmonella* was lower on egg shell that it was on other food matrices such as pig skins, poultry and fresh lettuce. This result may have been caused by the innate characteristics of egg shell. Translocation of *Salmonella* from the surface of the egg shell to its outer and inner membrane influence overall phage effectiveness. Guenther et al. (2011) evaluated a broad host range phage FO1-E2 for reduction of *S.* Typhimurium in few food products including egg yolk. The yolk was artificially contaminated with *S.* Typhimurium to reach a contamination level of 10^3 CFU/ml. After pre-incubation at 8 °C or 15 °C to allow bacteria adapt to the conditions, phage FO1-E2 was added at a concentration of 10^8 PFU/ml of the sample. Viable bacterial counts and phage titers were determined after certain days incubation. The authors found a reduction by at least 2.0 $\log_{10}$ CFU/ml in all tested food except egg yolk. The inhibitory effect of phage treatment in egg yolk we only significant during the first 2 days incubation at 15 °C, whereas *Salmonella* population subsequently reached the same level as control group where no phage was added. High viscosity and presence of compounds interfering with phage adsorption may explain the results observed in yolk. Since egg white and liquid whole egg are less viscous than egg yolk, the effectiveness of phage application may be different in these two food matrices. Hong et al. (2016) tested phage efficacy in liquid whole egg at a phage concentration of 10^8 PFU/ml. Fifteen ml liquid egg sample was inoculated with 10^7 CFU/ml *Salmonella* Typhimurium or Entritidis. After phage treatments, the concentrations of *S.* Typhimurium in liquid eggs were significantly lower than those in non-phage treated samples at room temperature. Especially after 24 h, viable bacterial counts were reduced by 3.2 $\log_{10}$

CFU/ml. However, *Salmonella* population recovered gradually after two days. On the other hand, no differences in *Salmonella* counts were observed between phage-treated and un-treated liquid egg samples at 4 °C incubation for 2 weeks. In conclusion, with the emergence of new phage applications in food, more attention is needed to mimic the real conditions encountered in food manufacturing processes in order to better utilize this novel tool for the biocontrol of foodborne pathogens. In addition, more studies should be done in unique food matrices such as shell egg and egg products.

1.6 Commercial *Salmonella* phage products and their applications

Considering the increasing interests in phage applications studied for improving food safety, corresponding regulatory approvals are needed for commercial bacteriophage preparations. For example, in 2006, FDA issued the first approval for a phage product, ListShield™ (Mai et al., 2010; Sulakvelidze, 2013), which could be directly used in food supply chain as a food additive. Shortly after, another *Listeria*-specific preparation, Listex™, was recognized as a Generally Recognized as Safe (GRAS) substance by FDA (Mathew, 2016). Moreover, in some USDA issued guidelines for the production of meat, poultry, and egg products, several phage preparations have already been included as safe ingredients (Moye, 2018). Basic information of few commercial *Salmonella* phage preparations is listed in **Table 1.2**.

Table 1.2 Some commercial *Salmonella* phage products.

Company	Phage product	Regulatory	Reference
THESEO (Laval, France)	SalmoFREE®	Not applicable	Clavijo et al., 2019
Intralytix, Inc. (Baltimore, MD, USA)	SalmoFresh™	FDA, GRN 435; USDA, FSIS Directive 7120.1	Sukumaran et al., 2016

Continued

Table 1.2 continued

Intralytix, Inc. (Baltimore, MD, USA)	SalmoLyse®	Designated as pet food safety product	Soffer et al., 2016
Micreos Food Safety (Wageningen, Netherlands)	PhageGuard S™	FDA, GRN 468	Grant et al., 2017

With the emergency of phage products on the market, several studies have reported the effectiveness of those phage products in some food applications. The efficacy of an approved *Salmonella* lytic bacteriophage preparations (SalmoFresh™) was evaluated by Sukumaran et al. (2016). In their study, chicken breast fillets were inoculated with *Salmonella* cocktail at a concentration of 10^3 CFU/ml. All samples were further treated with SalmoFresh™ which was adjusted to 10^9 PFU/ml as either a dip or surface treatment. The dipped-treated samples were stored 4 °C and microbiological analysis was carried out after 2 h and 24 h of storage. On the other hand, surface-treated samples were kept at room temperature (25 ± 2 °C) for 8 h to examine the efficacy of SalmoFresh™ in reducing *Salmonella* when chicken fillet was stored under temperature abused condition. *Salmonella* was significantly reduced by 0.9 $\log_{10}$ CFU/ml after one-day incubation, compared to untreated samples with immersion of chicken breast fillets in bacteriophage solution. At room temperature, surface treatment was the most effective approach to restrict *Salmonella* growth, causing 0.8, 0.9 and 0.4 $\log_{10}$ CFU/g reduction in *Salmonella* counts at 0, 4 and 8 h of incubation, respectively. Despite these results, the authors suggested that phage application may not be the most desirable method for poultry processing since chicken carcasses naturally carry various bacteria other than *Salmonella*, and, cross-contamination of other organisms on chicken prats could happen when all the poultry products are dipped with the same phage solution. Considering similar inhibition effects of *Salmonella* could be achieved by surface treatment with appropriate treatment conditions, it is recommended that surface treatment would be a better option when using commercial phage preparations in

poultry products. Another phage product, PhageGuard S™, was tested by Grant et al. (2017) in ground chicken. A *Salmonella* cocktail (Newport, Typhimurium, Thompson, Heidelberg and Enteritidis) inoculated skinless chicken meat was firstly treated with phage suspension on both sides. After incubating at 4 °C for 30 min to 8 h, samples were grounded using a benchtop grinder. Two different incubation times were chosen, considering that phage-bacteria interaction takes at least 30 min according to the manufacturer of this phage product, and 8 h is the maximum time to be practical in commercial poultry processing. The results showed that greater log reduction was observed after 8 h, which led to 0.9 $\log_{10}$ CFU/cm^2 reduction, at most, in *Salmonella* counts. Clavijo et al. (2019) assessed the relationship between the addition of a commercial phage preparation, SalmoFREE®, into animal feeds, then productivity such as feed conversion, weight gain and homogeneity of a broiler chicken farm were measured. They observed that the phage product (a) did not exhibit any negative effects on the production parameters; (b) caused the reduction in *Salmonella* incidence up to 100 % in phage-treated groups; (c) has the potential to be used at a large scale such as the poultry production system. Despite of the phage preparations for commercial applications to improve the safety of human foods, some preparations are available for pet food applications; one of these is SalmoLyse®. Applications at both low (2-4 × 10^6 PFU/g) and standard (9 × 10^6 PFU/g) concentrations significantly reduced at least 60 % of *Salmonella* contamination in all examined pet food ingredients (Soffer et al., 2016).

1.7 Phage as a part of hurdle technologies in the food industry

As previously described, plenty of evidence in literatures has supported the potential applications of bacteriophage biocontrol for targeting specific pathogenic bacteria in various foods. In most of the studies using phage applications on foods, it was obvious that significant

log reduction in targeted bacteria population can be observed initially, and very little or no further reduction occurs afterwards. This indicates that phage is able to reduce the bacterial contamination level in foods, but bacterial host cannot be eliminated all the time. Phage biocontrol typically reduces targeted bacteria by 1-3 logs, which is considerably lower than log reduction achieved by other, more harsh interventions. For example, a 3-min treatment with slightly acidic electrolyzed water containing 15 mg/l chlorine effectively inactivated the *S.* Enteritidis inoculated on the surface of shell eggs by 6.5 $\log_{10}$ CFU/g (Cao et al., 2008). Although very few, if any, foods are contaminated with more than 10^5 CFU of foodborne pathogens per gram/milliliter, and the targeted bacterium by bcteriophage is only reduced by 1 or 2 logs instead of being eliminated from foods, the treatment would still increase the safety of food.

In view of the relatively low efficiency of phage biocontrol, some studies proposed to include phage biocontrol as a part of hurdle technology to pursue optimal phage efficiency. In other words, combining bacteriophages with some other processing approaches could be a good solution to eliminate or control the growth of pathogens in foods due to the potential synergistic effects. Magnone et al. (2013) investigated the phage cocktail SalmoFresh™, a levulinic acid produce wash, and their combinations against *Salmonella* on broccoli, cantaloupe and strawberries. A 10-g of each food sample was inoculated with host bacteria inoculum by randomly dropping 10 spots on the surface. A portion (200 μl) of a phage preparation was sprayed, yielding approximately a phage concentration of 10^8 PFU/g. After phage treatment, samples were first held at room temperature for phage adsorption, followed by incubated at 10°C for 24 h. On the following day, all samples were immersed in prepared wash solution composed of water, levulinic acid and grapefruit oil terpenes for few seconds for up to 5 min. When the

phage treatment was combined with 5 min washing, *Salmonella* was reduced below detection limit on strawberries, whereas cantaloupe and broccoli still had a population of 3.6 and 4.55 log CFU/g, respectively. Although the growth of bacteria cannot be completely inhibited, the highest efficiency was observed in tested food samples with dual treatments. Hence, the authors concluded that the use of bacteriophage combined with produce wash would mitigate the risk of food pathogens in fresh fruit and vegetables. Sukumaran et al. (2015) combine the same phage preparation, SalmoFresh^{TM}, with lauric arginate (LAE), cetylpyridinium chloride (CPC), or peracetic acid (PAA) in order to reduce *Salmonella* population in chicken breast fillets and chicken skins. Inoculated chicken meat was surface-treated with 0.5 ml of the assigned antimicrobial (LAE or CPC), phage, or their combination. *Salmonella* counts were analyzed after 0, 1, and 7 days storage at 4 °C. Chicken skin was first immersed in 100 ml of selected chemical sanitizers for 20 s and then surface treated with 0.5 ml phage solution. All samples were stored at 4 °C and *Salmonella* counts was determined after 24 h. High concentration of phage treatment alone reduced *Salmonella* by 1.0 – 1.1 $\log_{10}$ CFU/g, and the application of LAE plus CPC alone reduced *Salmonella* by 0.7 – 0.9 $\log_{10}$ CFU/g, compare to the untreated samples. Greater log reduction (1.2 -1.4 $\log_{10}$ CFU/g) was achieved by combination treatment, yet there were no significant differences between combined and individual treatments. Similar results obtained from chicken skins; PAA (peracetic acid) treated samples yielded at most 1.5 -1.7 $\log_{10}$ CFU/g log reduction whereas the sequential application of phage after PAA reduced bacterial population by 2.2 – 2.5 $\log_{10}$ CFU/g. Some chemicals are capable of inactivating phages, thus, in order to achieve the optimal reductions, chemicals and phages need to be applied separately to ensure the phage viability.

Similar study has been done by Wang et al. (2016) where they used a single bacteriophage fmb-p1, nisin, and potassium sorbate (PS) along with their combinations to reduce *Salmonella* on fresh chilled pork. For the combined antimicrobial treatments, phage was applied first, followed by PS and nisin, while control samples were surface treated with distilled water. All the samples were stored at 4 °C for further microbiological, chemical and sensory evaluation. They found that *Salmonella* was undetectable in all phage treatments, whereas treatments with nisin and PS individually and in combination without phage did not yield significant reduction on chilled pork. The combination of the three antimicrobials could significantly reduce *Salmonella* by 2.3 $\log_{10}$ CFU/g at 7th day. Furthermore, combined treatment was able to not only reduce odor but also maintain good sensory of chilled pork, and no adverse effect on the qualities of chilled pork was observed in this study.

In conclusion, the combined treatment of bacteriophage and other chemical sanitizers or antimicrobials could be a potential answer to the challenges facing phage application. Use of phage alone may not eliminate targeted pathogens or reduce its population considerably (>3 log reduction is desirable). Further studies would ideally combine some physical treatments with phage biocontrol to improve the safety of certain food products. In addition, more studies are needed to determine changes of food properties after these combination treatments.

Reference

Abuladze, T., Li, M., Menetrez, M.Y., Dean, T., Senecal, A., Sulakvelidze, A., 2008. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 74, 6230–8. https://doi.org/10.1128/AEM.01465-08

Ackermann H.W. (2009) Phage Classification and Characterization. P127-140. In: Clokie M.R., Kropinski A.M. (eds) Bacteriophages. Methods and protocols, vol 1: Isolatin, Characterization, and Interation. Humana Press. https://doi.org/10.1007/978-1-60327-164-6_13

Andreatti Filho, R.L., Higgins, J.P., Higgins, S.E., Gaona, G., Wolfenden, A.D., Tellez, G., Hargis, B.M., 2007. Ability of bacteriophages isolated from different sources to reduce *Salmonella enterica* serovar Enteritidis *in vitro* and in vivo. Poult. Sci. 86 (9), 1904-1909. https://doi.org/10.1093/ps/86.9.1904

Atterbury, R.J., Van Bergen, M.A.P., Ortiz, F., Lovell, M.A., Harris, J.A., De Boer, A., Wagenaar, J.A., Allen, V.M., Barrow, P.A., 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl. Environ. Microbiol. 73, 4543–4549. https://doi.org/10.1128/AEM.00049-07

Badr, H.M., 2006. Effect of gamma radiation and cold storage on chemical and organoleptic properties and microbiological status of liquid egg white and yolk. Food Chem. 97, 285–293. https://doi.org/10.1016/j.foodchem.2005.05.004

Bao, H., Zhang, P., Zhang, H., Zhou, Y., Zhang, L., Wang, R., 2015. Bio-control of s*almonella* Enteritidis in foods using bacteriophages. Viruses 7, 4836–4853. https://doi.org/10.3390/v7082847

Bardina, C., Spricigo, D.A., Cortés, P., Llagostera, M., 2012. Significance of the bacteriophage treatment schedule in eeducing *Salmonella* colonization of Poultry. Appl. Environ. Microbiol. 78, 6600–6607. https://doi.org/10.1128/aem.01257-12

Bertani, G. 1973. Lysogenic Versus Lytic Cycle Phage Multiplication. Cold Spring Harb Symp Quant Biol 1953. 18: 65-70. https:// doi:10.1101/SQB.1953.018.01.014

Cao, W., Zhu, Z.W., Shi, Z.X., Wang, C.Y., Li, B.M., 2009. Efficiency of slightly acidic electrolyzed water for inactivation of *Salmonella* enteritidis and its contaminated shell eggs. Int. J. Food Microbiol. 130, 88–93. https://doi.org/10.1016/j.ijfoodmicro.2008.12.021

CDC, 2012. *Salmonella* Bareilly and Nchanga Infections Associated with a Raw Scraped Ground Tuna Product - *Salmonella* [WWW Document], n.d. URL https://www.cdc.gov/salmonella/bareilly-04-12/index.html (accessed 7.29.19).

CDC, 2017. Pathogens Transmitted by Food Contaminated by Infected Persons Who Handle Food, and Modes of Transmission of Such Pathogens.

CDC, 2018. Reports of *Salmonella* Outbreak Investigations from 2018. URL https://www.cdc.gov/salmonella/outbreaks-2018.html (accessed 5.18.19).

CDC, 2019. Outbreak of *Salmonella* Infections Linked to Frozen Raw Tuna - Outbreak of *Salmonella* Infections Linked to Frozen Raw Tuna. URL https://www.cdc.gov/salmonella/newport-04-19/index.html (accessed 7.29.19).

Chen, Y., Sun, E., Song, J., Tong, Y., Wu, B., 2018. Three *Salmonella enterica* serovar Enteritidis bacteriophages from the *Siphoviridae* family are promising candidates for phage therapy. Can. J. Microbiol. 64, 865–875. https://doi.org/10.1139/cjm-2017-0740

Clark, S., 2014. Food Processing: Principles and Applications. John Wiley & Sons, Ltd, Hoboken, NJ.

Clavijo, V., Baquero, D., Hernandez, S., Farfan, J.C., Arias, J., Arévalo, A., Donado-Godoy, P., Vives-Flores, M., 2019. Phage cocktail SalmoFREE® reduces Salmonella on a commercial broiler farm. Poult. Sci. https://doi.org/10.3382/ps/pez251

Clokie, M.R.J., Millard, A.D., Letarov, A. V., Heaphy, S., 2011. Phages in nature. Bacteriophage 1, 31–45. https://doi.org/10.4161/bact.1.1.14942

El-Dougdoug, N.K., Cucic, S., Abdelhamid, A.G., Brovko, L., Kropinski, A.M., Griffiths, M.W., Anany, H., 2019. Control of *Salmonella* Newport on cherry tomato using a cocktail of lytic bacteriophages. Int. J. Food Microbiol. 293, 60–71. https://doi.org/10.1016/j.ijfoodmicro.2019.01.003

Ellis E.L, Delbruck M. (1939) The growth of bacteriophage. J Gen Physiol. 22:365–84.

Eng, S.K., Pusparajah, P., Ab Mutalib, N.S., Ser, H.L., Chan, K.G., Lee, L.H., 2015. *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. Front. Life Sci. 8, 284–293. https://doi.org/10.1080/21553769.2015.1051243

European Food Safety Authority. 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16. https://doi.org/10.2903/j.efsa.2018.5500

Friesema, I.H., de Jong, A.E., Fitz James, I.A., Heck, M.E., van den Kerkhof, J.H., Notermans, D.W., van Pelt, W., Hofhuis, A., 2012. Outbreak of *Salmonella* Thompson in the Netherlands since July 2012. Eurosurveillance 17, 1–4.

Galarce, N.E., Bravo, J.L., Robeson, J.P., Borie, C.F., 2015. Bacteriophage cocktail reduces *Salmonella enterica* serovar Enteritidis counts in raw and smoked salmon tissues. Rev. Argent. Microbiol. 46, 333–337. https://doi.org/10.1016/s0325-7541(14)70092-6

Gantois, I., Eeckhaut, V., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2008. A comparative study on the pathogenesis of egg contamination by different serotypes of *Salmonella*. Avian Pathol. 37, 399–406. https://doi.org/10.1080/03079450802216611

Garcia-Gonzalez, L., Geeraerd, A.H., Elst, K., Van Ginneken, L., Van Impe, J.F., Devlieghere, F., 2009. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. J. Supercrit. Fluids 51, 74–82. https://doi.org/10.1016/j.supflu.2009.06.020

Grant, A., Parveen, S., Schwarz, J., Hashem, F., Vimini, B., 2017. Reduction of Salmonella in ground chicken using a bacteriophage. Poult. Sci. 96, 2845–2852. https://doi.org/10.3382/ps/pex062

Gu, L., Zhou, S., Zhu, L., Liang, C., Chen, X., 2015. Small-Molecule inhibitors of the type III secretion system. Molecules 20, 17659–17674. https://doi.org/10.3390/molecules200917659

Guard-Petter, J., 2001. Guard-Petter J: The chicken, the egg and *Salmonella* enteritidis. Environ. Microbiol. 3, 421–430.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol. 154, 66–72. https://doi.org/10.1016/j.ijfoodmicro.2011.12.023

Hanlon, G.W., 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int. J. Antimicrob. Agents 30, 118–128. https://doi.org/10.1016/j.ijantimicag.2007.04.006

Heyse, S., Hanna, L.F., Woolston, J., Sulakvelidze, A., Charbonneau, D., 2015. Bacteriophage Cocktail for Biocontrol of *Salmonella* in Dried Pet Food. J. Food Prot. 78, 97–103. https://doi.org/10.4315/0362-028x.jfp-14-041

Hong, Y., Schmidt, K., Marks, D., Hatter, S., Marshall, A., Albino, L., Ebner, P., 2016. Treatment of *Salmonella* -contaminated eggs and pork with a broad-spectrum, single bacteriophage: assessment of efficacy and resistance development. Foodborne Pathog. Dis. 13, 679–688. https://doi.org/10.1089/fpd.2016.2172

Huang, C., Virk, S.M., Shi, J., Zhou, Y., Willias, S.P., Morsy, M.K., Abdelnabby, H.E., Liu, J., Wang, X., Li, J., 2018. Isolation, characterization, and application of bacteriophage LPSE1 against *Salmonella enterica* in Ready to Eat (RTE) foods. Front. Microbiol. 9, 1–11. https://doi.org/10.3389/fmicb.2018.01046

Humphrey T.J., 1994. Contamination of egg shell and contents with *Salmonella* enteritidis: A review. Int J Food Microbiol 21:31–40.

Kang, H.-W., Kim, J.-W., Jung, T.-S., Woo, G.-J., 2013. wksl3, a new biocontrol agent for *Salmonella enterica* serovars Enteritidis and Typhimurium in foods: characterization, application, sequence analysis, and oral acute toxicity study. Appl. Environ. Microbiol. 79, 1956–1968. https://doi.org/10.1128/aem.02793-12

Kropinski, A.M., Anany, H., Kuhn, J.H., Tolstoy, I., Kutter, E., Adriaenssens, E.M., 2017. To create a new bacteriophage family, *Ackermannviridae*, containing two (2) new subfamilies including four (4) genera. ICTV Rep. 1–12. https://doi.org/10.13140/RG.2.2.29173.88800

Lechevalier, V., Guérin-Dubiard, C., Anton, M., Beaumal, V., David Briand, E., Gillard, A., Le Gouar, Y., Musikaphun, N., Tanguy, G., Pasco, M., Dupont, D., Nau, F., 2017. Pasteurisation of liquid whole egg: Optimal heat treatments in relation to its functional, nutritional and allergenic properties. J. Food Eng. 195, 137–149. https://doi.org/10.1016/j.jfoodeng.2016.10.007

Lenski R.E. (1988) Dynamics of interactions between bacteria and virulent bacteriophage. Adv Microb Ecol. 10:1–44.

Leverentz, B., Conway, W.S., Alavidze, Z., JanisiewicZ, W.J., Fuchs, Y., Camp, M.J., Chighladze, E., Sulakvelidze, A., 2016. Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: A model study. J. Food Prot. 64, 1116–1121. https://doi.org/10.4315/0362-028x-64.8.1116

Li, Meng, Li, Mengzhe, Lin, H., Wang, J., Jin, Y., Han, F., 2016. Characterization of the novel T4-like *Salmonella enterica* bacteriophage STP4-a and its endolysin. Arch. Virol. 161, 377–384. https://doi.org/10.1007/s00705-015-2647-0

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., Griffiths, M.W., 2016. Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. Int. J. Food Microbiol. 217, 49–58. https://doi.org/10.1016/j.ijfoodmicro.2015.10.011

López-Cuevas, O., Castro-del Campo, N., León-Félix, J., González-Robles, A., Chaidez, C., 2011. Characterization of bacteriophages with a lytic effect on various *Salmonella* serotypes and *Escherichia coli* O157:H7. Can. J. Microbiol. 57, 1042–1051. https://doi.org/10.1139/w11-099

Magnone, J.P., Marek, P.J., Sulakvelidze, A., Senecal, A.G., 2013. Additive Approach for Inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* spp. on contaminated fresh fruits and vegetables using bacteriophage cocktail and produce wash. J. Food Prot. 76, 1336–1341. https://doi.org/10.4315/0362-028x.jfp-12-517

Mai, V., Ukhanova, M., Visone, L., Abuladze, T., Sulakvelidze, A., 2010. Bacteriophage administration reduces the concentration of *Listeria monocytogenes* in the gastrointestinal

tract and its translocation to spleen and liver in experimentally infectedmice. Int. J. Microbiol. 2010. https://doi.org/10.1155/2010/624234

Mathew, S., 2016. A need for careful consideration of bacteriophage therapy. Indian J. Med. Res. 144, 482–483. https://doi.org/10.4103/0971-5916.198691

Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., Imai, S., 2005. Bacteriophage therapy: A revitalized therapy against bacterial infectious diseases. J. Infect. Chemother. 11, 211–219. https://doi.org/10.1007/s10156-005-0408-9

Monfort, S., Mañas, P., Condón, S., Raso, J., Álvarez, I., 2012. Physicochemical and functional properties of liquid whole egg treated by the application of Pulsed Electric Fields followed by heat in the presence of triethyl citrate. Food Res. Int. 48, 484–490. https://doi.org/10.1016/j.foodres.2012.04.015

Moye, Z.D., Woolston, J., Sulakvelidze, A., 2018. Bacteriophage applications for food production and processing. Viruses 10, 1–22. https://doi.org/10.3390/v10040205

Müller, A.J., Hoffmann, C., Galle, M., Van Den Broeke, A., Heikenwalder, M., Falter, L., Misselwitz, B., Kremer, M., Beyaert, R., Hardt, W.D., 2009. The *S.* Typhimurium effector sopE induces caspase-1 activation in stromal cells to initiate gut inflammation. Cell Host Microbe 6, 125–136. https://doi.org/10.1016/j.chom.2009.07.007

Nabil, N.M., Tawakol, M.M., Hassan, H.M., 2018. Assessing the impact of bacteriophages in the treatment of *Salmonella* in broiler chickens. Infect. Ecol. Epidemiol. 8. https://doi.org/10.1080/20008686.2018.1539056

Newase, S., Kapadnis, B.P., Shashidhar, R., 2019. Isolation and genome sequence characterization of bacteriophage vB_SalM_PM10, a Cba120virus, concurrently infecting *Salmonella enterica* serovars Typhimurium, Typhi, and Enteritidis. Curr. Microbiol. 76, 86–94. https://doi.org/10.1007/s00284-018-1588-8

Pinto, P., Ribeiro, R., Sousa, L., Verde, S.C., Lima, M.G., Dinis, M., Santana, A., Botelho, M.L., 2004. Sanitation of chicken eggs by ionizing radiation: Functional and nutritional assessment. Radiat. Phys. Chem. 71, 35–38. https://doi.org/10.1016/j.radphyschem.2004.03.065

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.P11101

Serrano, L.E., Murano, E.A., Shenoy, K., Olson, D.G., 1997. D values of *Salmonella* enteritidis isolates and quality attributes of shell eggs and liquid whole eggs treated with irradiation. Poult. Sci. 76, 202–206. https://doi.org/10.1093/ps/76.1.202

Soffer, N., Abuladze, T., Woolston, J., Li, M., Hanna, L.F., Heyse, S., Charbonneau, D., Sulakvelidze, A., 2016. Bacteriophages safely reduce *Salmonella* contamination in pet food and raw pet food ingredients. Bacteriophage 6, e1220347. https://doi.org/10.1080/21597081.2016.1220347

Spricigo, D.A., Bardina, C., Cortés, P., Llagostera, M., 2013. Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry. Int. J. Food Microbiol. 165, 169–174. https://doi.org/10.1016/j.ijfoodmicro.2013.05.009

SPX Process Bulletin: Pasteurized Liquid Egg, 2008. URL http;//www.spx.com (assessed 7.29.19).

Sukumaran, A.T., Nannapaneni, R., Kiess, A., Sharma, C.S., 2015. Reduction of *Salmonella* on chicken meat and chicken skin by combined or sequential application of lytic bacteriophage with chemical antimicrobials. Int. J. Food Microbiol. 207, 8–15. https://doi.org/10.1016/j.ijfoodmicro.2015.04.025

Sukumaran, A.T., Nannapaneni, R., Kiess, A., Sharma, C.S., 2016. Reduction of *Salmonella* on chicken breast fillets stored under aerobic or modified atmosphere packaging by the application of lytic bacteriophage preparation SalmoFresh^{TM}. Poult. Sci. 95, 668–675. https://doi.org/10.3382/ps/pev332

Sulakvelidze, A., 2013. Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. J. Sci. Food Agric. 93, 3137–3146. https://doi.org/10.1002/jsfa.6222

Sutterwala, F.S., Lara-Tejero, M., Flavell, R.A., Bertin, J., Galán, J.E., Ogura, Y., Coyle, A.J., Grant, E.P., 2006. Role of the caspase-1 inflammasome in *Salmonella* typhimurium pathogenesis. J. Exp. Med. 203, 1407–12. https://doi.org/10.1084/jem.20060206

Thung, T.Y., Krishanthi Jayarukshi Kumari Premarathne, J.M., San Chang, W., Loo, Y.Y., Chin, Y.Z., Kuan, C.H., Tan, C.W., Basri, D.F., Jasimah Wan Mohamed Radzi, C.W., Radu, S., 2017. Use of a lytic bacteriophage to control *Salmonella* Enteritidis in retail food. LWT - Food Sci. Technol. 78, 222–225. https://doi.org/10.1016/j.lwt.2016.12.044

Thung, T.Y., Lee, E., Mahyudin, N.A., Anuradha, K., Mazlan, N., Kuan, C.H., Pui, C.F., Ghazali, F.M., Mahmud Ab Rashid, N.K., Rollon, W.D., Tan, C.W., Radu, S., 2019. Evaluation of a lytic bacteriophage for bio-control of *Salmonella* Typhimurium in different food matrices. Lwt 105, 211–214. https://doi.org/10.1016/j.lwt.2019.02.033

Viator, C.L.; Muth, M.K.; Brophy, J.E. Costs ofFood Safety Investments; Report; RTI International: Research Triangle Park, NC, USA, 2015. Retrieved from https://www.fsis.usda.gov/wps/wcm/connect/0cdc568e- f6b1-45dc-88f1-45f343ed0bcd/Food-Safety-Costs.pdf?MOD=AJPERES (accessed 7.29.2019).

Wang, C., Yang, J., Zhu, X., Lu, Y., Xue, Y., Lu, Z., 2017. Effects of *Salmonella* bacteriophage, nisin and potassium sorbate and their combination on safety and shelf life of fresh chilled pork. Food Control 73, 869–877. https://doi.org/10.1016/j.foodcont.2016.09.034

Wittebole, X., De Roock, S., Opal, S.M., 2014. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. Virulence 5, 209–218. https://doi.org/10.4161/viru.25991

Wong, C.L., Sieo, C.C., Tan, W.S., Abdullah, N., Hair-Bejo, M., Abu, J., Ho, Y.W., 2014. Evaluation of a lytic bacteriophage, Φ st1, for biocontrol of *Salmonella enterica* serovar Typhimurium in chickens. Int. J. Food Microbiol. 172, 92–101. https://doi.org/10.1016/j.ijfoodmicro.2013.11.034

Wu, J. (2014). Eggs and Egg Products Processing. In Food Processing (eds S. Clark, S. Jung and B. Lamsal). https://doi.org/10.1002/9781118846315.ch19

Wu, V.C.H., 2008. A review of microbial injury and recovery methods in food. Food Microbiol. 25, 735–744. https://doi.org/10.1016/j.fm.2008.04.011

Xu, D., Jiang, Y., Wang, L., Yao, L., Li, F., Zhai, Y., Zhang, Y., 2018. Biocontrol of *Salmonella* Typhimurium in raw salmon fillets and scallop adductors by using bacteriophage SLMP1. J. Food Prot. 81, 1304–1312. https://doi.org/10.4315/0362-028x.jfp-17-525

Young, R., Wang, I.N., Roof, W.D., 2000. Phages will out: Strategies of host cell lysis. Trends Microbiol. 8, 120–128. https://doi.org/10.1016/S0966-842X(00)01705-4

Chapter 2: isolation and characterization of *Salmonella* bacteriophage from farm environments in Ohio

2.1 Abstract

Bacteriophage biocontrol has been recently accepted as an alternative tool to eliminate foodborne pathogens in various food matrices. In this study, a total of 31 *Salmonella* phages were isolated from chicken houses, and from farms of cattle, swine and goat. Among these isolates, two lytic phages, OSY-STA and OSY-SHC, were highly efficient in infecting their host strain, *Salmonella* Typhimurium ATCC33090 and *Salmonella* Heidelberg, respectively. In particular, phage OSY-STA could infect 20 out of 36 tested *Salmonella* strains, including 15 different serovars. However, the host range determination results suggested phage OSY-STA could not lyse *Salmonella* Enteritidis with high efficiency, whereas phage OSY-SHC lysed all of the tested *Salmonella* Enteritidis strains. Thus, phage OSY-STA and OSY-SHC were selected and characterized for further applications. Both isolates exhibited a distinctive two-phase growth curves in presence of their host bacterium. Phage OSY-SHC presented a slightly longer generation time (70 min) and greater burst size (256 PFU/cell) than these for OSY-STA (65 min, 176 PFU/cell, respectively). Moreover, the two phages remained stable at a pH range of 4 – 12, which covers the pH values of most of foods (pH 3.5 – 7.5). Additionally, phage titers did not change significantly when phage suspension were held at 4, 25, 37 or 55 °C. Transmission electron microscope revealed that phage OSY-STA and OSY-SHC belong to *Siphoviridae* family with icosahedral heads and rigid, non-contractile tails. Incubation of *Salmonella* Typhimurium in TSB for 24 h at 25 °C with a cocktail of these two phages reduced the

population of the pathogen by 4.0 $\log_{10}$ CFU/ml, a value greater than that achieved by the application of individual phages. Additionally, this phage cocktail reduced the population of *Salmonella* Enteritidis by 1.3 $\log_{10}$ CFU/ml under the conditions described above. At 4 °C, the phage cocktail significantly decreased viable population of *Salmonella* Enteritidis and an 0.8-log reduction in bacterial concentration was observed after 4 days (Tukey's Test, $P < 0.05$). These results demonstrated that a cocktail of OSY-STA and OSY-SHC phages is a promising biocontrol agent for controlling *Salmonella* contamination in food.

2.2 Introduction

Salmonella, a genus of gram-negative member of the family *Enterobacteriaceae*, is a major global health concern, causing considerable foodborne illnesses and mortalities worldwide. Currently, more than 2,650 serotypes of *Salmonella* have been identified, in which nontyphoidal *Salmonella* serovars are the principle pathogens involved in foodborne disease outbreaks (Leader et al., 2009). Nontyphoidal salmonellosis refers to illnesses caused by all serotypes of *Salmonella* except for Typhi, Paratyphi A, B, and C (CDC, 2018). The most common clinical presentation of nontyphoidal *Salmonella* infection is acute gastroenteritis. The incubation time is typically 6 – 72 h, while abnormal cases have been documented even 14 days after exposure. Common symptoms of salmonellosis are diarrhea, abdominal pain, fever, and vomiting. These symptoms usually last 4 to 7 days and patients recover from illnesses without special treatments. However, it estimated that 5 % of patients develop severe symptoms such as meningitis or osteomyelitis. Other illness manifestations are associated with some *Salmonella* serotypes. For example, *Salmonella* Dublin and Choleraesuis are more likely to cause invasive infections than other serotypes. According to a report published in 2011, nontyphoidal *Salmonella* was not only the second most problematic pathogen causing majority of cases of

foodborne diseases, but also the leading cause of hospitalization (Scallan et al., 2015). *Salmonell*a can be found in variety of foods. A study conducted in Malaysia identified 31 different *Salmonella* serotypes from buffalo, beef, poultry and pork meat. Moreover, the two most dominant serotypes were *S.* Typhimurium (12.7 %) and *S*. Enteritids (12.5 %) (Roseliza et al., 2011).

Various antimicrobial agents have been widely accepted to treat salmonellosis in food animals. However, using antimicrobial agents at a large scale without proper restrictions has greatly promoted the emergence of antimicrobial resistant microbes. In one study, 145 out of 309 *Salmonella* strains isolated from chicken carcasses during poultry processing harbored the gene *blaCMY*, which implied that penicillin and ceftiofur class of antimicrobials would be ineffective against these strains (Grant et al., 2016). Furthermore, tetracycline-resistant *Salmonella* was found in fresh produce such as lettuce, carrots and bell peppers. Despite using antimicrobials in food primary production, other intervention strategies during post-harvest food processing are generally based on chemical and physical treatments. However, these post-harvest processes may cause food quality deterioration. The increasing appearance of antibiotic-resistant microbes and the need for maintaining food quality prompted investigators for developing safe and effective alternative strategies for controlling foodborne pathogens.

One of the promising approaches for improving food safety is to use bacteriophages as biocontrol agents. Lytic phages are viruses that infect their bacterial hosts and multiply inside bacterial cells without integrating their genomes into bacterial DNA; and the phage progeny bursts from the host cell causing its death. Since phage particles infect bacterial cells with high specificity, they are able to replicate rapidly in the presence of susceptible bacteria and lyse their targets regardless of the antimicrobial resistance profiles of these bacteria (Moye et al., 2018). In

addition, phages are environmentally friendly, economical to use and show no or minimal effects on food properties (Carter et al., 2012). The use of bacteriophages to control foodborne pathogens have been reported in various food products and at different points in processing. These foods include chicken (Thung et al., 2019), duck meat (Wang et al., 2017), raw and cooked beef (Hudson et al., 2013), pasteurized cheese (Modi et al., 2001), chocolate milk (Guenther et al., 2012) and fresh produce including sprouts (Ye et al.,2010), lettuce (Sharma et al., 2009; Carter et al., 2012), and cut cantaloupe (Sharma et al., 2009). The safety of phage biocontrol has been supported by most of the published results and some commercial phage preparations have been granted Generally Recognized as Safe (GRAS) by the FDA.

The performance of a phage is highly dependent on its biological and physiological characteristics. Therefore, the aim of this study was to isolate *Salmonella*-specific lytic bacteriophages from farm environments and to characterize them in terms of host range, multiplication curve, morphology, genome size, and pH and thermal stability. The study also was aimed to determining the effectiveness of most promising bacteriophages and their cocktails against selected *Salmonella* serotypes.

2.3 Materials and methods

2.3.1 Sample collecting

A total of nine farms located in Ohio were sampled for *Salmonella* phages between May 2018 and September 2018. Among these, five were poultry farms (two broiler chicken houses, one egg laying farm, one quail farm and one farm with both quail and chicken), two were cattle farms, and two were swine and sheep farms. Samples were collected only once and a total of 68 samples were collected from all the nine farms. Information of sampling farms are listed in **Table 2.1**.

Upon sampling, sample supplies (coolers, gloves, sample bags, sterilized tubes…) were placed in the vehicle or a separated room to avoid contamination. All non-disposable supplies were autoclaved or sanitized with 70 % ethanol when they were taken to the next sample site. Solid sample (soil, feed, feather…) was scooped up with a clean trowel and transferred to a sample bag (> 10 g), same sample was obtained from randomly determined sample sites within the farm. Liquid samples were transferred into a 50-ml centrifuge tube with disposable syringes and filled up at least the 40 ml mark on the tube. Clean non-cotton wipes were used for collecting surface samples, sampling area were larger than 100 cm^2. After sampling, wipes were moistened with 10 ml sterilized Buffered Peptone and sealed in clean sample bags. All samples were placed in an insulated foam box with ice and shipped back to lab within author's vehicle. Sample was mixed thoroughly, and 10 grams (or milliliters) of each sample was prepared for phage amplification.

Table 2.1 Locations, operation types and sampling information of farms.

Farm ID	County	Type	Sampling date	Sample type
A	Wayne	broiler chicken house	6/25/18	bedding material; feeds; drinking water; manure; feather; organ; cage surface; slaughter house rack
B	Franklin	poultry farm	6/29/18	quail feces; quail feeds; quail cage; quail bedding; quail compost; quail drinking water; partridge feeds; partridge cage; partridge bedding; partridge drinking water
C	Licking	egg laying farm	7/20/18	slat; nesting box; drinking water; feather; manure; feeds; ash; soil; fly
D	Franklin	swine farm	8/1/2018	cleaning water; drinking water; waste water; feed; manure; cage surface; scale area; pig body(dead)
E	Franklin	poultry farm	8/1/2018	drinking water-quail; drinking water-chicken; bedding/manure/feather-chicken; feed-quail; feed-chicken; manure-quail; feather-quail; cage surface-quail; cage surface-chicken; drain-quail; ash-chicken

Continued

Table 2.1 continued

F	Franklin	cattle farm	8/8/2018	drinking water-barn1; drinking water-barn; feeding facility-barn1; feeding facility-barn2; hay; manure; gate-barn1
G	Franklin	sheep farm	8/8/2018	drinking water-sheep; hay-goat; manure-goat; manure-sheep; drinking water-goat; gate + feeding facility-sheep; gate + feeding facility-goat
H	Tuscarawas	cattle farm	9/6/18	gate; milking facility; floor; manure; feeds; drinking water
I	Tuscarawas	chicken house	9/6/18	wall; feed

2.3.2 Culture preparation

Salmonella Enteritidis 99-30581-13, *Salmonella* Typhimurium ATCC33090 and *Salmonella* Heidelberg (unspecified strain) were obtained from Dr. Yousef's laboratory master collection (Department of Food Science and Technology, The Ohio State University, OH). Bacterial strains were cultured using Trypticase Soy Broth medium (TSB; Becton, Dickinson and Company, Sparks, MD) unless otherwise mentioned. To prepare fresh culture, a loop-full of bacterial frozen stock was transferred to a fresh Trypticase Soy Agar plate (one Liter of TSB supplemented with 1.5 % agar, VWR Chemicals, Solon, OH) by three phase streaking and incubated at 37 °C for 16 – 18 h. A single colony from this agar plate was transferred to another fresh TSA plate by streaking. One colony form the second TSA plate was transferred into five ml fresh TSB and incubated under the same conditions. The resulting culture was used in subsequent experiments. In particular, five ml of equal volume of three overnight bacteria culture were mixed in order to prepare a bacteria cocktail for bacteriophage screening.

2.3.3 Buffer preparation

To make 1M Tris-Cl stock solution (pH = 7.5), 12.1 g of Tris base (Fisher Scientific, Fairlawn, NJ) was dissolved in 80 ml H_2O. Concentrated hydrochloric acid (HCl; Fisher

Scientific, Fairlawn, NJ) was added until the desired pH was reached. Finally, the volume was adjusted to 100 ml with water and final solution was autoclaved for future use.

Sodium chloride/Magnesium sulfate (SM) buffer was prepared based on cold spring harbor protocols (http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8111.full?text_only=true). Briefly, 2.9 g of NaCl (Fisher Scientific, Belgium) and 1 g of $MgSO_4 \bullet 7H_2O$ ((Fisher Scientific, Belgium) were dissolved in 400 ml of H_2O, then, 25 ml Tris-Cl (1M, pH 7.5) was added and volume was adjusted to 500 ml. SM buffer was autoclaved and stored at room temperature until use.

For preparing 0.1 M ammonium acetate (pH = 7), 7.7 g of solid Ammonium Acetate (Fisher Scientific, Fairlawn, NJ) was dissolved in 1L H_2O and pH was determined by a pH meter (Corning, New York, USA). The buffer solution was autoclaved and stored at refrigeration temperature.

2.3.4 Bacteriophage screening

Salmonella phages were isolated after phage enrichment with a multi-strain *Salmonella* cocktail, following previous publications (Moreno Switt et al., 2016; Andreatti Filho et al., 2007) with some modifications. Ten g or ten ml of farm environmental sample was mixed with 90 ml TSB to obtain the ratio of 1:10, followed by adding 1 ml of overnight cocktail of the three *Salmonella* serotypes (volume ratio: 1:1:1) described in 2.3.2. The mixture was incubated for 24 h in a shaker (New Brunswick Scientific CO., INC, Edison, NJ) set at 37 °C and 120 rpm. Samples after one-day enrichment were then filtered by a three steps filtration process in the following order: filter paper (Whatman Limited, England); 0.45 μm filter (Merck Millipore Ltd, Tullagreen, Carrigtwohill, CO. Cork, Ireland); 0.22 μm filter (Merck Millipore Ltd, Tullagreen, Carrigtwohill, CO. Cork, Ireland). For preparing a single serotype *Salmonella* bacterial lawn,

overnight culture was diluted in 0.9 % saline solution to obtain the final concentration of approximately 10^8 CFU/ml. A portion (300 µl) of the diluted culture was mixed with 4 ml of soft TSA. Soft TSA was prepared in advance by adding agar to dissolved TSB until agar final concentration is 0.75 %. The soft TSA-*Salmonella* suspension mixture was then poured onto a TSA plate, filtrates were applied after soft agar layer was solidified. Ten µl of the final filtrate was spotted onto three host bacterial lawn grown on Trypticase Soy Agar plate and all plates were placed on bench until the filtrates were dried, followed by overnight incubation in a 37°C incubator (Isotemp® Oven Model 630F, Fisher Scientific, Fairlawn, NJ). After incubation, filtrate that yielded a lysis zone on any one of the three bacterial lawns (*S.* Enteritidis, *S.* Typhimurium or *S.* Heidelberg) was considered as positive sample for further isolation and purification.

2.3.5 Bacteriophage isolation, purification and propagation

Phage isolation and purification procedures were done as described in previous publications (Andreatti Filho et al., 2007; Bielke et al., 2007). A sample (100 µl) of any positive filtrate was serially diluted in saline solution (0.9 % NaCl) and appropriate dilutions were mixed with 300 µl aliquots of 1:10 dilutions overnight culture of different *Salmonella* host strains. A filtrate is considered positive if it exhibited lysis reaction on the bacterial lawns of these strains in 2.3.2. The filtrate-bacteria mixture was transferred to a soft TSA tube, prepared as described before. Contents were thoroughly mixed by slightly tilting the tube few times, and the mixture was poured onto TSA plate, followed by incubation at 37 ℃ for 16 – 18 h. The incubated TSA plates with separated plaques were saved for phage isolation. The plaques with unique morphologies were picked up by sterile pipette tips (VWR International, LLC, Radnor, PA) and suspended in 100 µl of saline solution 0.9 % NaCl. Ten-fold serial dilutions (100 µl) of the suspension were mixed with 300 µl host bacteria in a 4 ml soft TSA tube. The plaque

suspension-bacteria mixture was poured plated onto TSA plates and same procedure was repeated for at least three times, until a single plaque morphology was observed on one TSA plate. After final serial passage procedure, collected phage suspension was mixed with bacteria culture and soft agar as previously described and directly pour-plating on a TSA plate to obtain highest concentration of phage particles on a single plate. In some case, more than one high concentration plates are needed if phage titer is low. Incubated plates from each phage suspension after 24 h at 37 ℃ were flooded with 10 ml of SM buffer, followed by incubation at room temperature for at least 3 h with shaking. SM buffer from incubated plates was collected in a sterilized 300 ml centrifuge bottle, chloroform (Fisher Scientific, Fairlawn, NJ) was subsequently added to a final concentration of 0.2 % v/v. Meanwhile, the soft, top layer agar of the same incubated plates were scraped off by disposable loops (FIsherBrand®, Fisher Scientific, Fairlawn, NJ), and, transferred into the centrifuge bottle along with SM buffer. All centrifuge bottles were labeled carefully according to assigned sample IDs and held at room temperature for 40 min with shaking. Next, the mixture was centrifuged at 8,500 × *g* for 10 min (Centra® MP4R; International Equipment Company, Boston, MA) to remove soft agar as well as bacterial cells. Supernatant was pipetted out, followed by passing through a 0.22 μm filter. The resulting transparent, yellowish solution was crude phage lysate. Collected crude lysate was further ultra-centrifuged at 58,000 × *g* for 3 h (Beckman L8-55; Beckman Coulter, Indianapolis, IN) to sediment phage particles. The supernatant was discarded, and phage pellet was resuspended in SM buffer for the second ultra-centrifugation under the same condition to wash the phage pellet. After at least two times of washing, pellet was mixed thoroughly with 5 ml SM buffer to make pure phage stock, which was stored at 4 °C for titer determination.

Phage titer was determined by double-layer agar overlay assay (Kropinski et al.2009). Briefly, pure phage stock was serially diluted in SM buffer, and 100 μl of the appropriate dilution was combined with 300 μl of host bacterial cell suspension (10^8 CFU/ml). The phage-host preparation was mixed in a molten 4-ml soft TSA and poured on a TSA plate. After overnight incubation at 37 ℃, phage plaques were counted to determine phage titer as plaque forming units (PFU/ml).

2.3.6 Host range determination

For each phage isolate, lysis profile was determined using 2 *Escherichia coli* strains and 37 *Salmonella* isolates that represented 28 serotypes (**Table 2.2**).

Table 2.2 *Salmonella enterica* and *Escherichia coli* strains used in host range determination analysis for phage isolates.

Serotype	Strain ID[a]	Source
Salmonella enterica		
Kentucky	unspecified	unspecified
Juviana	7N	orange juice
Weltevreden	4N	tomato
Tenessee	2053H	thyme
Newport	H9113	mango
Montenido	unspecified	unspecified
Typhimurium	ATCC14028	unspecified
Anatm	unspecified	unspecified
Poona	unspecified	unspecified
Muechen	ATCC8388	unspecified
Seftenberg	OSU836	unspecified
4,5,12:i:-	S5-390	human
Agona	S5-667	bovine
Corvallis	R8-092	human
Braenderup	S5-373	human
Weltevreden	R8-798	human

Continued

Table 2.2 continued

Enteritidis	S5-371	human
Heidelberg	S5-455	human
Infantis	S5-506	human
Newport	S5-515	human
Oranienburg	R8-376	bovine
Panama	S5-454	human
Stanley	S5-464	human
Typhimurium	S5-370	human
Virchow	S5-961	human
Saintpaul	S5-369	human
Montevideo	S5-0264	chicken
Kentucky	S5-0276	chicken
Enteritidis	S5-0282	chicken
Seftenberg	S5-0288	chicken
Newport	R8-4315	cloacal swab
Kiambu	R8-8389	colon
unspecified	R8-0257	egg yolk sac
C2/C3	R6-0578	cloacal
Group B	R9-1348	cloacal swab
Typhimurium var 5-	R9-4864	egg yolk sac
Enteritidis	R9-1219	feces
Escherichia coli		
	K12	unspecified
	EDL933	unspecified

Host range determination was performed as described in a previous publication (Snyder et al., 2016). Briefly, overnight host strain culture (10^9) in TSB was diluted in saline solution (0.9 % NaCl) to obtain concentration of 10^8 CFU/ml. A portion (300 µl) of diluted culture was mixed with soft TSA and poured on a fresh TSA plate for the formation of bacterial lawn. Host range of each phage isolate was determined by spotting 10 µl of pure phage suspension (phage concentration has been adjusted to approx.10^5 PFU/ml) on each bacterial lawn. After 16 – 18 h incubation at 37 ℃, all plates were examined for the presence of host inhibition zones. The

degree of phage infection activity was assessed by clearing where the lysate was spotted and characterized as described by Peters et al. (2010). The presence of confluent inhibition zones by a phage isolate exhibiting the strongest activity against the corresponding host was assigned a triple-plus sign (+++); phage isolate yielded semi-confluent inhibition zones was assigned with two plus signs (++) and one plus sign (+) was assigned to phage isolate which only yielded separated plaques on bacterial lawn . Phage isolate did not yield clearing zone on a given bacterial lawn was assigned a minus sign (-). Host range determination was conducted in two independent replicates.

2.3.7 Phage replication kinetics

The One-step growth curve of selected bacteriophages was constructed as previously described with some modifications (Amarillas et al.,2017), *Salmonella* Typhimurium ATCC33090 and *Salmonella* Heidelberg were used as the host strains for phage OSY-STA and phage OSY-SHC, respectively. In brief, *Salmonella* was grown to exponential phase (12 h) in 10 ml TSB. The culture was centrifuged at 8,500 × *g* for 5 min and supernatant was discarded. Cell pellet was resuspended in fresh TSB and OD_{600} was adjusted to 0.5 (*Salmonella* concentration: ~10^8 CFU/ml). Aliquot (10 µl) of pure phage stock (10^8 PFU/ml) was mixed with 1 ml of *Salmonella* cell suspension (10^8 CFU/ml) to a multiplicity of Infection (MOI) of 0.01, and the phage was allowed to be absorbed to host cells for 10 min at room temperature. The mixture was then centrifuged at 7000 × *g* for 3 min in a bench-top centrifuge (Biofuge A; Scientific Instrument Center Inc., Columbus, OH) and the supernatant was discarded. The bacteria-phage pellet was washed twice with fresh TSB and resuspended in 20 ml of TSB, followed by incubation at 37 ℃ with constant shaking. Aliquots of 1 ml were removed every 10 min and immediately centrifuged at 13,000 × *g* for 3 min and then the supernatant was diluted and plated

for phage titration by double-layer agar plate agar overlay method as previously described. All experiments were performed in triplicates. Calculations of burst size of a single phage isolate were performed as below (El-Dougdoug et al.,2019):

$$\text{Burst size } (\frac{\text{PFU}}{\text{ml}}) = \frac{\text{phage titer at the end of the burst cycle} - \text{initial phage titer}}{\text{initial phage titer}}$$

2.3.8 pH stability

To prepare Buffered Peptone Water (BPW; Fisher Scientific, Fairlawn, NJ) at a pH range of 2 -13, pH of BPW was adjusted by either hydrochloric acid (HCl) or sodium hydroxide (NaOH; Fisher Scientific, Fairlawn, NJ).Aliquots (100 μl) of pure phage stock was resuspended in 900 μl of corresponding pH adjusted BPW Buffered Peptone Water (BPW; Fisher Scientific, Fairlawn, NJ) at a pH range of 2 -13, followed by incubation at 37 ℃ for 2 h with shaking. Thereafter, phage suspensions were serially diluted in BPW (pH = 7.2 ± 0.2) and the change of titer under different pH conditions was evaluated by double agar overlay method and represented by plaque forming unit (PFU/ml). pH of BPW was adjusted by either hydrochloric acid (HCl) or sodium hydroxide (NaOH; Fisher Scientific, Fairlawn, NJ) in advance. The pH stability of each phage isolate was calculated as formula below:

$$\text{Phage pH stability } (\%) = \frac{\text{Viable phage titers after treatment}}{\text{Phage titers before treatment}} \times 100\%$$

2.3.9 Thermal stability

To test the stability of selected bacteriophages (OSY-STA and OSY-SHC) held at various temperatures, 100 μl of phage suspension was transferred to the pre-heated microcentrifuge tubes containing 900 μl of BPW, and heat treated at 4, 25, 37 or 55℃ for 30 or 60 min by submerging the phage-containing microcentrifuge tubes in water bath (Thermo Scientific Model 2864, Thermo Fisher Scientific, Marietta, OH). After each treatment, heated-

suspension was immediately cooled on ice for 5 min. Phage titer was determined with double agar overlay method.

2.3.10 Phage morphology

Sample preparation for examining phage morphology was adopted from Ackermann (2009) with some changes. Freshly-prepared pure phage suspension (approx. 10^9 PFU/ml) was ultra-centrifuged at 58,000 × *g* for 1 h to sediment phage particles. The supernatant was discarded and replaced with 0.1 M ammonium acetate solution. Phage suspension was subsequently ultra-centrifuged again under the same conditions and resulting pellet was suspended in 1 ml of SM buffer.

For phage fixation, 1 ml of high-titer phage stock was centrifuged at 10,000 × *g* for 1h at 4°C, and only 50 – 70 µl of supernatant was kept with phage pellet. The phage particles were resuspended using 100 µl fresh 2.5% Glutaraldehyde in SM buffer.

Negative staining method was conducted for observing phage morphology. Briefly, a 200-mesh carbon-coated Formvar covered grid (Electron Microscopy Sciences, Hatfiled, PA) was discharged using the glow discharge instrument (PELCO easiGlow™; Ted Pella, INC., Redding, CA). Approximately 12.5 µl of prepared phage suspension and 1 % Uranyl Acetate (UA) were applied to a staining surface, separately. The dark, shiny side of the grid was inverted onto phage droplet and incubated for 1 min. Before transferring the grid to UA stain droplet, the excess sample solution was blotted, and grid was gently rinsed by distilled water. After UA staining for about 1 min, the excess liquid was drawn off again by blotting with filter paper. The staining grid was further allowed to dry at room temperature and was then inserted into a transmission electron microscopy (TEM; Hitachi H-7500; Hitachi High-Technologies Corporation, Tokyo, Japan) for morphology examination. The TEM images were taken with

SIA-L12C digital camera and analyzed using ImageJ (https://imagej.nih.gov/ij/index.html). Briefly, three complete, individual phage images were randomly picked up and all measurements were taken three times with ImageJ in terms of the scale of the images, average and standard deviation of the three independent measurements were calculated as the final results.

2.3.11 Efficiency of phage and phage cocktail against *Salmonella* in TSB

To examine the efficacy of a single phage and a phage cocktail on the viability of *S.* Enteritidis and *S.* Typhimurium, bacterial challenge test was performed based on previously published methods (Duc et al., 2018; Huang et al., 2018). To prepare a cocktail of OSY-STA and OSY-SHC phages (10^5 PFU/ml), equal volumes were mixed and stored at 4 °C for further experiments.

Efficiency of a single phage isolate against *Salmonella* was evaluated by inoculating equal *volumes* of overnight *S.* Enteritids or *S.* Typhimurium (10^3 CFU/ml) suspended in fresh TSB with a phage suspension (10^5 PFU/ml) to obtain a MOI of 100. For phage cocktail treatment, the single phage suspension was replaced by phage cocktail suspension (10^5 PFU/ml), then, mixed with equal volume of *S.* Enteritids or *S.* Typhimurium culture. The control groups were only bacterial culture added with same volume of BPW (pH = 7.2 ± 0.2). All treatments and controls were mixed thoroughly and subsequent incubated at 4 °C and 25 °C. At 0, 2, 4, 6, 8, 10, and 24 h of incubation, 100 μl of the samples was collected and diluted in BPW. The dilutions were then spread on TSA plates and incubated at 37 °C for 24 h. Samples stored at 4 °C were incubated up to 5 days and Salmonella population was enumerated at each day. The viable counts of *Salmonella* on TSA plates were enumerated after 24 h incubation.

2.3.12 Statistical analysis

All experiments were independently performed in triplicates unless indicated otherwise. The data significant difference between non-phage treated control and phage treated experimental groups were analyzed by one-way analysis of variance (ANOVA) with 95 % confidence interval using (JMP 14, SAS Institute, Inc., Cary, NC). All results were presented as means, and error bars indicated the standard deviation. Statistical differences between the mean values were analyzed using Tukey's test. A value of $P < 0.05$ was considered a statistically significant difference.

2.4 Results

2.4.1 Screening, isolation and purification of bacteriophages from environmental samples

A total of 68 samples were obtained from 9 livestock farms and 31 *Salmonella*-infecting phages that yielded inhibition zones on bacterial host lawns were isolated for further examination (**Table 2.3).**

Table 2.3 Number of positive samples with potential phage candidates and ID of each isolated phage.

Farm ID	Total Sample	Number of positive samples on specific serovar			Isolated phage ID
		Typhimurium	Enteritidis	Heidelberg	
A	8	2/8	2/8	5/8	A-DW-SE; A-Organ-ST; A-Bed-ST
B	9	1/9	2/9	8/9	B-Cage-P-SH; B-Cage-Q-SH
C	9	3/9	4/9	5/9	C-Feather-SH
D	8	1/8	5/8	8/8	D-Cage-SH; D-CW-SH; D-Body-SH; D-WW-SH; D-Scale-SH; D-DW-SH
E	11	1/11	1/11	11/11	E-Feather-Q-SH; E-DW-C-SH; E-Cage-Q-SH; E-Drain-Q-SH; E-Cage-C-SH; E-Scale-SE
F	7	2/7	3/7	7/7	F-Cattle-FFB1-SH; F-Cattle-Gate-SH

Continued

Table 2.3 continued

G	7	1/7	3/7	5/7	G-Sheep-Gate-SH; G-Goat-DW-SH; G-Goat-Gate-SH; G-Goat-Hay-SH
H	6	0/6	6/6	6/6	H-DW-SH; H-Gate-SH; H-MF-SH; H-Floor-SH; H-Manure-SH
I	6	0/6	5/6	6/6	I-Pipe-SH; I-Wall-SH

Among the three *Salmonella* serotypes used for phage isolation on all samples, serovar Heidelberg yielded the most phage isolates, whereas only few isolates were obtained on serovar Enteritidis. Although there was a considerable number of positive samples (103) which were able to exhibit inhibition reaction on any of the three *Salmonella* strains, some positive sample only yielded turbid lysis zones and others were hard to be propagated.

(a) (b) (c)

Figure 2.1 Examples of phage lysis reactions on specific *Salmonella* serovars. Sample types are labeled on the bottom of the Petri dish. Host: (a) *S.* Typhimurium; (b) *S.* Enteritidis; (c) *S.* Heidelberg. All samples in this figure were obtained from A farm. Positive lysis reactions were circled in red.

As shown in **Figure 2.1**, bedding materials, organ and drinking water samples yielded inhibition zones on S. Typhimurium. Organ and drinking water samples also produced phages that lysed *S.* Enteritids albeit weakly. Seven of the 8 samples exhibited positive lysis reaction on S. Heidelberg, yet most of the inhibition zones were unclear. In fact, only three isolates were obtained from farm A: A-DW-SE, A-Organ-ST, A-Bed-ST with a phage titer of 6.0×10^7, 1.3×10^9 and 7.2×10^8 PFU/g, respectively. Other putative phage isolates from farm A yielded low phage titer ($< 10^4$ PFU/g) or showed very weak lysis ability.

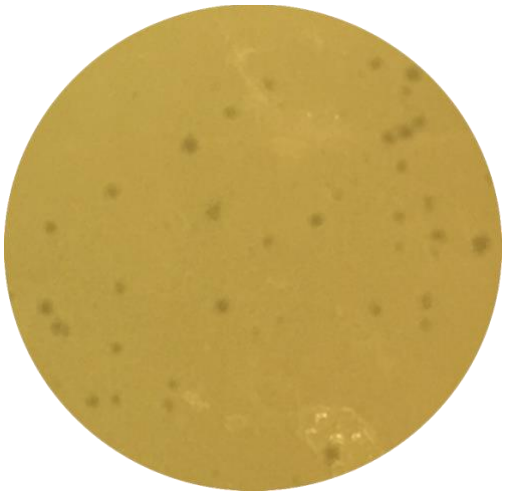


Figure 2.2 An example of phage-forming plaques on TSA.

2.4.2 Host range determination

The results of phage host ranges of all 31 isolates are shown in the **Appendix**. While 3 phage isolates (A-DW-SE, A-Organ-ST and A-Bed-ST) were characterized by a broad host range (defined as lysing between 9-15 different *Salmonella* serovars), the other 28 phage isolates

were characterized by a narrow host range (lysing 1-6 different *Salmonella* serovars). Among the host strains tested, the most sensitive serotypes are Typhimurium, Enteritidis, Heidelberg and 4,5,12:i:-, whereas the most resistant serotypes are Antam, Poona, Muechen, Seftenberg, Agona, Braenderup, infantis, Panama and Kiambu. These serovars were found to be resistant against all phage isolates. Based on their lysis profiles, phage isolates A-Organ-ST (designated as OSY-STA) and C-Feather-SH (designated as OSY-SHC) were selected as the most promising candidates for the following reasons. OSY-STA has the broadest host range, which can infect 15 *Salmonella* serovars; however, this isolate could not lyse *Salmonella* Enteritids strains tested in this study. OSY-SHC has a broad host range and is potentially effective against one of the most problematic serotypes, *Salmonella* Enteritidis; therefore, this isolate was selected as the second phage candidate for further investigation. Detailed host range observations for these two selected phages are shown in **Table 2.4**.

Table 2.4 Host range of phage OSY-STA and OSY-SHC determined against 39 strains of *Salmonella enterica* and *Escherichia coli*.

***Salmonella* strain ID**	**Phage ID**	
	A-Organ-ST (OSY-STA)	**C-Feather-SH (OSY-SHC)**
unspecified (Kentucky)	+++[a]	-[d]
7N	+++	-
4N	++[b]	-
2053H	+++	-
H9113	++	-
unspecified (Montenido)	+++	-
ATCC14028	-	-
unspecified (Anatm)	-	-
unspecified (Poona)	-	-
ATCC8388	-	-
OSU836	-	-

Continued

Table 2.4 continued

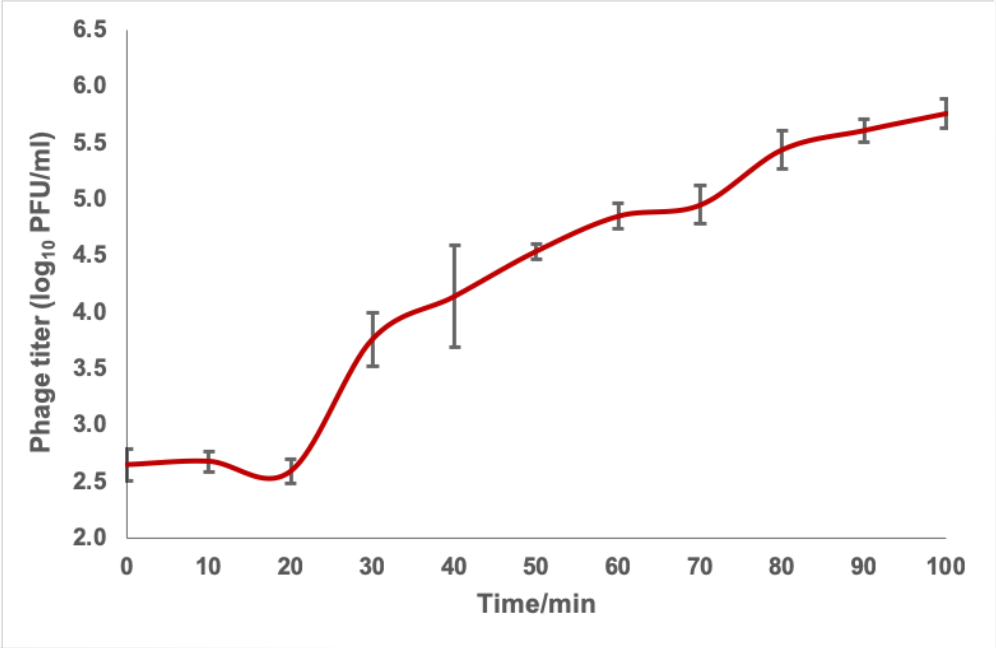
S5-390	+++	+++
S5-667	-	-
R8-092	++	-
S5-373	-	-
R8-798	+++	-
S5-371	-	+
S5-455	++	+++
S5-506	-	-
S5-515	+++	+
R8-376	+++	-
S5-454	-	-
S5-464	-	+[c]
S5-370	-	-
S5-961	+++	-
S5-369	+++	-
S5-0264	-	-
S5-0276	+++	-
S5-0282	-	+++
S5-0288	-	-
R8-4315	++	-
R8-8389	-	-
R8-0257	+	-
R6-0578	+++	-
R9-1348	+++	-
R9-4864	+++	+
R9-1219	-	+++
K12 (*E.coli*)	+++	-
EDL933 (*E.coli*)	-	-

[a] +++: confluent lysis;
[b] ++: semi-confluent lysis;
[c] +: individual plaques;
[d] -: no lysis.

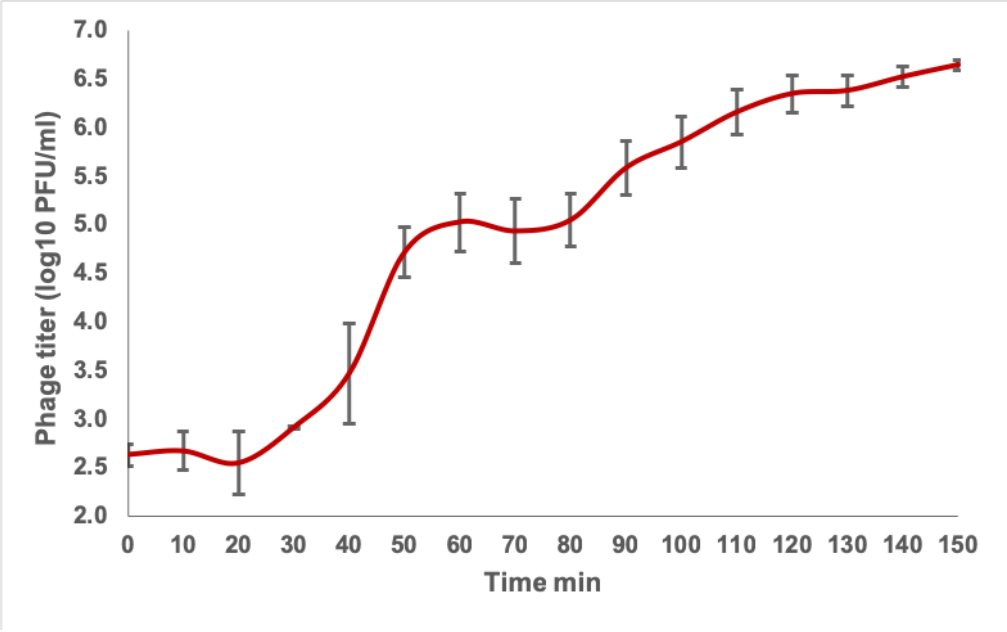
2.4.3 Phage replication kinetics

Burst size and latent periods were determined for phage OSY-STA and OSY-SHC at MOI of 0.01(**Figure 2.3**). In this study, replication curves of phage OSY-STA and SHCI were

determined with *Salmonella* Typhimurium ATCC33090 and *Salmonella* Heidelberg (unspecified strain), respectively.



(a)



(b)

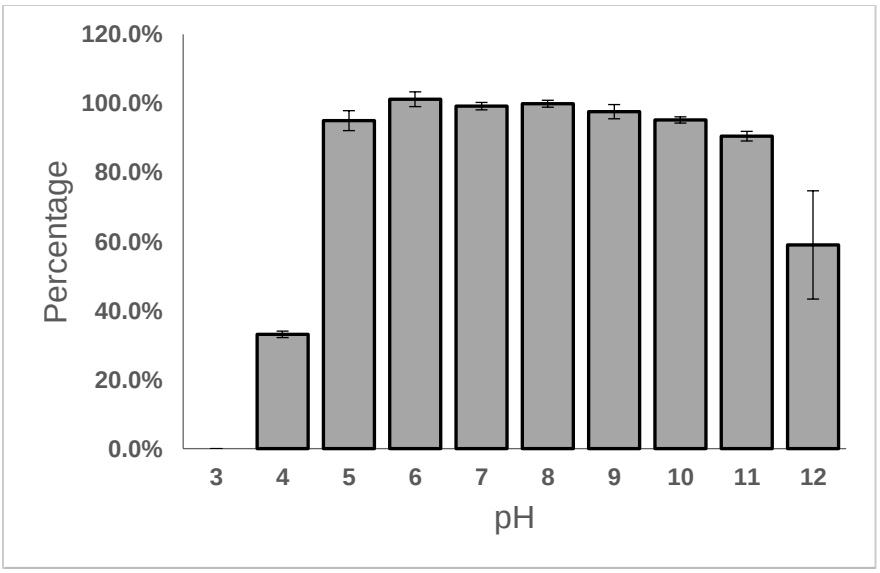
Figure 2.3 Replication curves (changes in phage titer) for two phages determined against selected host bacteria. (a) Phage OSY-STA tested against *Salmonella* Typhimurium LT2; (b) Phage OSY-SHC tested against *Salmonella* Heidelberg (unspecified strain). Experiments were

independently performed in triplicates. Data were reported are means ± standard deviations of three independent trials. Standard deviation of each time point is indicated by error bars.

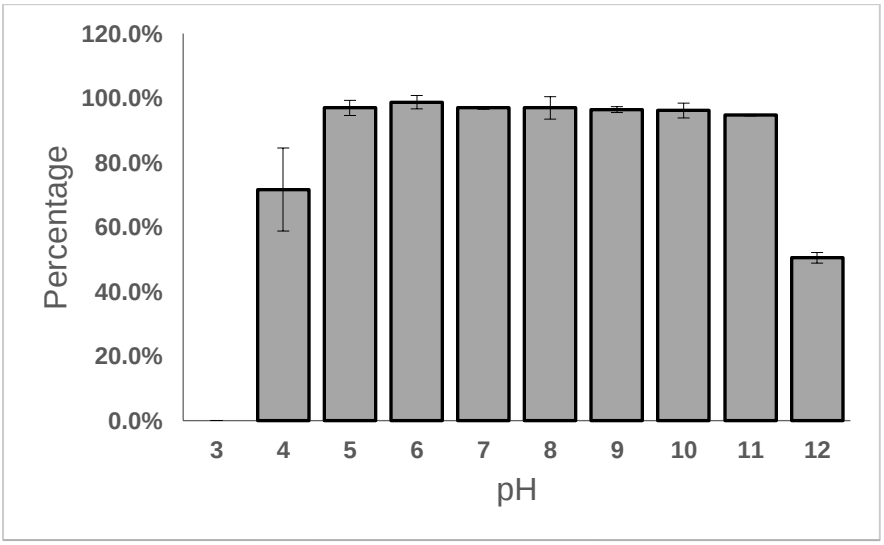
The latent period of phage OSY-STA was approximately 20 min, followed by the generation time of approximately 65 min, while phage OSY-SHC had a same latent period (20 min) and longer generation time (70 min). The burst sizes of OSY-STA and OSY-SHC were 176 PFU/cell and 256 PFU/cell, respectively. Each of the two curves could be divided into two phases and a higher bust size was observed in the first phase. During the first burst, the titer of phage OSY-STA increased from 2.6 to 5.0 $\log_{10}$ PFU/ml and eventually reached 5.8 $\log_{10}$ PFU/ml after 100 min. Similarly, the titer of phage OSY-SHC sharply increased from 2.6 to 4.9 $\log_{10}$ PFU/ml during the first burst phase, whereas after 150 min, phage titer was stable at approximately 6.5 $\log_{10}$ PFU/ml.

2.4.4 pH stability

Phage isolates OSY-STA and OSY-SHC were suspended in BPW of different pH values (range from pH 3 to pH 12) and the ratio between viable phage titers after pH treatments and initial phage titers in neutral BPW was recorded. Results are demonstrated in **Figure 2.4**.



(a)



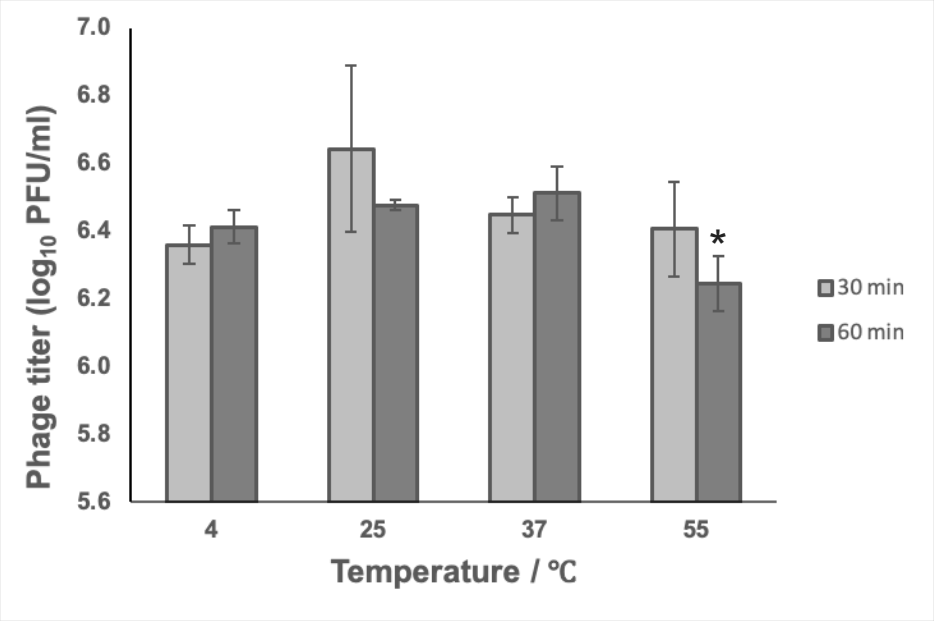
（b）

Figure 2.4 Stability of selected phage isolates at different pH values. (a) Phage OSY-STA tested against *Salmonella* Typhimurium LT2; (b) Phage OSY-SHC tested against *Salmonella* Heidelberg (unspecified strain). Experiments were independently performed in triplicates. Data reported are means ± standard deviations of three independent trials. Standard deviation of each pH point is indicated by error bars.

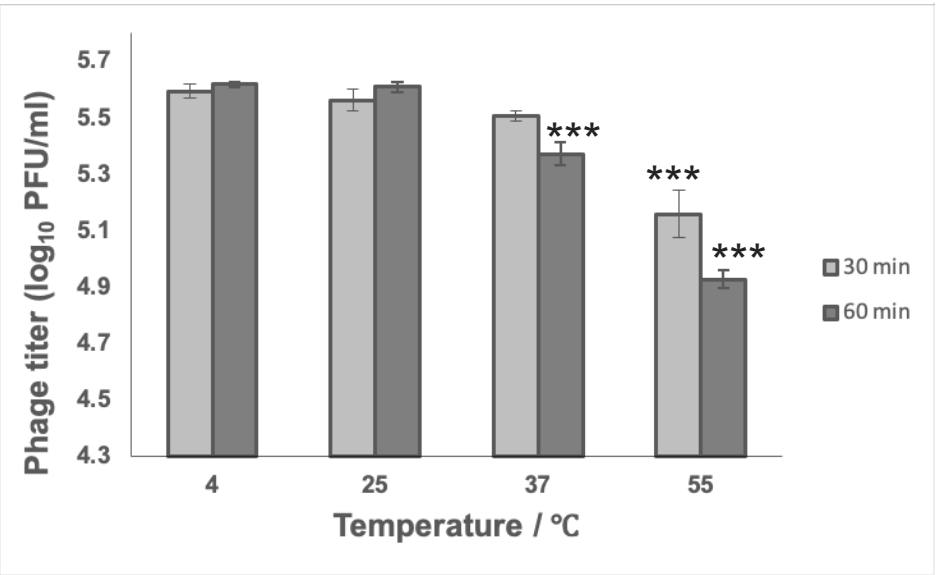
Phage OSY-STA and OSY-SHC were very stable at pH range from 5–11 during 2 h incubation at 37 °C. Within this pH range, over 95 % of titers were recoverable for both phages, except at pH 11, the recoverable titer of phage OSY-STA was 90 % approximately. Titers declined considerably when the phages were exposed to pH values lower than 5 or greater than 11. Phage OSY-STA was less stable than SHC at pH 4, while showing more resistant at pH 12. At extremely acidic environment (pH = 3), no phage was detectable.

2.4.5 Thermal stability

Log reductions in phage titers after 30- or 60-min treatments at 4℃ to 55°C are shown in **Figure 2.5**. Treatment at 4 °C was considered as a control group.



(a)



(b)

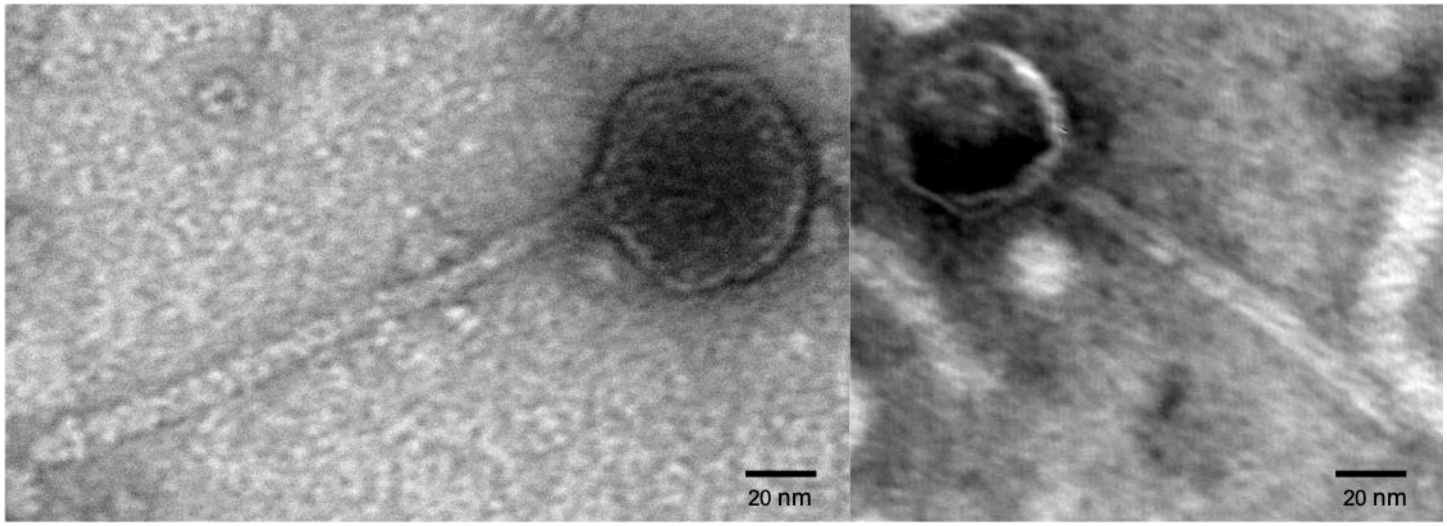
Figure 2.5 Stability of phage isolates upon holding at different temperatures. (a) Phage OSY-STA tested against *Salmonella* Typhimurium LT2; (b) Phage OSY-SHC tested against *Salmonella* Heidelberg (unspecified strain). Experiments were independently performed in triplicates. Data reported are means ± standard deviations of three independent trials. Standard deviation of each temperature point is indicated by error bars.

There was no reduction in the titer of OSY-STA when incubated at 25 °C and 37 °C up to 60 min. No reduction can be observed at 55 °C for 30 min incubation, however, there was a significant reduction in phage titer after 60 min incubation at 55 °C (approx. 0.2 $\log_{10}$ units, $P < 0.05$).

On the other hand, phage OSY- SHC was stable at 25°C and 37°C after 30 min treatment, the greatest decrease was observed at 37 °C, in which phage titer was reduced by 0.2 $\log_{10}$ PFU/ml (not significant, $P > 0.5$) compare to phage titer at 4 °C. At 55 °C, phage titer was significantly reduced by approximately 0.4 ($P < 0.001$) and 0.7($P < 0.001$) log after 30 and min and 60 min, respectively.

2.4.6 Phage morphology

When examined by Transmission Electron Microscope (TEM), both OSY-STA and OSY-SHC phages exhibited icosahedral heads and long, non-contractile tails (**Figures 2.6**). Phage OSY-STA had an isomeric head of 62.5 ± 4.3 nm in diameter and a rigid tail of 177.2 ± 7.0 nm in length and around 8.7 ± 1.8 nm in width (**Figure 2.6 a**). Phage OSY-SHC was smaller in size with a head of 41.84 ± 1.2 nm in diameter, a tail of 130.1 ± 5.3 nm in length and 10.2 ± 2.0 nm in width (**Figure 2.6 b**). Based on their morphological properties, the phages were assigned to the *Caudovirales* order and the *Siphoviridae* family. *Salmonella* phages of *Siphoviridae* family have been frequently isolated from sewage, poultry house and poultry-associated products (Wang et al., 2017; Huang et al., 2018; Kang et al., 2013). The morphology of phage OSY-STA was similar to phage *fmb-p1* (head: 57.2 ± 2.0 nm; tail: 171.2 ± 9.0 nm) (Wang et al., 2017), whereas phage OSY-SHC was smaller than most of *Salmonella* phages belonging to the same family.

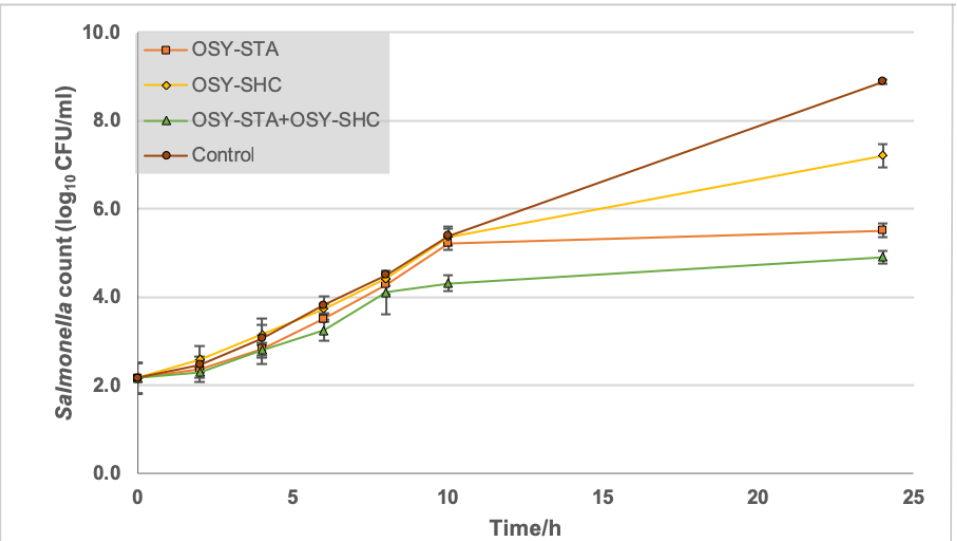


(a) (b)

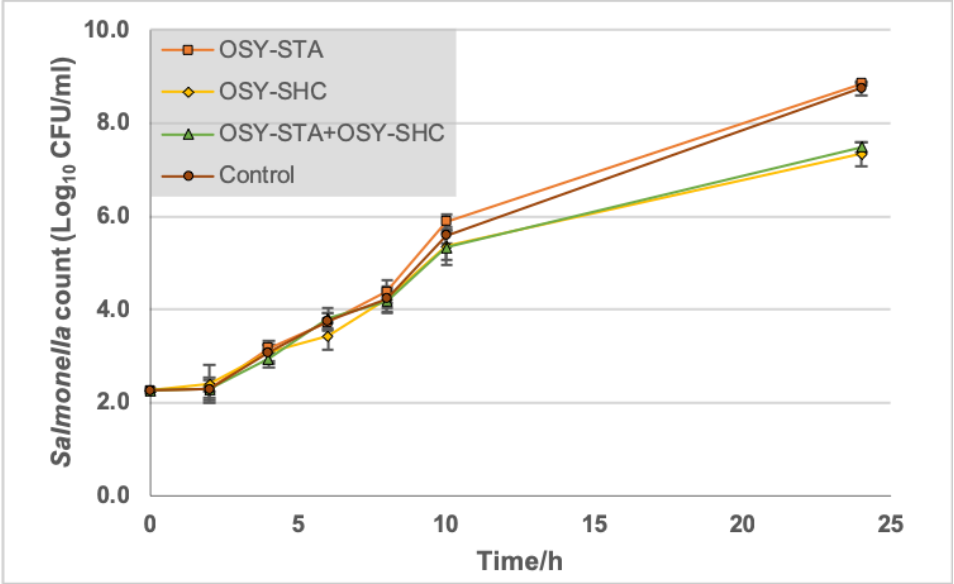
Figure 2.6 Morphology of bacteriophages examined by TEM. (a) phage OSY-STA; (b) phage OSY-SHC.

2.4.7 Inactivation of *Salmonella* serotypes in TSB by phages and a phage cocktail

The efficacy of two selected phage isolates, OSY-STA and OSY-SHC, as well as their mixture, against *S.* Typhimurium and *S.* Enteritidis in broth medium was investigated at 4°C and 25°C.



(a)



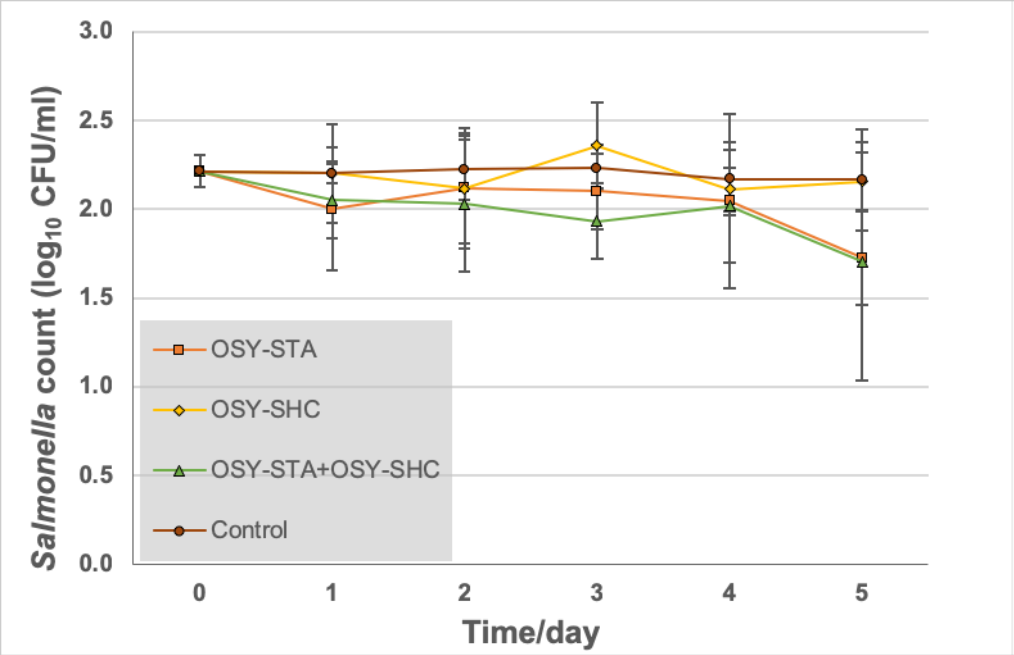
(b)

Figure 2.7 Lytic effects of single phage isolate OSY-STA, OSY-SHC and the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes in liquid cultures (TSB) held at 25 °C. (a) *Salmonella* Typhimurium LT2; (b) *Salmonella* Enteritidis 99-30581-13. Experiments were independently performed in triplicates. Data reported are means ± standard deviations of three independent trials. Standard deviation of each time point is indicated by error bars.

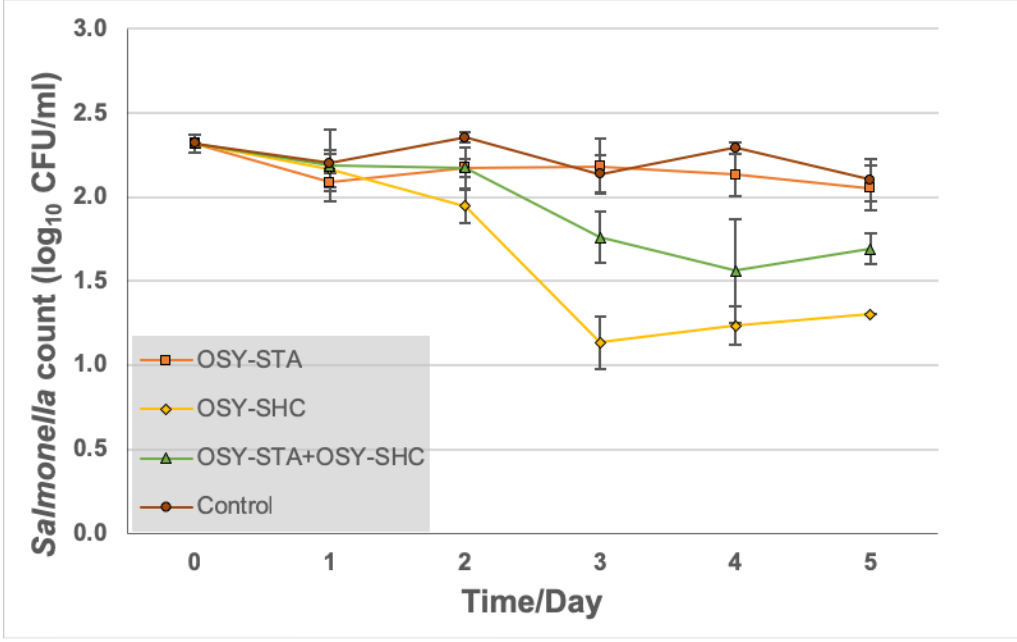
As shown in **Figure 2.7 (a)**, *Salmonella* population continuously increased at 25 °C with the addition of either single phage or phage cocktail at MOI of 10^2 within 24 h. Significant decrease ($P < 0.01$) in viable population of *S.* Typhimurium was observed at 6 h treatment of phage cocktail compare to the phage-free control. The number of viable bacterial counts was reduced by 0.5 $\log_{10}$ CFU/ml with OSY-STA phage cocktail treatments. However, no significant differences in bacterial counts was observed between phage cocktail treatments and any other treatments at 8 h. After 10 h, 1.1 log reduction was achieved in *Salmonella* population samples treated with phage cocktail, which is significant different from that in control and single phage treated samples ($P < 0.001$). Furthermore, all bacterial population were significantly different from control group after 24 h ($P < 0.001$). Especially with phage cocktail treatment, population of *S.* Typhimurium was reduced by 4.0 log whereas only 1.7 $\log_{10}$ unit reduction resulted from phage OSY-SHC treatment individually.

Similarly, **Figure 2.7 (b)** shows the reduction of *S.* Enteritidis growth compared to phage-free control when single phage isolates and phage cocktail were added at MOI of 10^2 to host cells initially present at approximately 1.9×10^2 CFU/ml. In this case, any of the phage treatments was not able to cause significant log reductions in bacterial counts within 10 h, yet the greatest reduction achieved during this time period was 0.3 $\log_{10}$ with phage cocktail treatment. Further incubation up to 24 h demonstrated that the efficiency phage OSY-STA treatment alone at MOI of 10^2 could not remarkably kill the host, which was consistent with the host range of OSY-STA. On contrary, OSY-SHC and phage cocktail achieved a peak reduction of 1.5 and 1.3 $\log_{10}$ CFU/ml after 24 h, respectively, while the number of *Salmonella* in control has reached 8.8 $\log_{10}$ CFU/ml.

In summary, the growth of both *S.* Typhimurium and *S.* Enteritidis could be inhibited by phage treatments individually or phage cocktail. In this study, viable *S.* Typhimurium count reached 4.9 $\log_{10}$ CFU/ml at 24 h in the presence of phage cocktail, compared to 8.9 $\log_{10}$ CFU/ml for the control. Phage cocktail exhibited highest efficiency against *S.* Typhimurium. However, high log reduction was not achieved when the phage treatment targeted *S.* Enteritidis although the reductions were still significant with phage cocktail treatments or phage OSY-SHC treatments. In this study, single phage OSY-SHC treatment and phage cocktail treatment exhibited similar efficiency against *S.* Enteritidis after 24 h at 25 °C, whereas phage cocktail showed distinct effect against *S.* Typhimurium, which indicated that *S.* Typhimurium was more sensitive to phage treatments under current experimental conditions.



(a)



(b)

Figure 2.8 Lytic effects of single phage isolate OSY-STA, OSY-SHC and the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes in tryptic soy broth (TSB) held at 4 °C. (a) *Salmonella* Typhimurium LT2; (b) *Salmonella* Enteritidis 99-30581-13. Experiments

were independently performed in triplicates. Data reported are means ± standard deviations of three independent trials. Standard deviation of each time point is indicated by error bars.

Figure 2.8 describes the same experiments applying phage alone or in a mixture to control *Salmonella* growth in TSB. Instead of 25 °C treatments, all samples were incubated at 4 °C for 5 days to simulate food storage conditions. As shown in **Figure 2.8 (a)**, population of *S.* Typhimurium remained relatively stable (approx.10^2 CFU/ml) over 5 days in all phage treatments and phage-free control due to the refrigeration temperature. The largest reduction, 0.5 $\log_{10}$ CFU/ml, was observed on day 5 in phage cocktail treatment, compare to the control group. However, phage cocktail did not significantly reduce the counts of *Slamonella* Typhimurium during the 5-day experiment. In addition, a high standard deviation of phage mixture treatment on day 5 indicated that viable bacterial counts from three independent experiments were spread out over a wider range of values. It is possible that mobility of phages was restricted under low temperature, hence, uneven phage distribution influenced phage-bacterium interactions, resulting in large deviations in results.

The results of effects of phage treatments on *S.* Enteritidis at 4 °C for 5 days are shown in **Figure 2.8 (b)**. Since OSY-STA was not effective against *S.* Enteritidis, the treatments with OSY-STA individually showed very similar pattern with controls. In contrast, OSY-SHC and phage cocktail displayed their ability to reduce *S.* Enteritidis counts at MOI of 10^2 under this storage condition. Greatest reduction (1 $\log_{10}$ CFU/ml) was achieved on day 3, caused by individual phage OSY-SHC treatment. Moreover, significant decreasing in viable *Salmonella* resulted from OSY-SHC and phage cocktail was recorded every day after day 2.

Considering the overall efficiency of both phage isolates and the phage cocktail at 4°C and 25°C, mixing OSY-STA and OSY-SHC as a phage cocktail not only successfully limited the growth *Salmonella*, but also effectively mitigated *S.* Enteritidis contamination. Therefore, phage cocktail was selected in further food applications.

2.5 Discussion

To develop new biocontrol strategies for improve the safety of food, we isolated, purified, and characterized *Salmonella* phages from samples collected from few farms in Ohio. Our data indicated that *Salmonella* bacteriophages are widely distributed in farm environments; almost all types of samples showed signs of phages when tested against *Salmonella* serovars. The diversity and abundance of bacteriophages have been confirmed by plenty of studies. Recently, *Salmonella* phages were isolated from municipal wastewater plants (Hong et al., 2015; Huang et al., 2018; El-Dougdoug et al., 2019), commercial broiler houses (Andreatti Filho et al., 2007), swine farms (Lettini et al., 2014), raw chicken skin and gizzard (Duc et al., 2018), and others. It has been reported that phage isolated from farm environments not only exhibited considerable phenotypic and genetic diversity, but also lysed various *Salmonella* serovars associated with human salmonellosis (Moreno Switt at al., 2013). Accordingly, the present study used farm samples as a source of *Salmonella* phages.

Although the majority of farm samples were positive for phages, not all samples yielded phages isolates that could be further propagated. For example, it was difficult to isolate phages from the tiny plaques (diameter < 0.05 mm) produced by many of these samples. Similar difficulties for propagating bacteriophages were reported for isolating *E.coli* phages from beef samples (Imamovic and Muniesa, 2011). Besides, turbid lysis resulted from few phage isolates

indicated that they may not be highly specific for *Salmonella* and could not completely eliminate target bacterium, causing the limitations of their potential applications.

Some studies reported the wide-host-range of *Salmonella* phages that could infect more than one bacterial genus, including *Klebsiella* and *Escherichia* (Bielke et al., 2007). Phage OSY-STA isolated in the current study showed similar ability to lyse *E.coli* K12 and *Salmonella*. The importance of high specificity of bacteriophages was emphasized in some phage therapies since targeted pathogens and other gut microbiota would all be exposed to phage particles, hence, some studies suggested that phages with genus specificity pose low risks to natural microflora in the gastrointestinal tract (Huang et al.,2012). On contrary, in some other studies, non-genera-restricted phages could be propagated with non-pathogenic hosts, allowing for improving personnel safety during propagation and purification.

Burst size is and indicator of phage effectiveness. In the present study, the burst size of phage OSY-STA was 139 116 PFU/cell, a value similar to that of phage FGCSSa1 (Carey-Smith et al., 2006). Phage OSY-SHC had greater burst size (235 PFU/ml), which is close to that of phage STS9 (209 PFU/cell; Duc et al., 2018). Besides, lysis process of both phages comprised two phases, and a higher production of PFU per cell occurred in the first burst compared to the second burst. This phenomenon could be explained by the higher availability of susceptible bacteria during the first burst (Abedon et al., 2003), or that a large amount of phage particles lysed bacteria from outside before phage replication occurs during the second phase. Furthermore, the length of latent period and generation time depends on phage replication rate, host generation time, incubation conditions and the physiological conditions. Abedon et al. (2001) described that latent period could be diminished at high host quantity and good quality of

host. Also, it has been suggested that phages with short latency are better suited as biocontrol agent since they can infect more bacterial cells in less time.

The isolated phages in this study presented high stability over a wide range of pH and incubation temperatures. Previously, pH values from 6 to 8 were considered optimal pH conditions for long term storage while it has been reported that a T2 phage lost 50 % of its infectivity after 2 weeks in pH 5 – 9 range (Jonczyk et al., 2011). In the current study, no significant reduction in the titer of the two isolated phages when incubated at 4, 25, 37 and 55 °C. As a result, these phage isolates could be applied in a wide range of foods owing to their broad pH stability and thermal stabilities.

To simulate the general processing and storage conditions in the food industry, 25°C and 4 °C were chosen in the present study. Experiments revealed that cell lysis by a mixture of the phage OSY-STA and OSY-SHC was more efficient than that resulting from the application of individual phages. Phages absorb onto the surface of bacteria by identifying specific receptors, and different serotypes may restrict the ability of phage to locate the target bacteria. Additionally, *Salmonella* often develops resistance to phage infection by modifying surface receptors (Labrie et al., 2010). Few studies suggested that mixing phages targeting different receptors is a potential strategy to improve the efficiency of phage treatments as well as to avoid the development of phage-resistant bacteria. Bai et al. (2019) reported a phage cocktail consisting of three phages, resulting in a significant reduction in the development of bacterial resistance. Besides, over 5 $\log_{10}$ CFU/cm^2 reduction was achieved in iceberg lettuce by applying this phage cocktail. Similarly, a phage cocktail developed by Chen et al. (2018) effectively inhibited the growth of *Salmonella* Enteritidis while reducing the biofilm formation of *Salmonella* Typhimurium.

It was noticed in the current study that the effectiveness of individual phage isolates or a phage cocktail was relatively lower at 4°C compared with that at 25°C. Similar results have been reported in previous studies (Bigwood et al., 2008; Huang et al., 2018), where a lower reduction in bacterial counts was observed when the incubation temperature was 4 or 5 °C, in contrast to room temperature. The low temperature not only prevents the propagation of bacteria, it also inhibits the regrowth of bacteria after phage treatment, thus, the reduction in bacterial counts could persist during storage for a longer time period (Soni et al., 2010).

The present work was limited to analysis of lytic effects of phage isolates or phage cocktail on representative *Salmonella* serovars in TSB medium at a fixed MOI. Apparently, phage treatments conducted at MOI of 100 in this study only inhibited the growth of *Salmonella* rather than elimination of targeted host. to achieve higher efficiency of applications, it is desirable to examine the lytic effects of the phage cocktail when applied at higher MOI. Nevertheless, the findings of this studies suggested the potential of our phage cocktail to mitigate *Salmonella* risk and provided a solid basis for further research on the phage cocktail and its combination of other food decontamination strategies for *Salmonella* biocontrol in foods.

Reference

Abedon, S.T., Hyman, P., Thomas, C., 2001. Bacteriophage latent-period evolution as a response to bacterial availability. Appl. Environ. Microbiol. 69, 7499–7506. https://doi.org/10.1128/AEM.69.12.7499-7506.2003

Abedon, S.T., Hyman, P., Thomas, C., 2003. Experimental examination of bacteriophage latent-period evolution as a response to bacterial availability. Appl. Environ. Microbiol. 69, 7499–506. https://doi.org/10.1128/AEM.69.12.7499

Ackermann H.W. (2009) Phage Classification and Characterization. P127-140. In: Clokie M.R., Kropinski A.M. (eds) Bacteriophages. Methods and protocols, vol 1: Isolatin, Characterization, and Interation. Humana Press. https://doi.org/10.1007/978-1-60327-164-6_13

Amarillas, L., Rubí-Rangel, L., Chaidez, C., González-Robles, A., Lightbourn-Rojas, L., León-Félix, J., 2017. Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrug-resistant *Escherichia coli*. Front. Microbiol. 8, 1–18. https://doi.org/10.3389/fmicb.2017.01355

Bai, J., Jeon, B., Ryu, S., 2019. Effective inhibition of *Salmonella* Typhimurium in fresh produce by a phage cocktail targeting multiple host receptors. Food Microbiol. 77, 52–60. https://doi.org/10.1016/j.fm.2018.08.011

Bielke, L., Higgins, S., Donoghue, A., Donoghue, D., Hargis, B.M., 2007. *Salmonella* host range of bacteriophages that infect multiple genera. Poult. Sci. 86, 2536–2540. https://doi.org/10.3382/ps.2007-00250

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G. V., Heinemann, J.A., 2008. Phage inactivation of foodborne pathogens on cooked and raw meat. Food Microbiol. 25, 400–406. https://doi.org/10.1016/j.fm.2007.11.003

Carey-Smith, G. V., Billington, C., Cornelius, A.J., Hudson, J.A., Heinemann, J.A., 2006. Isolation and characterization of bacteriophages infecting *Salmonella* spp. FEMS Microbiol. Lett. 258, 182–186. https://doi.org/10.1111/j.1574-6968.2006.00217.x

Carter, C.D., Parks, A., Abuladze, T., Li, M., Woolston, J., Magnone, J., Senecal, A., Kropinski, A.M., Sulakvelidze, A., 2012. Bacteriophage cocktail significantly reduces *Escherichia coli* O157. Bacteriophage 2, 178–185. https://doi.org/10.4161/bact.22825

CDC, 2018. Salmonellosis (Nontyphoidal) - Chapter 3, 2018 Yellow Book -Travelers' Health | CDC [WWW Document], n.d. URL

https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/salmonellosis-nontyphoidal (accessed 6.6.19).

Chen, Y., Sun, E., Song, J., Tong, Y., Wu, B., 2018. Three *Salmonella enterica* serovar Enteritidis bacteriophages from the *Siphoviridae* family are promising candidates for phage therapy. Can. J. Microbiol. 64, 865–875. https://doi.org/10.1139/cjm-2017-0740

Duc, H.M., Son, H.M., Honjoh, K. ichi, Miyamoto, T., 2018. Isolation and application of bacteriophages to reduce *Salmonella* contamination in raw chicken meat. LWT - Food Sci. Technol. 91, 353–360. https://doi.org/10.1016/j.lwt.2018.01.072

El-Dougdoug, N.K., Cucic, S., Abdelhamid, A.G., Brovko, L., Kropinski, A.M., Griffiths, M.W., Anany, H., 2019. Control of *Salmonella* Newport on cherry tomato using a cocktail of lytic bacteriophages. Int. J. Food Microbiol. 293, 60–71. https://doi.org/10.1016/j.ijfoodmicro.2019.01.003

Grant, A., Hashem, F., Parveen, S., 2016. *Salmonella* and *Campylobacter*: Antimicrobial resistance and bacteriophage control in poultry. Food Microbiol. 53, 104–109. https://doi.org/10.1016/j.fm.2015.09.008

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol. 154, 66–72. https://doi.org/10.1016/j.ijfoodmicro.2011.12.023

Hong, Y., Schmidt, K., Marks, D., Hatter, S., Marshall, A., Albino, L., Ebner, P., 2016. Treatment of *Salmonella* -contaminated eggs and pork with a broad-spectrum, single bacteriophage: assessment of efficacy and resistance development. Foodborne Pathog. Dis. 13, 679–688. https://doi.org/10.1089/fpd.2016.2172

Huang, C., Shi, J., Ma, W., Li, Z., Wang, J., Li, J., Wang, X., 2018. Isolation, characterization, and application of a novel specific *Salmonella* bacteriophage in different food matrices. Food Res. Int. 111, 631–641. https://doi.org/10.1016/j.foodres.2018.05.071

Hudson, J.A., Billington, C., Cornelius, A.J., Wilson, T., On, S.L.W., Premaratne, A., King, N.J., 2013. Use of a bacteriophage to inactivate *Escherichia coli* O157: H7 on beef. Food Microbiol. 36, 14–21. https://doi.org/10.1016/j.fm.2013.03.006

Imamovic, L., Muniesa, M., 2011. Quantification and evaluation of infectivity of shiga toxin-encoding bacteriophages in beef and salad. Appl. Environ. Microbiol. 77, 3536–3540. https://doi.org/10.1128/aem.02703-10

Jończyk, E., Kłak, M., Międzybrodzki, R., Górski, A., 2011. The influence of external factors on bacteriophages-review. Folia Microbiol. (Praha). 56, 191–200. https://doi.org/10.1007/s12223-011-0039-8

Kang, H.-W., Kim, J.-W., Jung, T.-S., Woo, G.-J., 2013. wksl3, a New Biocontrol Agent for *Salmonella* enterica serovars Enteritidis and Typhimurium in foods: characterization, application, sequence analysis, and oral acute toxicity study. Appl. Environ. Microbiol. 79, 1956–1968. https://doi.org/10.1128/aem.02793-12

Kropinski, A.M., Anany, H., Kuhn, J.H., Tolstoy, I., Kutter, E., Adriaenssens, E.M., 2017. To create a new bacteriophage family, *Ackermannviridae*, containing two (2) new subfamilies including four (4) genera. ICTV Rep. 1–12. https://doi.org/10.13140/RG.2.2.29173.88800

Labrie, S.J., Samson, J.E., Moineau, S., 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. 8, 317.

Leader, B.T., Frye, J.G., Hu, J., Fedorka-Cray, P.J., Boyle, D.S., 2009. High-throughput molecular determination of *Salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. J. Clin. Microbiol. 47, 1290–1299. https://doi.org/10.1128/JCM.02095-08

Lettini, A.A., Saccardin, C., Ramon, E., Longo, A., Cortini, E., Dalla Pozza, M.C., Barco, L., Guerra, B., Luzzi, I., Ricci, A., 2014. Characterization of an unusual Salmonella phage type DT7a and report of a foodborne outbreak of salmonellosis. Int. J. Food Microbiol. 189, 11–17. https://doi.org/10.1016/j.ijfoodmicro.2014.07.021

M. Donoghue, A., Bielke, L.R., E. Higgins, S., J. Donoghue, D., M. Hargis, B., Tellez, G., 2009. Use of wide-host-range bacteriophages to reduce *Salmonella* on poultry products. Int. J. Poult. Sci. 6, 754–757. https://doi.org/10.3923/ijps.2007.754.757

Modi, R., Hirvi, Y., Hill, A., Griffiths, M.W., 2016. Effect of Phage on Survival of *Salmonella* Enteritidis during manufacture and storage of cheddar cheese made from raw and pasteurized milk. J. Food Prot. 64, 927–933. https://doi.org/10.4315/0362-028x-64.7.927

Moreno Switt, A.I., den Bakker, H.C., Vongkamjan, K., Hoelzer, K., Warnick, L.D., Cummings, K.J., Wiedmann, M., 2013. *Salmonella* bacteriophage diversity reflects host diversity on dairy farms. Food Microbiol. 36, 275–285. https://doi.org/10.1016/j.fm.2013.06.014

Moye, Z.D., Woolston, J., Sulakvelidze, A., 2018. Bacteriophage applications for food production and processing. Viruses 10, 1–22. https://doi.org/10.3390/v10040205

Peters, T., Hopkins, K.L., Lane, C., Nair, S., Wain, J., De Pinna, E., 2010. Emergence and characterization of *Salmonella enterica* serovar typhimurium phage type DT191a. J. Clin. Microbiol. 48, 3375–3377. https://doi.org/10.1128/JCM.00109-10

Roseliza, R., Maswati, M.A., Hasnah, Y., Ramlan, M., 2011. Identification of *Salmonella* serotypes. Malaysian J. Vet. Res. 59–64.

Scallan, E., Hoekstra, R.M., Mahon, B.E., Jones, T.F., Griffin, P.M., 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. Epidemiol. Infect. 143, 2795–2804. https://doi.org/10.1017/s0950268814003185

Sharma, M., Patel, J.R., Conway, W.S., Ferguson, S., Sulakvelidze, A., 2016. Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and Lettuce. J. Food Prot. 72, 1481–1485. https://doi.org/10.4315/0362-028x-72.7.1481

Snyder, A.B., Perry, J.J., Yousef, A.E., 2016. Developing and optimizing bacteriophage treatment to control enterohemorrhagic *Escherichia coli* on fresh produce. Int. J. Food Microbiol. 236, 90–97. https://doi.org/10.1016/j.ijfoodmicro.2016.07.023

Soni, K.A., Nannapaneni, R., Hagens, S., 2009. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100 . Foodborne Pathog. Dis. 7, 427–434. https://doi.org/10.1089/fpd.2009.0432

Thung, T.Y., Lee, E., Mahyudin, N.A., Anuradha, K., Mazlan, N., Kuan, C.H., Pui, C.F., Ghazali, F.M., Mahmud Ab Rashid, N.K., Rollon, W.D., Tan, C.W., Radu, S., 2019. Evaluation of a lytic bacteriophage for bio-control of *Salmonella* Typhimurium in different food matrices. Lwt 105, 211–214. https://doi.org/10.1016/j.lwt.2019.02.033

Vital, P.G., Caballes, M.B.D., Rivera, W.L., 2017. Antimicrobial resistance in *Eescherichia coli* and salmonella spp. Isolates from fresh produce and the impact to food safety. J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes 52, 683–689. https://doi.org/10.1080/03601234.2017.1331676

Wang, C., Yang, J., Zhu, X., Lu, Y., Xue, Y., Lu, Z., 2017. Effects of *Salmonella* bacteriophage, nisin and potassium sorbate and their combination on safety and shelf life of fresh chilled pork. Food Control 73, 869–877. https://doi.org/10.1016/j.foodcont.2016.09.034

Ye, J., Kostrzynska, M., Dunfield, K., Warriner, K., 2016. Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on

antagonistic bacteria and lytic bacteriophages. J. Food Prot. 73, 9–17. https://doi.org/10.4315/0362-028x-73.1

Additional tables

Table 2.5 Host range of 31 phage isolates determined against 39 bacterial strains (From A-DW-SE to D-CW-SH).

***Salmonella* strain**	**Phage ID**							
	A-DW-SE	A-Organ-ST[a]	A-Bed-ST	B-Cage-P-SH	B-Cage-Q-SH	C-Feather-SH[b]	D-Cage-SH	D-CW-SH
unspecified (Kentucky)	-	+++	+++	-	-	-	-	-
7N	-	+++	+++	-	-	-	-	-
4N	-	++	++	-	-	-	-	-
2053H	-	+++	+++	-	-	-	-	-
H9113	-	++	++	-	-	-	-	-
unspecified (Montenido)	-	+++	+++	-	-	-	-	-
ATCC14028	-	-	-	-	-	-	-	-
unspecified (Anatm)	-	-	-	-	-	-	-	-
unspecified (Poona)	-	-	-	-	-	-	-	-
ATCC8388	-	-	-	-	-	-	-	-
OSU836	-	-	-	-	-	-	-	-
S5-390	-	+++	+++	+++	++	+++	++	++
S5-667	-	-	-	-	-	-	-	-
R8-092	-	++	++	-	-	-	-	-

Continued

Table 2.5 Continued

S5-373	-	-	-	-	-	-	-	-
R8-798	+++	+++	++	-	-	-	-	-
S5-371	+	-	-	+	+	+	+	+
S5-455	+++	++	++	+++	+++	+++	+++	+++
S5-506	-	-	-	-	-	-	-	-
S5-515	++	+++	+++	+	+	+	+	-
R8-376	++	+++	+++	-	-	-	-	-
S5-454	-	-	-	-	-	-	-	-
S5-464	-	-	-	+	+	+	-	-
S5-370	-	-	-	-	-	-	-	-
S5-961	++	+++	+++	-	-	-	-	-
S5-369	+++	+++	-	-	-	-	-	-
S5-0264	-	-	-	-	-	-	-	-
S5-0276	++	+++	+++	-	-	-	-	-
S5-0282	++	-	-	+++	+++	+++	+++	+++
S5-0288	-	-	-	-	-	-	-	-
R8-4315	-	++	++	-	-	-	-	-
R8-8389	-	-	-	-	-	-	-	-

Continued

Table 2.5 Continued

R8-0257	+	+	-	-	-	-	-	-
R6-0578	+++	+++	+++	-	-	-	-	-
R9-1348	-	+++	+++	-	-	-	-	-
R9-4864	-	+++	-	+	+	+	+	+
R9-1219	++	-	-	+++	+++	+++	+++	+++
K12 (*E.coli*)	-	+++	+++	-	-	-	-	-
EDL933 (*E.coli*)	-	-	-	-	-	-	-	-

[a] A-Organ-ST: phage OSY-STA;
[b] C-Feather-SH: phage OSY-SHC.

Table 2.6 Host range of 31 phage isolates determined by against 39 bacterial strains (From D-Body-SH to E-Drain-Q-SH).

Salmonella strain ID	Phage ID							
	D-Body-SH	D-WW-SH	D-Scale-SH	D-DW-SH	E-Feather-Q-SH	E-DW-C-SH	E-Cage-Q-SH	E-Drain-Q-SH
unspecified (Kentucky)	-	-	-	-	-	-	-	-
7N	-	-	-	-	-	-	-	-
4N	-	-	-	-	-	-	-	-
2053H	-	-	-	-	-	-	-	-
H9113	-	-	-	-	-	-	-	-
unspecified (Montenido)	-	-	-	-	-	-	-	-
ATCC14028	-	-	-	-	-	-	-	-
unspecified (Anatm)	-	-	-	-	-	-	-	-
unspecified (Poona)	-	-	-	-	-	-	-	-
ATCC8388	-	-	-	-	-	-	-	-
OSU836	-	-	-	-	-	-	-	-
S5-390	++	++	++	++	++	++	++	++
S5-667	-	-	-	-	-	-	-	-
R8-092	-	-	-	-	-	-	-	-
S5-373	-	-	-	-	-	-	-	-

Continued

Table 2.6 Continued

R8-798	-	-	-	-	-	-	-	-
S5-371	+	+	+	+	+	+	+	+
S5-455	+++	+++	+++	-	-	-	+++	+++
S5-506	-	-	-	-	-	-	-	-
S5-515	+	+	-	-	-	-	-	-
R8-376	-	-	-	-	-	-	-	-
S5-454	-	-	-	-	-	-	-	-
S5-464	-	-	-	-	-	+	-	-
S5-370	-	-	-	-	-	-	-	-
S5-961	-	-	-	-	-	-	-	-
S5-369	-	-	-	-	-	-	-	-
S5-0264	-	-	-	-	-	-	-	-
S5-0276	-	-	-	-	-	-	-	-
S5-0282	+++	+++	+++	+++	+++	+++	+++	+++
S5-0288	-	-	-	-	-	-	-	-
R8-4315	-	-	-	-	-	-	-	-
R8-8389	-	-	-	-	-	-	-	-
R8-0257	-	-	-	-	-	-	-	-

Continued

Table 2.6 Continued

R6-0578	-	-	-	-	-	-	-	-
R9-1348	-	-	-	-	-	-	-	-
R9-4864	+	+	+	+	+	+	+	+
R9-1219	+++	+++	+++	+++	+++	+++	+++	+++
K12 (*E.coli*)	-	-	-	-	-	-	-	-
EDL933 (*E.coli*)	-	-	-	-	-	-	-	-

Table 2.7 Host range of 31 phage isolates determined by against 39 bacterial strains (From E-Cage-C-SH to G-Gost-Hay-SH).

***Salmonella* ID**	**Phage ID**							
	E-Cage-C-SH	E-Scale-SE	F-Cattle-FFB1-SH	F-Cattle-Gate-SH	G-Sheep-Gate-SH	G-Goat-DW-SH	G-Goat-Gate-SH	G-Goat-Hay-SH
unspecified (Kentucky)	-	-	-	-	-	-	-	-
7N	-	-	-	-	-	-	-	-
4N	-	-	-	-	-	-	-	-
2053H	-	-	-	-	-	-	-	-
H9113	-	-	-	-	-	-	-	-
unspecified (Montenido)	-	-	-	-	-	-	-	-
ATCC14028	-	-	-	-	-	-	-	-
unspecified (Anatm)	-	-	-	-	-	-	-	-
unspecified (Poona)	-	-	-	-	-	-	-	-
ATCC8388	-	-	-	-	-	-	-	-
OSU836	-	-	-	-	-	-	-	-
S5-390	-	-	+	+	-	+	+	+
S5-667	-	-	-	-	-	-	-	-
R8-092	-	-	-	-	-	-	-	-

Continued

Table 2.7 Continued

S5-373	-	-	-	-	-	-	-	-
R8-798	-	-	-	-	-	-	-	-
S5-371	+	+	+	+	+	+	+	+
S5-455	++	-	++	-	-	-	-	-
S5-506	-	-	-	-	-	-	-	-
S5-515	-	-	-	-	-	-	-	-
R8-376	-	-	-	-	-	-	-	-
S5-454	-	-	-	-	-	-	-	-
S5-464	-	-	-	-	-	-	-	-
S5-370	-	-	-	-	-	-	-	-
S5-961	-	-	-	-	-	-	-	-
S5-369	-	-	-	-	-	-	-	-
S5-0264	-	-	-	-	-	-	-	-
S5-0276	-	-	-	-	-	-	-	-
S5-0282	+++	+++	+++	+++	+++	+++	+++	+++
S5-0288	-	-	-	-	-	-	-	-
R8-4315	-	-	-	-	-	-	-	-
R8-8389	-	-	-	-	-	-	-	-

Continued

Table 2.7 Continued

R8-0257	-	-	-	-	-	-	-	-
R6-0578	-	-	-	-	-	-	-	-
R9-1348	-	-	-	-	-	-	-	-
R9-4864	+	-	+	++	++	++	++	++
R9-1219	+++	-	+++	+++	+++	+++	+++	+++
K12 (*E.coli*)	-	-	+	-	-	-	-	-
EDL933 (*E.coli*)	-	-	-	-	-	-	-	-

Table 2.8 Host range of 31 phage isolates determined by 39 bacterial strains (From H-DW-C-SH to I-Wall-SH).

***Salmonella* ID**	**Phage ID**						
	H-DW-SH	H-Gate-SH	H-MF-SH	H-Floor-SH	H-Manure-SH	I-Pipe-SH	I-Wall-SH
unspecified (Kentucky)	-	-	-	-	-	-	-
7N	-	-	-	-	-	-	-
4N	-	-	-	-	-	-	-
2053H	-	-	-	-	-	-	-
H9113	-	-	-	-	-	-	-
unspecified (Montenido)	-	-	-	-	-	-	-
ATCC14028	-	-	-	-	-	-	-
unspecified (Anatm)	-	-	-	-	-	-	-
unspecified (Poona)	-	-	-	-	-	-	-
ATCC8388	-	-	-	-	-	-	-
OSU836	-	-	-	-	-	-	-
S5-390	++	+	++	-	+	+	++
S5-667	-	-	-	-	-	-	-
R8-092	-	-	-	-	-	-	-
S5-373	-	-	-	-	-	-	-

Continued

Table 2.8 Continued

R8-798	-	-	-	-	-	-	-
S5-371	+	+	+	+	+	+	+
S5-455	++	-	++	-	-	-	++
S5-506	-	-	-	-	-	-	-
S5-515	-	-	-	-	-	-	-
R8-376	-	-	-	-	-	-	-
S5-454	-	-	-	-	-	-	-
S5-464	-	-	-	-	-	-	-
S5-370	-	-	-	-	-	-	-
S5-961	-	-	-	-	-	-	-
S5-369	-	-	-	-	-	-	-
S5-0264	-	-	-	-	-	-	-
S5-0276	-	-	-	-	-	-	-
S5-0282	+++	+++	+++	+++	+++	+++	+++
S5-0288	-	-	-	-	-	-	-
R8-4315	-	-	-	-	-	-	-
R8-8389	-	-	-	-	-	-	-
R8-0257	-	-	-	-	-	-	-

Continued

Table 2.8 Continued

R6-0578	-	-	-	-	-	-	-
R9-1348	-	-	-	+	-	-	+
R9-4864	+	++	+	++	++	++	++
R9-1219	+++	+++	+++	+++	+++	+++	+++
K12 (*E.coli*)	-	-	-	-	-	-	-
EDL933 *(E.coli*)	-	-	-	-	-	-	-

Chapter 3: Bioinformatic analyses of *Salmonella* phage OSY-STA

3.1 Abstract

The use of lytic bacteriophages as biocontrol agents has been increasingly accepted in food preservation, agriculture production and disease control. In concert with these developments, there is a need for research to ensure that the newly-introduced phages do not contain genes encoding for bacterial virulence factors or other deleterious agents such as immune reactive allergens. Whole genome sequencing (WGS) technology is a common way to reveal these potential hazards. Additionally, WGS provides information about important phage-encoded proteins, such as phage tail fiber protein and lytic enzymes.

In present study, complete genome sequencing was carried out for *Salmonella* phage OSY-STA, which was found in this study to infect a broad range of *Salmonella* strains. Phage DNA was extracted and its whole genome was sequenced. The phage genome comprised 111,373 base pairs with 169 open reading frames; the functions of 62 of these were assigned. Conserved regions in OSY-STA genome was observed by gene alignment with its homologous phages and further analyses indicated the important role of tRNAs in phage protein translation. Studies like this are essential for the development and use of phages as efficient antimicrobial agents in commercial applications or in phage therapeutic applications against *Salmonella*.

3.2 Introduction

Bacteriophages occupy a distinctive biological position on earth and offer us a particular view on the diversity, origins and evolution of viruses (Hendrix, 2002). It has been estimated that more than 10^{31} phage particles exist in the biosphere, and, their origins can be traced back to three billion years ago. Phage is relatively easy to be sequenced due to its small genome size and relatively simple isolation procedures. The first complete genome ever obtained was bacteriophage øX174 in 1977, which contains 5,386 bp single stranded DNA (Sanger et al., 1977). Subsequently, phage lambda was completely sequenced with 48,502 bp double-stranded DNA (Sanger et al., 1982). The first complete sequenced double-stranded tailed-phage was mycobacteriophage L5 in 1993 (Hatfull et al., 1993). Thereafter, numerous phage genomes were elucidated along with the development of DNA sequencing technologies.

The data contained in genetic material, which differs extensively among bacteriophages, represent not only gene-encoded protein synthesis, but also sequences important for the taxonomic classification of viruses into orders, families, subfamilies, genus and species (Klumpp et al., 2012). However, it needs to be emphasized that obstacles are still present regarding bacteriophage DNA sequencing despite of the advanced sequencing technologies. Firstly, optimization and improvements are needed for phage DNA isolation and purification protocols to obtain pure phage genomic material. Moreover, the intrinsic characteristics of phage genome, such as methylated bases and repetition regions, are difficult to sequence and determine (Klumpp et al., 2013).

Bacteriophage sequencing study is essential for understanding functional genomics. Meanwhile, whole genome sequencing has been considered as a security measure to ensure the safety of bacteriophage products. Phage genome should be free of bacterial virulence factors, and as a potential food additive, phage should not be able to induce allergies. Although it is rare to find allergen-related genes in phage genome, a study published in 2018 confirmed that a tail measure protein of *E.coli* phage ØKP26, a putative tail length tape measure protein precursor and a hypothetical protein of *E.coli* phage ØC119 were classified as probable allergen by their analyses (Ramirez et al., 2018). The present work aims to provide detailed information of a broad host range *Salmonella* phage OSY-STA at the molecular level, and to enhance our understanding of safe phage applications.

3.3 Materials and methods

3.3.1 DNA extraction, library preparation and whole genome sequencing

Phage OSY-STA was selected for whole genome sequencing and bioinformatics analysis based on its broad host range pattern and strong lytic activity compared to other phage isolates. Bacteriophage DNA was extracted using Norgen's Phage DNA Isolation Kit, according to the manufacturer's instructors (Norgen Biotek Crop. Thorold, ON, Canada) with few optimizations. A portion (3 ml) of high titer phage suspension (approx. 10^9 PFU/ml) was mixed with 30 μl (20 units) of DNase I (New England Biolabs, Ipswich, MA) and incubated at 25 °C for 15 min, followed by thermal inactivation of DNase I at 75 °C for 10 min. The heat-treated suspension was mixed with 1500 μl of lysis buffer, included in the DNA extracted kit, and 12 μl of Proteinase K (20 mg/ml;

Invitrogen, Waltham, MA). The mixture was incubated for 30 min at 55 °C and subsequently for 15 min at 65 °C. Phage DNA was purified using spin columns provided by the kit after addition of 960 µl of isopropanol. DNA concentration (ng/µl) and purity was determined spectrophotometrically (Nanovue plus; Biochrom USA, Holliston, MA). The purity of DNA was assessed by the ratio of absorbance at 260 nm and 280 nm.

The phage genome was sequenced using an Illumina MiniSeq platform with pair-end read sizes of 150 bp (The Sequencing Company, Fort Collins, CO).Raw sequencing data was in FASTQ format and the quality of raw reads was checked with FastQC software and additional preprocessing steps including BBDuk adapter/quality trimming, Dedupe duplicate read remover and BBNorm error correction and read normalization were conducted before calling the *de novo* assembly using Geneious Assembler (https://www.geneious.com/commercial/features/assembly-mapping/).

3.3.2 Bioinformatic analyses of phage genome

Potential open reading frames (ORFs) were predicted using MyRast (http://rast.theseed.org/FIG/rast.cgi) and GeneMarks (http://exon.gatech.edu/GeneMark/genemarks.cgi). Gene annotation was done using MyRast to determine gene-encoding proteins and tRNAScanSE (http://lowelab.ucsc.edu/tRNAscan-SE/index.html) to determine gene-encoding tRNAs. Functional annotation was screened using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) against the non-redundant protein database at NCBI to find similar, characterized proteins.

Additionally, comparative genomic analysis of phage isolated with homologous phages was conducted with progressive Mauve alignment (http://darlinglab.org/mauve/mauve.html) to determine conserved sequence segments of the phage genomes. A genome map with nucleotide positions, translation directions and putative functions was prepared using SnapGene (4.3.10).

3.4 Results

3.4.1 General features of the phage genome

Phage OSY-STA was sequenced to further understand its biological characteristics. A *de novo* genome assembly based on 113,404 reads yielded a single contig. Genetic annotation including positions, directions and putative functions of each gene were listed in **Table 3.1**.

The whole genome sequencing revealed a double-stranded DNA genome consisting of 111,373 bp and a GC content of 40.0 %. A total of 169 ORFs were identified with 122 ORFs on the positive strand and 47 ORFs on the negative strand. Among all putative ORFs, only 62 ORFs were determined to be functional, whereas the majority of ORFs was predicted as hypothetical proteins (**Figure 3.1**). Furthermore, BLAST analyses revealed a high similarity to T5-like phages, especially to *Salmonella* phage 3-29 (nucleotide identity: 98.9 %; query cover: 94.0 %; accession number: MK393882.1) and *Salmonella* phage BSP22A (nucleotide identity: 98.9 %; query cover: 93.0 %; accession number: KY787212.1). Three phages were obtained from different regions while sharing high similarities in their genomes, indicating the complex evolutionary relationships among these phages. Additionally, conserved sequence regions

among three phages were determined in Mauve (**Figure 3.2**) and results suggested that some regions are highly homologous whereas rearrangements of regions exist between phages with high nucleotide identity.

Bioinformatic studies also indicated that no gene products of phage OSY-STA showed similarities to any other known virulent, toxin, and pathogen-associated proteins or specific *Salmonella* proteins. Amino acids sequence corresponding to DNA sequences of phage OSY-STA was analyzed with allergenic protein sequence using Allergenic Protein Sequence Searches (http://www.allergenonline.org/). All 169 predicted protein products were not matched with any allergenic proteins with a cut-off of 0.01.

Based on the results of gene functional annotation, the OSY-STA genes could be categorized into three groups: phage structural genes, cell wall lysis genes and metabolism-related genes.

Table 3.1 Features of the open reading frames of bacteriophage OSY-STA and homology to protein databases.

ORF	Start	Stop	Strand	Homology	Query (%)	E-value	Identity (%)
1	243	995	+	phosphate starvation inducible protein [*Salmonella* virus Stitch]	100	0	100
2	997	1239	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$8.00E^{-52}$	100
3	1363	3693	+	ribonucleoside diphosphate reductase alpha subunit [*Salmonella* phage LVR16A]	100	0	99.61
4	3810	4946	+	ribonucleoside diphosphate reductase beta subunit [*Salmonella* virus Stitch]	100	0	100
5	4946	5476	+	dihydrofolate reductase [*Salmonella* phage 3-29]	100	$2.00E^{-126}$	100
6	5473	6327	+	thymidylate synthase [*Salmonella* phage 100268_sal2]	100	0	100
7	6331	6684	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$3.00E^{-66}$	100
8	6783	7361	+	putative proteasome [*Salmonella* phage Sw2]	100	$3.00E^{-138}$	98.96
9	7364	7633	+	hypothetical protein AGC_0102 [*Escherichia* virus EPS7]	100	$8.00E^{-57}$	100
10	7633	8109	+	ribonuclease H [*Escherichia* virus EPS7]	100	$4.00E^{-115}$	100
11	8186	8464	+	hypothetical protein CPT_Stitch95 [*Salmonella* virus Stitch]	100	$5.00E^{-59}$	100
12	8548	9063	+	hypothetical protein AGC_0099 [*Escherichia* virus EPS7]	100	$3.00E^{-121}$	100
13	9125	9340	+	hypothetical protein AGC_0098 [*Escherichia* virus EPS7]	100	$4.00E^{-41}$	100
14	9382	9594	+	hypothetical protein [*Salmonella* phage S126]	100	$6.00E^{-42}$	97.14

Continued

Table 3.1 continued

15	9625	10326	+	putative metallopeptidase [*Salmonella* phage BSP22A]	100	$2.00E^{-175}$	99.57
16	10397	10579	+	hypothetical protein *[Salmonella* phage 100268_sal2]	100	$1.00E^{-33}$	100
17	10634	11272	+	tail fiber protein [*Salmonella* phage STG2]	100	$4.00E^{-148}$	98.58
18	11716	12033	+	hypothetical protein CPT_Stitch87 [*Salmonella* virus Stitch]	100	$1.00E^{-71}$	100
19	12039	12488	+	spore cortex-lytic enzyme precursor [*Salmonella* virus Stitch]	100	$5.00E^{-107}$	100
20	12557	12727	+	hypothetical protein AGC_0090 [*Escherichia* virus EPS7]	100	$1.00E^{-30}$	100
21	12727	13170	+	recombination related exonuclease [*Salmonella* phage SH9]	100	$3.00E^{-102}$	99.32
22	14168	14368	+	hypothetical protein AGC_0088 [*Escherichia* virus EPS7]	100	$2.00E^{-38}$	100
23	14380	15324	+	hypothetical protein CPT_Stitch82 [*Salmonella* virus Stitch]	100	0	100
24	15588	16643	+	NadR transcriptional regulator [*Salmonella* phage S113]	100	0	100
25	16645	17322	+	PnuC-like ribosyl nicotinamide transporter [*Bacteriophage* T5-like chee130_1]	100	$9.00E^{-158}$	100
26	17429	17725	+	hypothetical protein AGC_0084 [*Escherichia* virus EPS7]	100	$6.00E^{-65}$	98.98
27	17971	18156	+	hypothetical protein CPT_Stitch76 [*Salmonella* virus Stitch]	100	$3.00E^{-37}$	100
28	18158	18496	+	hypothetical protein BSP22A_0085 [*Salmonella* phage BSP22A]	100	$8.00E^{-75}$	99.11
29	18597	19719	+	hypothetical protein CPT_Stitch74 [*Salmonella* virus Stitch]	100	$1.00E^{-20}$	97.5
30	18733	18939	+	hypothetical protein AGC_0079 [*Escherichia* virus EPS7]	100	$5.00E^{-40}$	100
31	19030	19305	+	hypothetical protein CPT_Stitch72 [*Salmonella* virus Stitch]	100	$2.00E^{-59}$	100

Continued

Table 3.1 continued

32	19854	20126	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$3.00E^{-61}$	100
33	20177	20401	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$9.00E^{-46}$	100
34	20404	20589	+	hypothetical protein AGC_0075 [*Escherichia* virus EPS7]	100	$1.00E-^{36}$	98.36
35	20692	20817	-	hypothetical protein AGC_0074 [*Escherichia* virus EPS7]	100	$2.00E-^{20}$	97.56
36	20845	21030	+	hypothetical protein CPT_Stitch68 [*Salmonella* virus Stitch]	100	$2.00E^{-33}$	100
37	22371	22535	+	hypothetical protein STG2_90 [*Salmonella* phage STG2]	100	$1.00E^{-30}$	98.15
38	22634	22951	+	hypothetical protein CPT_Stitch66 [*Salmonella* virus Stitch]	100	$3.00E^{-73}$	100
39	22951	23094	+	hypothetical protein CPT_Stitch65 [*Salmonella* virus Stitch]	100	$2.00E-^{24}$	95.74
40	23184	23537	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$4.00E^{-79}$	99.15
41	23909	24370	+	hypothetical protein [*Salmonella* phage S124]	100	$1.00E^{-101}$	90.85
42	24570	24746	+	hypothetical protein CPT_Stitch62 [*Salmonella* virus Stitch]	89	$6.00E^{-23}$	75
43	24839	25042	+	hypothetical protein [*Salmonella* phage SH9]	100	$4.00E^{-42}$	100
44	25544	25744	+	hypothetical protein [*Salmonella* phage 100268_sal2]	54	$1.00E^{-12}$	86.49
45	26007	26228	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$4.00E^{-40}$	100
46	26221	26385	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$1.00E^{-46}$	100
47	26546	26839	+	hypothetical protein CPT_Stitch56 [*Salmonella* virus Stitch]	100	$2.00E^{-64}$	100
48	27368	27736	+	ribonucleotide reductase subunit [*Salmonella* virus Stitch]	100	$1.00E^{-83}$	100
49	27818	28117	+	hypothetical protein BSP22A_0061 [*Salmonella* phage BSP22A]	100	$8.00E^{-69}$	100

Continued

Table 3.1 continued

50	28182	28367	+	hypothetical protein CPT_Stitch53 [*Salmonella* virus Stitch]	100	$3.00E^{-36}$	100
51	28426	28821	+	hypothetical protein CPT_Stitch52 [*Salmonella* virus Stitch]	100	$7.00E^{-91}$	100
52	28828	29325	+	MULTISPECIES: hypothetical protein [*Bacillus*]	97	$2.00E^{-44}$	50
53	29402	29683	+	hypothetical protein [*Salmonella* phage LVR16A]	100	$1.00E^{-59}$	97.85
54	29670	29891	+	hypothetical protein [*Salmonella* phage LVR16A]	100	$4.00E^{-45}$	100
55	29884	30183	+	hypothetical protein [*Salmonella* phage LVR16A]	100	$2.00E^{-65}$	100
56	30176	30595	+	hypothetical protein [*Salmonella* phage LVR16A]	100	$9.00E^{-101}$	100
57	30549	30645	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$7.00E^{-66}$	98.98
58	30842	31126	+	hypothetical protein AGC_0051 [*Escherichia* virus EPS7]	100	$5.00E^{-60}$	98.94
59	31243	31590	+	hypothetical protein CPT_Sw2_049 [*Salmonella* phage Sw2]	100	$2.00E^{-78}$	100
60	31736	32434	+	hypothetical protein AGC_0049 [*Escherichia* virus EPS7]	100	$5.00E^{-167}$	96.98
61	32431	32928	+	H-N-H-endonuclease F-TflVI [*Escherichia* phage OSYSP]	100	$1.00E^{-119}$	100
62	32928	33371	+	putative membrane protein [*Escherichia* phage OSYSP]	100	$3.00E^{-104}$	100
63	33302	33655	+	hypothetical protein AGC_0047 [*Escherichia* virus EPS7]	100	$8.00E^{-83}$	100
64	33655	34407	+	deoxynucleoside-5'-monophosphate kinase [*Escherichia* virus EPS7]	100	0	100
65	34420	35019	+	putative ATP-dependent Clp protease [*Escherichia* virus EPS7]	100	$2.00E^{-148}$	100
66	35176	35832	+	holin [*Salmonella* virus Stitch]	100	$2.00E^{-159}$	100
67	35829	36242	+	endolysin *[Salmonella* virus Stitch]	100	$2.00E^{-96}$	99.27

Continued

Table 3.1 continued

68	36318	36734	+	hypothetical protein CPT_Stitch38 [*Salmonella* virus Stitch]	100	$4.00E^{-95}$	100
69	36810	37220	+	hypothetical protein CPT_Stitch37 [*Salmonella* virus Stitch]	100	$6.00E^{-94}$	99.26
70	37213	37503	+	thioredoxin [*Salmonella* virus Stitch]	100	$7.00E^{-51}$	100
71	37606	37986	+	hypothetical protein [*Salmonella* phage 3-29]	100	$7.00E^{-87}$	100
72	37986	38849	+	serine/threonine protein phosphatase [*Salmonella* phage 3-29]	100	0	99.3
73	38849	39217	+	hypothetical protein [*Salmonella* phage LVR16A]	100	$4.00E^{-83}$	100
74	39217	39423	+	hypothetical protein BSP22A_0040 [*Salmonella* phage BSP22A]	100	$1.00E^{-41}$	100
75	39423	40013	+	putative serine/threonine protein phosphatase [*Salmonella* phage BSP22A]	100	$1.00E^{-142}$	100
76	40006	40107	+	hypothetical protein CPT_Stitch30 [*Salmonella* virus Stitch]	100	$7.00E^{-12}$	100
77	40176	40607	+	hypothetical protein BSP22A_0037 [*Salmonella* phage BSP22A]	100	$6.00E^{-101}$	100
78	40686	40937	+	hypothetical protein CPT_Stitch28 [*Salmonella* virus Stitch]	100	$3.00E^{-52}$	100
79	40937	41335	+	hypothetical protein BSP22A_0035 [*Salmonella* phage BSP22A]	100	$7.00E^{-91}$	99.24
80	41332	41613	+	hypothetical protein CPT_Stitch26 [*Salmonella* virus Stitch]	100	$2.00E^{-54}$	97.85
81	41610	41855	+	hypothetical protein AGC_0030 [*Escherichia* virus EPS7]	100	$1.00E^{-49}$	100
82	41845	42177	+	hypothetical protein BSP22A_0032 [*Salmonella* phage BSP22A]	100	$2.00E^{-74}$	100
83	42278	42478	+	hypothetical protein CPT_Stitch23 [*Salmonella* virus Stitch]	100	$1.00E^{-41}$	100
84	42475	42942	+	hypothetical protein CPT_Stitch22 [*Salmonella* virus Stitch]	100	$2.00E^{-112}$	100

Continued

Table 3.1 continued

85	42917	43267	+	hypothetical protein [*Salmonella* phage S132]	100	$4.00E^{-50}$	98.8
86	43312	43830	+	hypothetical protein CPT_Sw2_024 [*Salmonella* phage Sw2]	99	$2.00E^{-132}$	100
87	44041	44274	+	hypothetical protein [*Salmonella* phage SH9]	100	$1.00E^{-42}$	93.51
88	44274	44459	+	hypothetical protein [*Salmonella* phage S113]	100	$2.00E^{-31}$	100
89	44459	45070	+	hypothetical protein [*Salmonella* phage S113]	100	$1.00E^{-151}$	100
90	45070	45318	+	hypothetical protein SB9_137 [*Salmonella* phage vB_SenS_SB9]	98	$1.00E^{-49}$	93.83
91	45388	46275	+	hypothetical protein STG2_138 [*Salmonella* phage STG2]	100	0	98.31
92	47019	47132	-	hypothetical protein CPT_Sw2_016 [*Salmonella* phage Sw2]	100	$1.00E^{-17}$	100
93	47909	48148	-	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$8.00E^{-52}$	98.73
94	48276	48488	-	hypothetical protein [*Salmonella* phage 1-23]	100	$3.00E^{-42}$	100
95	48485	48829	-	hypothetical protein AGC_0013 [*Escherichia* virus EPS7]	100	$1.00E^{-76}$	98.25
96	48939	49151	-	hypothetical protein BN79_010 [*Yersinia* phage phiR201]	100	$7.00E^{-43}$	100
97	49205	49435	-	hypothetical protein Sepoy_014 [*Salmonella* phage Sepoy]	100	$5.00E^{-45}$	94.74
98	49543	50037	-	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$8.00E^{-118}$	99.39
99	50101	51096	-	hypothetical protein [*Salmonella* phage S132]	100	0	98.49
100	52188	52406	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$1.00E^{-44}$	100
101	52572	52823	+	hypothetical protein CPT_Stitch7 [*Salmonella* virus Stitch]	100	$7.00E^{-52}$	97.59
102	52924	53340	+	A2 protein [*Salmonella* phage S126]	100	$1.00E^{-92}$	100

Continued

Table 3.1 continued

103	53381	53626	+	A1 protein precursor [*Salmonella* virus Stitch]	100	$3.00E^{-44}$	100
104	53735	55399	+	A1 protein [*Salmonella* phage Seabear]	100	0	99.82
105	55452	55709	+	hypothetical protein BSP22A_0003 [*Salmonella* phage BSP22A]	100	$8.00E^{-56}$	100
106	55746	56138	+	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$2.00E^{-90}$	99.23
107	56226	56960	+	deoxynucleoside-5'-monophosphatase [*Salmonella* phage 100268_sal2]	100	0	100
108	57119	57334	-	hypothetical protein CPT_Sw2_205 [*Salmonella* phage Sw2]	100	$1.00E^{-42}$	97.18
109	57331	57576	-	hypothetical protein CPT_Sw2_204 [*Salmonella* phage Sw2]	100	$2.00E^{-47}$	96.3
110	57753	58067	-	hypothetical protein [*Salmonella* phage S116]	100	$3.00E^{-13}$	97.06
111	58142	58408	-	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$1.00E^{-67}$	100
112	58492	60273	+	receptor-blocking protein [*Salmonella* virus Stitch]	100	$2.00E^{-58}$	98.66
113	60284	60766	+	receptor-binding protein [*Salmonella* phage S116]	100	0	99.49
114	60766	62082	+	terminase large subunit [*Salmonella* phage SH9]	100	0	100
115	62197	62634	+	hypothetical protein [*Salmonella* phage S114]	91	$2.00E^{-28}$	96.08
116	62593	62799	+	hypothetical protein [*Salmonella* phage SP1a]	95	$8.00E^{-40}$	96.92
117	62877	63275	+	portal protein [*Salmonella* phage SP1a]	85	$4.00E^{-74}$	98.23
118	63256	63555	+	portal protein [*Escherichia* virus EPS7]	100	0	100
119	63552	64034	+	putative tail protein [*Salmonella* phage BSP22A]	100	$1.00E^{-111}$	100
120	64038	64670	+	probable prohead protease [*Escherichia* virus EPS7]	100	$2.00E^{-154}$	100

Continued

Table 3.1 continued

121	64688	65860	+	major cpasid protein *[Salmonella* phage Sw2]	100	$3.00E^{-130}$	96.95
122	65920	66432	+	hypothetical protein [*Salmonella* phage 3-29]	100	$8.00E^{-124}$	99.41
123	66432	67199	+	hypothetical protein CPT_Stitch151 [*Salmonella* virus Stitch]	100	0	100
124	67203	67688	+	tail terminator [*Salmonella* phage S114]	100	$2.00E^{-116}$	100
125	67715	69124	+	major tail protein [*Salmonella* virus Stitch]	100	0	100
126	69129	70028	+	minor tail protein [*Salmonella* phage LVR16A]	100	0	100
127	70025	70429	+	hypothetical protein AGC_0154 [*Escherichia* virus EPS7]	100	$3.00E^{-94}$	100
128	70491	70859	+	tape measure chaperone [*Salmonella* phage Sw2]	100	$1.00E^{-84}$	100
129	70940	74650	+	pore-forming tail tip protein [*Salmonella* phage BSP22A]	100	0	100
130	74759	75373	+	hypothetical protein AGC_0150 [*Escherichia* virus EPS7]	100	$1.00E^{-145}$	100
131	75370	78219	+	tail protein [*Salmonella* phage LVR16A]	100	0	99.89
132	78220	80277	+	phage tail protein [*Salmonella* enterica subsp. enterica]	100	0	99.4
133	80283	80705	+	putative phage tail protein [*Escherichia* virus EPS7]	100	$9.00E^{-95}$	97.86
134	80705	83647	+	tail protein [*Salmonella* phage S116]	100	0	95.22
135	83915	84166	-	hypothetical protein P132_0129 [*Bacteriophage* T5-like saus132]	100	$1.00E^{-52}$	98.8
136	84144	84590	-	putative deoxyUTP pyrophosphatase [*Escherichia* virus EPS7]	100	$3.00E^{-106}$	100
137	84587	85462	-	flap endonuclease [*Escherichia* virus EPS7]	100	0	100
138	85462	85944	-	D14 protein [*Escherichia* virus EPS7]	100	$9.00E^{-116}$	100

Continued

Table 3.1 continued

139	85948	87786	-	exonuclease [*Salmonella* virus Stitch]	100	0	99.35
140	87767	88744	-	recombination nuclease [*Salmonella* virus Stitch]	100	0	100
141	88785	89558	-	hypothetical protein [*Salmonella* phage 100268_sal2]	100	0	100
142	89551	89913	-	hypothetical protein [*Salmonella* phage 100268_sal2]	100	$7.00E^{-82}$	100
143	90054	91406	-	helicase [*Salmonella* virus Stitch]	100	0	99.78
144	91403	91900	-	hypothetical protein CPT_Stitch129 [*Salmonella* virus Stitch]	100	$7.00E^{-117}$	100
145	91893	94460	-	DNA polymerase [*Salmonella* phage Sw2]	100	0	99.88
146	94523	95413	-	DNA primase [*Salmonella* virus Stitch]	100	0	100
147	95410	96882	-	DNA helicase [*Salmonella* phage Sepoy]	100	0	99.41
148	96965	97732	-	D5 protein [*Escherichia* virus EPS7]	100	0	100
149	97725	98504	-	DNA ligase [*Salmonella* phage SH9]	100	0	99.23
150	98707	99678	-	DNA ligase [*Salmonella* phage SH9]	100	0	100
151	99671	99871	-	hypothetical protein CPT_Stitch122 [*Salmonella* virus Stitch]	100	$3.00E^{-39}$	100
152	99957	100265	-	transcriptional regulator [*Salmonella* virus Stitch]	100	$5.00E^{-70}$	100
153	100316	100612	-	hypothetical protein CPT_Stitch120 [*Salmonella* virus Stitch]	100	$6.00E^{-66}$	100
154	100649	101059	-	D3 protein [*Escherichia* virus EPS7]	100	$2.00E^{-94}$	100
155	101167	101403	-	hypothetical protein [*Salmonella* phage SH9]	100	$1.00E^{-48}$	98.72
156	101396	102100	-	D2 protein [*Escherichia* virus EPS7]	100	$3.00E^{-171}$	100

Continued

Table 3.1 continued

157	102169	102402	-	hypothetical protein [*Salmonella* phage S113]	100	$2.00E^{-47}$	100
158	102386	105175	-	DNA primase C [*Salmonella* phage S113]	100	0	99.78
159	105792	106187	-	hypothetical protein AGC_0122 [*Escherichia* virus EPS7]	100	$2.00E^{-88}$	99.24
160	106197	106625	-	hypothetical protein BSP22A_0123 [*Salmonella* phage BSP22A]	100	$1.00E^{-99}$	100
161	106628	107143	-	hypothetical protein CPT_Stitch112 [*Salmonella* virus Stitch]	100	$1.00E^{-122}$	99.42
162	107121	107306	-	hypothetical protein [*Escherichi*a virus AKFV33]	100	$4.00E^{-34}$	100
163	107287	107454	-	hypothetical protein CPT_Stitch110 [*Salmonella* virus Stitch]	100	$2.00E^{-35}$	100
164	107447	108298	-	Sir2-like protein [*Salmonella* virus Stitch]	100	0	99.65
165	108298	108492	-	hypothetical protein CPT_Stitch108 [*Salmonella* virus Stitch]	100	$3.00E^{-38}$	100
166	108461	208664	-	hypothetical protein CPT_Stitch107 [*Salmonella* virus Stitch]	100	$8.00E^{-39}$	100
167	108674	108955	-	hypothetical protein SPC35_0091 [*Salmonella* virus SPC35]	100	$1.00E^{-62}$	100
168	109054	110928	-	anaerobic ribonucleoside-triphosphate reductase [*Salmonella* phage BSP22A]	100	0	100
169	111281	>111373	+	phosphate starvation-inducible protein [*Salmonella* phage LVR16A]	100	$2.00E^{-11}$	100

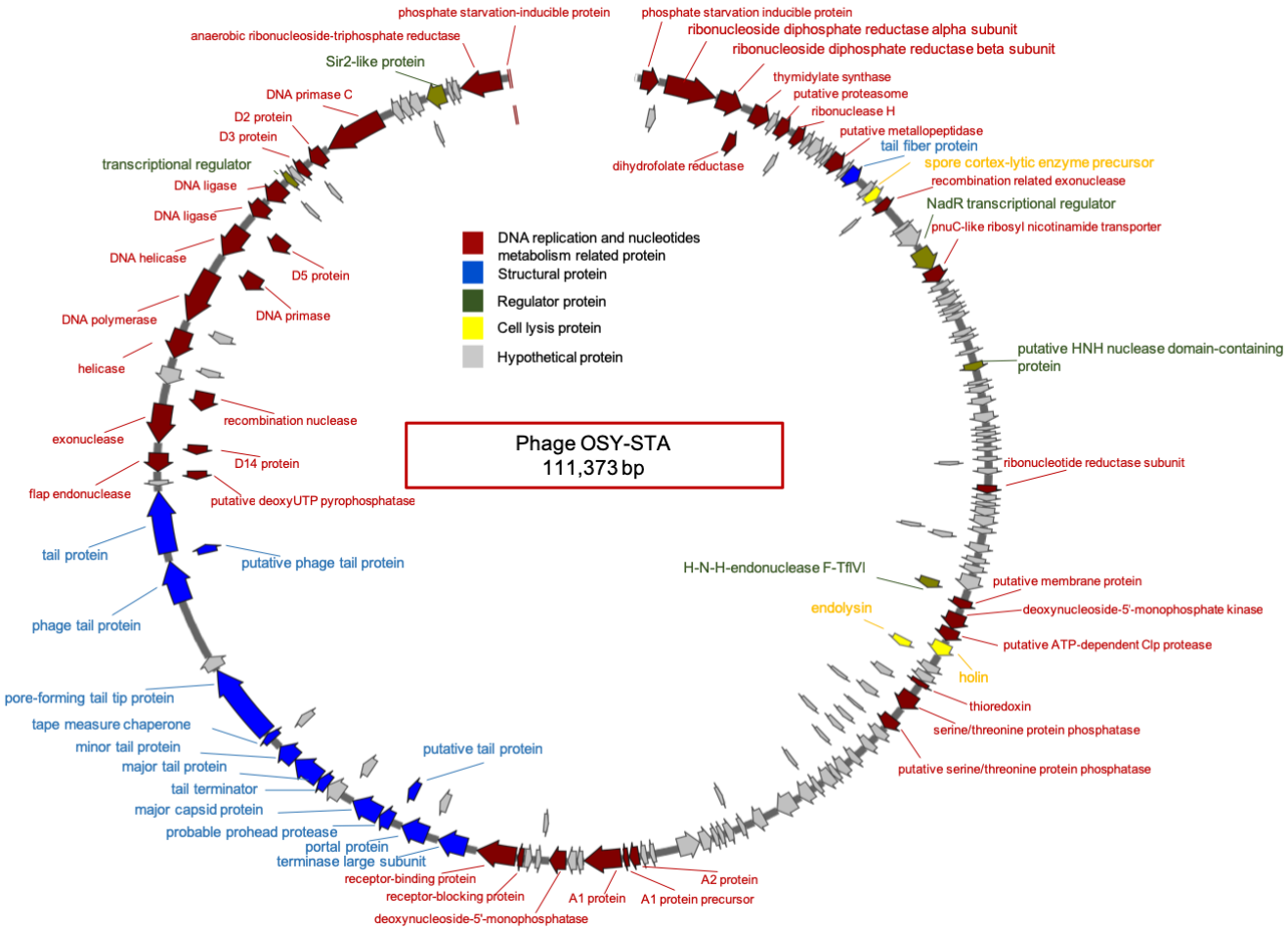


Figure 3.1 Schematic diagram of the OSY-STA genome and its open reading frames, colored-coded by protein functional categories. Red: DNA replication and nucleotides metabolism related proteins; blue: structural proteins; green: regulator protein; yellow: cell lysis protein; grey: hypothetical protein. Possible functions are annotated, respectively.

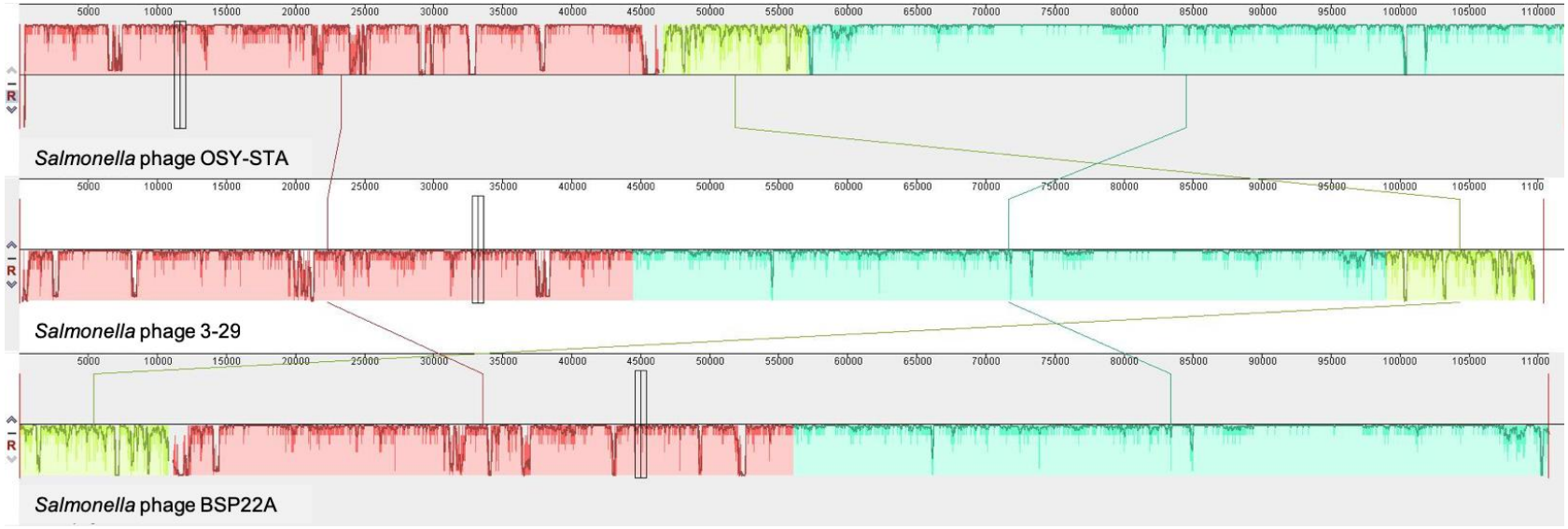


Figure 3.2 Mauve genome alignment of *Salmonella* phage OSY-STA, 3-29 and BSP22A. Boxes with identical colors represent local colinear blocks (LCB), indicating homologous genomic regions shared by phage chromosomes.

3.4.2 Regulatory sequences

Genes identified to be involved in encoding transcriptional regulators include NadR transcriptional regulator (ORF 24), H-N-H-endonuclease F-TflVI (ORF 61), transcriptional regulator (ORF 152) and Sir2-like protein (ORF 164).

3.4.3 DNA replication and nucleotide metabolism related sequence

Several genes predicted to be involved in nucleotide metabolism were identified in phage OSY-STA genome. The identified ORFs include ribonucleoside diphosphate reductase alpha/beta subunit (ORF 3 and 4), dihydrofolate reductase (ORF 5), thymidylate synthase (ORF 6), ribonuclease H (ORF 10), recombination related exonuclease (ORF 21), ribonucleotide reductase subunit (ORF 48), deoxynucleoside-5'-monophosphate kinase (ORF 64), flap endonuclease [(ORF 137), exonuclease (ORF 139), recombination nuclease (ORF 140), anaerobic ribonucleotide-triphosphate reductase (ORF 168). , whereas genes involved in DNA replication include D5, D14 protein (ORF 148 and 138), DNA helicase (ORF 143 and 147), DNA polymerase (ORF 145), DNA primase (ORF 146 and 158), DNA ligase (ORF 149 and 150). Putative orthologous of thioredoxin (ORF 70) was identified and its protein product act as hydrogen donor in ribonucleotide synthesis.

3.4.4 Structure and cell wall lysis related sequence

Genetic sequences encoding tail proteins (ORF 125, ORF 126, ORF 131, ORF 132 and ORF 134) showed 100 % similarity to *Salmonella* phage LVR16A tail protein and exhibited 95 % similarity to the tail protein of *Salmonella* phage S116. Tape measure chaperone facilitates DNA transit to bacterial cytoplasm during infection (Mahony et al.,

2016) was also identified in the present phage genome (ORF 128). Researchers' found that the genome injection process of *E.coli* phage HK97 requires an inner membrane glucose transporter protein PtsG and a tape measure chaperone FkpA to work together, in order to transmit its DNA into bacterial cytoplasm (Cumby et al., 2015). The lengths of the tape measure protein of phage OSY-STA are similar to other *Siphoviridae* phages such as *Salmonella* phage Sw2 (100 %) and *Salmonella* phage LVR16A (100 %). ORF 117 and 118 were predicted to be portal proteins, which play important role in phage assembly. BLAST analysis of ORF 121 showed significant homology to the capsid protein of *Salmonella* phage Sw2 (100 %). Additionally, ORF 119 showed sequence similarity to putative tail protein of *Salmonella* phage BSP22A (100 %).

Phage OSY-STA genome was expected to encode a spore cortex-lytic enzyme precursor (ORF 19), a holin (ORF 66) and an endolysin (ORF 67). All three ORFs would affect the host cell wall lysis ability of phage. The existence of both holing and endolysin encoding regions suggested current phage was involved in holin-endolysin system whereas some other phages such as *Salmonella* phage SE2 (Tiwari, et al., 2013) depends on only holin system to disrupt host cell membrane. Regarding the position of predicted prompter and terminator regions of the three cell lysis proteins, though with the same direction of gene transcription, the spore cortex-lytic enzyme precursor and holin-endolysin systems seemed to act independently on host cell lysis.

3.4.5 tRNA coding genes

A total of 24 tRNAs was identified in phage genome. Predicted positions and anticondons of tRNAs are summarized in **Table 3.2**.

Table 3.2 tRNA coding regions in phage genome.

tRNA	Begin	End	Type	Anticondon
1	14067	14141	Arg	TCT
2	17876	17953	Met	CAT
3	18505	18581	Leu	TAA
4	19506	19580	Tyr	GTA
5	19594	19670	Glu	TTC
6	19682	19755	Trp	CCA
7	19762	19836	Phe	GAA
8	20576	20651	Cys	GCA
9	20659	20741	Asn	GTT
10	21040	21116	Asp	GTC
11	21193	21268	Lys	CTT
12	22189	22265	Pro	TGG
13	22272	22350	Met	CAT
14	22538	22616	Lys	TTT
15	23085	23158	Val	TAC
16	23821	23895	Ala	TGC
17	24365	24441	Leu	TAG
18	25362	25438	His	GTG
19	25445	25519	Arg	ACG
20	25752	25827	Gln	CTG
21	25834	25909	Gln	TTG
22	25916	25990	Gly	TCC
23	26396	26470	Thr	TGT
24	26841	26916	Ile	GAT

3.5 Discussion

The phage OSY-STA showed high nucleotide similarity to *Salmonella* phage 3-29 and phage BSP22A. Conserved core genes related to replication and morphogenesis modules were observed in the genome of these three phages, which indicates these phages may share similar biological properties. However, pair-wise analysis of OSY-STA with one of the closest phylogenetic relatives, phage 3-29, revealed that phage OSY-STA has more than 35 % unique regions. Genes encoding regions present in phage OSY-STA are missing in 3-29 genome and vice versa, which may affect their infection ability. For example, the tail fiber protein was absent in phage 3-29 genome while present in phage OSY-STA (ORF 17). It has been reported that tail fiber protein is involved in both adsorption to the surface and degradation of the polysaccharide capsule, resulting in the penetration of host cells (Scholl et al., 2001). In other word, host reorganization and phage host specificity both highly depend on the expression of tail fiber protein genes. Regardless of the high DNA sequence homology shared between phages, small differences in tail fiber proteins could be associated with significant different host ranges.

It must be noted that a number of conserved hypothetical proteins were found in present study (107 ORFs out of 169 ORFs). Most of the hypothetical proteins from OSY-STA share homology with *Salmonella* bacteriophages while several hypothetical proteins may have been acquired from *Escherichia coli* virus. Particularly, in OSY-STA genome, one hypothetical protein (ORF 96) exhibited homology with *Yersinia* phage phiR201. This provides an indication of the evolution and the ancestry of this phage.

A comprehensive search of *Salmonella* phage with complete genome documented in NCBI Genebank database revealed that *Salmonella* phages have very genome sizes ranging from 21,437 bp (*Salmonella* phage 39, accession number: KR296693) to 240,413 bp (*Salmonella* phage SPN3US, accession number: JN641803.1). Typically, phages within the *Siphoviridae* family have an average genome size of 53.70 kb (Amarillas et al., 2017), therefore, the large genome size of phage OSY-STA is not common among members of this bacteriophage family. Few *Salmonella* phages belong to the same family with over 100 kb genome size were reported; these include *Salmonella* phage S116 (MH370369.1), *Salmonella* phage 3-29 (MK393882.1), *Salmonella* phage BSP22A (KY787212.1) and *Salmonella* phage Seafire (MK050846.1). It has been assumed that larger phage genome corresponds to more complex virion structure as well as phage metabolism process during the infection and replication cycle (Brown, 2012).

Compared to average GC content of *Salmonella* sp. (52.2 %), the molecular GC content of phage OSY-STA was calculated at 40.0 %, which is significantly lower than its host. Some studies suggested that GC content is associated with the codon usage of virus to optimize gene expression (Zuber et al., 2007). More specifically, lower GC content and higher AT content minimize the cost for living in the host cell and this feature also contributes to activate gene transcription of virus genome (Rocha and Danchin, 2002).

Furthermore, phage OSY-STA genome contains 24 tRNAs with various anticodons. Few studies have reported that partial phage-encoded tRNAs corresponds to codons seem to be more common in the phage than in the host (Amarillas et al., 2017;

Bailly-Bechet, et al., 2007). They claimed that the abundancy of certain tRNAs in phages allow them to gain a considerable benefit over their competitors by efficient translation of proteins, reduction of latency time and facilitation of reproduction rate. On the other hand, excessive codon usage in the phage compared to the host could supply particular tRNAs on its own, in case of the tRNA deficiency during translation. An assumption regarding the presence of tRNAs in virulent/lytic phage is that they are more likely to retain tRNA genes they acquire because of the lack of the ability to integrate phage genome to their host (Delesalle et al., 2016).

According to the prediction in PHACTS (Phage Classification Tool Set; http://edwards.sdsu.edu/PHACTS/upload.php), phage OSY-STA was considered as a lytic phage with a PHACTS output value of 0.504. In comparison, *Salmonella* phage BSP22A, which shares high sequence similarity with phage OSY-STA, was reported having strong lytic activity (Bai et al., 2019).

In conclusion, the assessment of phage genome revealed detailed information concerning its biology. Phage OSY-STA exhibited a modular organization which has not been observed in identified enterobacteriophages. Besides, several genes associated with cell lysis (endolysin and holin) were determined, and the exploration of those proteins could be useful as novel antimicrobials to control pathogenic bacteria.

Reference

Amarillas, L., Rubí-Rangel, L., Chaidez, C., González-Robles, A., Lightbourn-Rojas, L., León-Félix, J., 2017. Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrug-resistant *Escherichia coli*. Front. Microbiol. 8, 1–18. https://doi.org/10.3389/fmicb.2017.01355

Bai, J., Jeon, B., Ryu, S., 2019. Effective inhibition of *Salmonella* Typhimurium in fresh produce by a phage cocktail targeting multiple host receptors. Food Microbiol. 77, 52–60. https://doi.org/10.1016/j.fm.2018.08.011

Bailly-Bechet, M., Vergassola, M., Rocha, E., 2007. Causes for the intriguing presence of tRNAs in phages. Genome Res. 17, 1486–1495. https://doi.org/10.1101/gr.6649807

Brown, T. (2012). Introduction to Genetics: AMolecular Approach. New York, NY: Garland Science.

Cumby, N., Reimer, K., Mengin-Lecreulx, D., Davidson, A.R., Maxwell, K.L., 2015. The phage tail tape measure protein, an inner membrane protein and a periplasmic chaperone play connected roles in the genome injection process of *E.coli* phage HK97. Mol. Microbiol. 96, 437–447. https://doi.org/10.1111/mmi.12918

Delesalle, V.A., Tanke, N.T., Vill, A.C., Krukonis, G.P., 2016. Testing hypotheses for the presence of tRNA genes in mycobacteriophage genomes. Bacteriophage 6, e1219441. https://doi.org/10.1080/21597081.2016.1219441

Hatfult, G.F., Sarkis, G.J., 1993. DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol. Microbiol. 7, 395–405. https://doi.org/10.1111/j.1365-2958.1993.tb01131.x

Hendrix RW., 2002. Bacteriophages: evolution of the majority. Theor Popul Biol. 61:471–480.

Mahony, J., Alqarni, M., Stockdale, S., Spinelli, S., Feyereisen, M., Cambillau, C., Van Sinderen, D., 2016. Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. Sci. Rep. 6, 1–10. https://doi.org/10.1038/srep36667

Ramirez, K., Cazarez-Montoya, C., Lopez-Moreno, H.S., Castro-del Campo, N., 2018. Bacteriophage cocktail for biocontrol of *Escherichia coli* O157:H7: Stability and potential allergenicity study. PLoS One 13, 1–19. https://doi.org/10.1371/journal.pone.0195023

Rocha, C., Danchin, A., 2002. Base composition bias might result from competition for. Trends Genet. 18, 291–294.

Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchison, C.A., Slocombe, P.M., Smith, M., 1977. Nucleotide sequence of bacteriophage φX174 DNA. Nature 265, 687–695. https://doi.org/10.1038/265687a0

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F., Petersen, G.B., 1982. Nucleotide sequence of bacteriophage λ DNA. J. Mol. Biol. 162, 729–773. https://doi.org/https://doi.org/10.1016/0022-2836(82)90546-0

Scholl, D., Rogers, S., Adhya, S., Merril, C.R., 2002. Bacteriophage K1-5 encodes Two two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of *Escherichia coli*. J. Virol. 75, 2509–2515. https://doi.org/10.1128/jvi.75.6.2509-2515.2001

Tiwari, B.R., Kim, S., Kim, J., 2013. A virulent *Salmonella enterica* serovar Enteritidis phage SE2 with a strong bacteriolytic activity of planktonic and biofilmed cells. J. Bacteriol. Virol. 43, 186–194. https://doi.org/10.4167/jbv.2013.43.3.186

Zuber, S., Ngom-Bru, C., Barretto, C., Bruttin, A., Brüssow, H., Denou, E., 2007. Genome analysis of phage JS98 defines a fourth major subgroup of T4-like phages in *Escherichia coli*. J. Bacteriol. 189, 8206–8214. https://doi.org/10.1128/JB.00838-07

Chapter 4: Use of phage cocktail alone or in combination with heat against *Salmonella* Typhimurium and Enteritidis in liquid whole egg

4.1 Abstract

Egg and egg products were commonly identified as one of the vehicles in salmonellosis outbreaks. Several egg decontamination methods have proposed or implemented commercially to improve egg safety. However, the severity of some of these methods may negatively affect egg quality and functionality. Hence, a mild decontamination method, employing *Salmonella* phages, has been investigated in this study. Specifically, the effect of a phage cocktail (OSY-STA + OSY-SHC) i in combination with thermal treatment on the survival of *Salmonella* in liquid whole egg (LWE) was investigated. Compared with the initial phage titers, 82.5 % of Phage OSY-STA and 80.6 % of OSY-SHC were successfully recover from LWE after 24 h incubation at 4°C. Recovery tests of bacteriophages in LWE showed that phage titers would not be significantly infected when phages were incubated in liquid egg at 4°C for 24 h. No significant reduction in the population of *Salmonella* Typhimurium-contaminated LWE was observed, whereas phage cocktail resulted in 0.4 log reduction compared to non-phage treatment samples. When contaminated LWE was treated with the phage cocktail followed with a thermal treatment at 55 °C, this resulted in higher log reductions in *Salmonella* counts, compared to that resulting from the phage treatment or thermal treatment individually. Significant differences between thermal treatments and phage-thermal treatments was observed after samples were heated at 55 °C for 13 min, at

least. In particular, population of *Salmonella* Enteritidis was reduced from 2.8 $\log_{10}$ CFU/ml to undetectable level in LWE with the phage-thermal combined treatments. Our research shows that the phage cocktail has a potential effectiveness as a biocontrol agent of *Salmonella* in liquid whole eggs. In addition, phage application prior to thermal treatment could possibly reduce the severity of heat used in pasteurization yet achieve greater efficiency in eliminating *Salmonella* in egg products.

4.2 Introduction

Eggs have high nutritional values and play an important role as an ingredient in food industry. USDA Food Safety and Inspection Service estimated that an individual consumes on average 230 eggs per year without considering egg-containing products where eggs are consumed as a part of cake mixes, noodles, etc., and this number is continuously increasing year by year (FSIS, 2005). A USDA report has revealed that of the 76.2 billion eggs consumed in 2009, 30 % were in the form of egg products. Nowadays, egg products are used widely by the food industry in the form of liquid, dried or frozen products (USDA, 2009). Eggs have been considered as one of the most nutritious food that can be obtained at a low price. However, Eggs also are the most commonly implicated food in human salmonellosis outbreaks worldwide. As a common vehicle for *Salmonella* transmission, many researchers investigated the prevalence of *Salmonella* in commercial eggs. Vergara et al. (2016) reported that out of 341 samples, 10 different *Salmonella* isolates were obtained from the surface of eggs marketed at Ibague, Colombia. Moreover, *S.* Enteritidis (80 %) and *S.* Paratyphi B (10%) were the major serovars of *Salmonella* isolates. A similar study demonstrated that *Salmonella*

contamination was recorded in 6.1 % of egg shells and 1.8 % of egg contents in India, whereas no contamination was detected in Poland (Jamshidi et al., 2009). Although *Salmonella* prevalence in eggs may vary considerably between different countries and geographical regions, it is still the major concern for egg producers around the world. From 1985 to 2002, CDC estimated that approximately 53 % of the *Salmonella* outbreak cases were associated with egg contamination. There are two pathways for eggs to be internally contaminated with *Salmonella*. Direct contamination occurs in the reproductive organs during egg formation. *S.* Enteritidis was identifies as the most prevalence serotype isolated from egg contents, followed by *S.* Typhimurium (Whiley and Ross, 2015). On the other hand, *Salmonella* outside eggs may penetrate egg shell and membranes, reaching internal egg contents. Some studies suggested that high *Salmonella* incidence in egg may be due to the longtime of interactions between the hen and the egg after it has been laid (Namata et al., 2007).

Food industry put plenty of efforts to explore effective methods to control *Salmonella* through egg production processes. During primary production, daily cleaning and disinfection between flocks is the most common strategy. However, some studies pointed that most chicken houses cannot be cleaned effectively due to the insufficient application of disinfection agents, resulting in the rapid repopulation of *Salmonella* in a short time after routine cleaning (Davies and Breslin, 2004; Wales et al., 2007). In fact, areas such as cages, feeders of hen house are prone to residual contamination, which pose an early challenge to a new flock (Wales et al., 2007). In addition, Long et al. (2016) reported that *S.* Enteritidis and *S.* Typhimurium isolated from chicken and egg production

chains in Sichuan Province, China, exhibited high resistance to benzalkonium chloride and benzalkonium bromide, which are common disinfection agents used in egg production process. Similarly, the effectiveness of vaccination of hens can be highly variable since it only contributes to increasing the immunity of the hens against particular *Salmonella* serotypes (Berghaus et al., 2011). The problems resulting from antibiotic treatments in hen house could be more controversial. Diarra et al. (2014) investigated the antibiotic resistance and genotype of *Salmonella* isolated from broiler production facilities in Canada, and the results showed that more than 43 % of the isolates were simultaneously resistant to 5 different antibiotics.

In addition to the efforts aimed at controlling *Salmonella* preventions conducted in chicken houses, other strategies at the post-harvest stages have been explored. For example, egg washing technology has been optimized by adjusting washing temperature and pressure (Hutchison et al., 2004), or enhanced by combining chemical compound in washing solutions (Wang and Slavik, 1998). However, some researchers debated that this process may transfer *Salmonella* from the surface to the egg contents and cause cross-contamination. Thermal and irradiation treatments were tested in both shell eggs and egg products to control *Salmonella*. Although these processes were considered as the most potential methods for egg decontamination, yet, the adverse effects on egg properties resulted from irradiation and thermal pasteurization are considerable.

Recently, phage application in the egg production chains has attracted the attention of many investigators. Some researchers reported that a minor reduction of bacterial concentration could be observed in liquid egg products after phage treatments

(Spricigo et al., 2013; Hong et al., 2016). Obviously, the effectiveness of phage application in liquid egg is not as high as that could be achieved in other food matrices because of the high viscosity of eggs. Hence, we hypothesize that the overall efficacy of phage biocontrol in liquid egg products could be improved with the combination of mild heating.

The purpose of this study was to evaluate the effectiveness of a phage cocktail treatments with subsequent heating process against *S.* Enteritidis and *S.* Typhimurium in artificially contaminated liquid whole egg. To the best of author's knowledge, there has been no work reported on the application of bacteriophages and thermal treatments together for egg decontamination.

4.3 Materials and methods

4.3.1 Recovery test

To determine the viability of phages in liquid whole egg, 100 μl of pure phage suspension was added into 900 μl liquid whole egg, followed by incubation at 4 °C for 24 h. Samples were serially diluted and viable phage titers in liquid egg after incubation were determined using double agar overlay assay. Initial phage titers before the addition of phage to liquid egg were measured as controls. Viability of phage titer was calculated as follow:

$$\text{Phage Viability (\%)} = \frac{\text{Viable phage titers after treatment}}{\text{Phage titers before treatment}} \times 100\%$$

4.3.2 Egg inoculation

Eggs used in this study were obtained from local egg producers (Three Family Farm, OH). Prior to the assays, 5 eggs were randomly sampled, and eggs were soaked in 70 % ethanol for few seconds. Air-dried shell eggs were broken into a sterile stomacher bag (FIsherBrand®, Fisher Scientific, Fairlawn, NJ) and homogenized for 40 s using a bench-top homogenizer (Masticator, IUL Instruments, Barcelona, Spain). Aliquots (10 ml each) of homogenized liquid egg samples transferred to 15 ml centrifuge tube, and the samples were inoculated with 100 µl of 10^5 CFU of *S.* Typhimurium or *S.* Enteritidis suspended in of 0.9 % NaCl. The mixture was vortexed (Votex-genie G-500, Scientific industries, INC., Bohemia, NY). All samples were placed at room temperature for 20 min to allow the bacterium to adapt to the liquid egg and distribute evenly in the product.

4.3.3 Phage cocktail treatment

Ten ml of liquid whole egg samples assigned for phage treatments were treated with 10^7 of phage cocktail OSY-STA and OSY-SHC (1:1) in 100 µl of BPW, resulting in the final phage concentration of 10^5 in experimental samples, and control samples received 100 µl of BPW only. Samples were vortexed after inoculation and incubated at 4 °C for 24 h. For enumeration of *Salmonella* population, 100 µl of inoculated sample was plated onto Xylose-Lysine-Tergitol 4 agar plates (XLT-4; Becton, Dickinson and Company, Sparks, MD), followed by incubation at 37 °C for 16 -18 h.

4.3.4 Phage treatment combined with mild heating

Liquid whole egg samples were prepared as described in the previous section. Samples undergone phage treatment were inoculated with *S.* Typhimurium or *S.* Enteritidis and phage cocktail suspension, whereas control samples received the same

volume of BPW. After 24 h incubation at 4 °C, 10 ml of each samples in 15 ml centrifuge tubes were moved to a water bath set at 55°C and heated for 3.5, 7, 10 and 13 min. At each designed heating time, one phage-treated sample and one untreated sample (control) were transferred from the water bath to ice bath and held for 5 min. Surviving *Salmonella* populations were enumerated after spreading on XLT-4 plates and incubating at xxx37°C for xxx 24 h.. Flow chart of phage cocktail treatment and combined phage and heating treatments in liquid whole egg are shown in **Figure 4.1**.

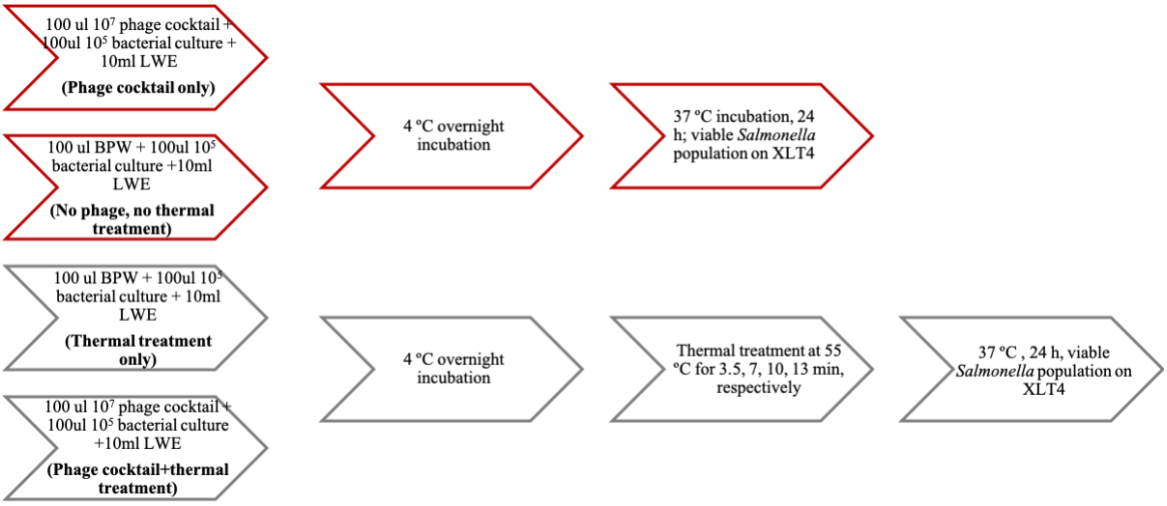


Figure 4.1 Flow chart of phage cocktail and phage cocktail-heatl treatments in liquid whole eggs.

4.3.5 Statistical analysis

Phage titer and bacterial population were determined by double agar overlay method and plate counting, respectively. All experiments were independently performed

in triplicates. The data obtained were analyzed by one-way analysis of variance (ANOVA) when phage was the only factor or two-way analysis of variance (ANOVA) if both time and phage treatments influenced experimental results. Analyses was carried out using (JMP 14, SAS Institute, Inc., Cary, NC). All results were presented as means, and error bars indicated the standard deviation. Statistical differences between the mean values were analyzed using Tukey's test. A value of $P < 0.05$ was considered a statistically significant difference.

4.4 Results

4.4.1 Recovery test

Table 4.1 Phage titer in liquid whole egg after 24 h incubation at 4°C

Phage ID	Viable phage titer after 24 h (log_{10} PFU/ml)		Percentage of viable phage titer in LWE and control
	Control[a]	LWE[b]	
OSY-STA	5.2±0.01	5.1±0.05	82.5 %
OSY-SHC	6.9±0.1	6.8±0.2	80.6 %

[a] Initial phage titer, phage suspended in Buffered Peptone Water (BPW).
[b] Phage titer was measured after 24 h incubation at 4 °C, phage suspended in liquid whole egg
[c] Data reported are means ± standard deviations of three independent trial.

Viable phage titers after incubation in liquid whole egg for 24 h at 4 °C are presented in **Table 4.1**. Approximately 82.5% of OSY-STA and 80.6 % of OSY-SHC were still detectable after 24 h. Both isolates survived in the food matrices without a significant reduction in their titers, which indicated the applicability of the phage cocktail intended to be used as biocontrol tools in liquid whole egg.

4.4.2 Application of the phage cocktail as a biocontrol agent against *Salmonella* in liquid whole egg

An initial trial was performed to evaluate the ability of phage cocktail (OSY-STA+OSY-SHC) to lyse *S.* Typhimurium and *S.* Enteritidis in liquid whole egg. Ten ml liquid egg sample was inoculated with 10^5 CFU/ml *S.* Typhimurium or *S.* Enteritidis. After 1-day incubation at 4 °C, phage cocktail significantly reduced *S.* Enteritidis counts by 0.4 $\log_{10}$ CFU/ml, compared to phage-free control at a MOI of 10^2 ($P < 0.05$). In contrast, phage cocktail only caused a minor reduction in the population of *S.* Typhimurium (**Figure 4.2**). Therefore, the results suggested that when presented at a MOI of 100, phage cocktail could not efficiently eliminate *Salmonella* cells in liquid egg samples. Additionally, *S.* Enteritidis strains used in this study seemed to be more susceptible than *S.*Typhimurium to phage treatment.

Low MOI of phage application would be preferred in the food industry for economic reasons. We further investigated effectiveness of the combination of phage application with thermal treatment – a traditional liquid egg decontamination method that has been widely implemented.

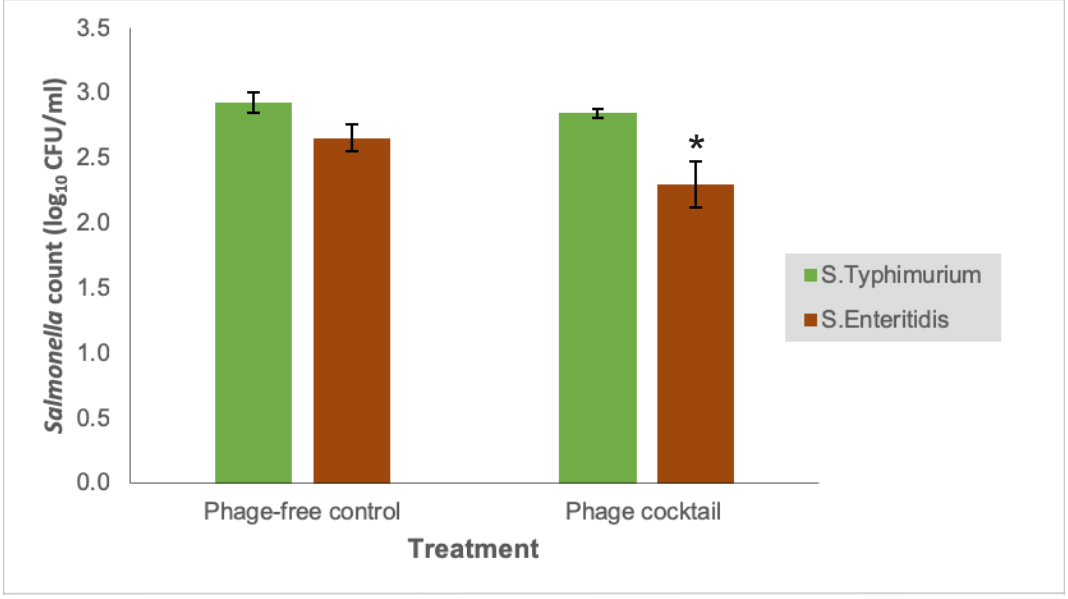
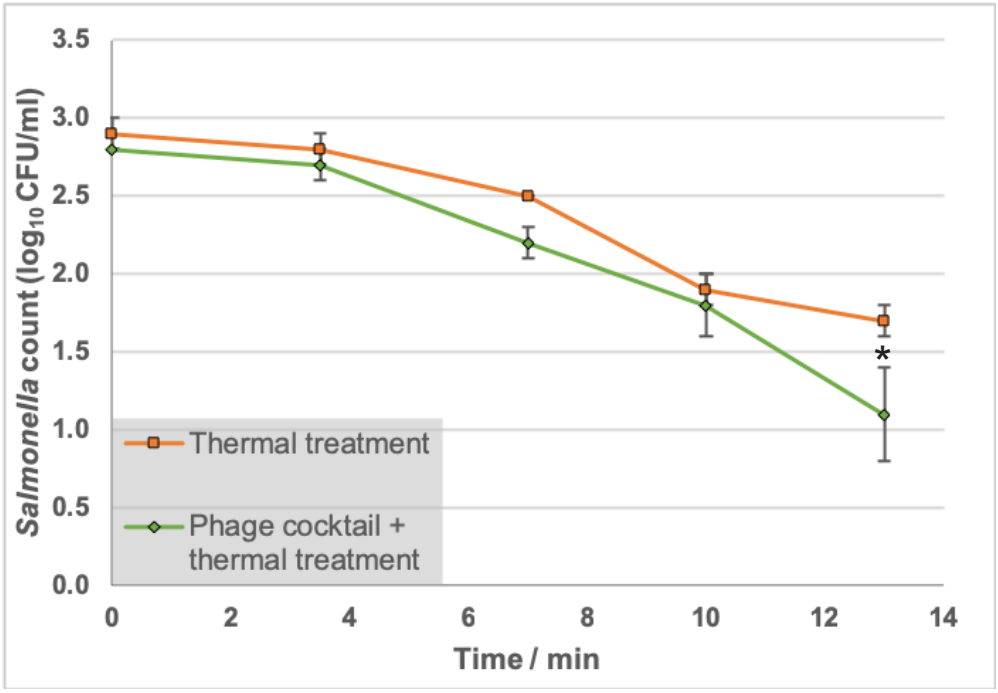


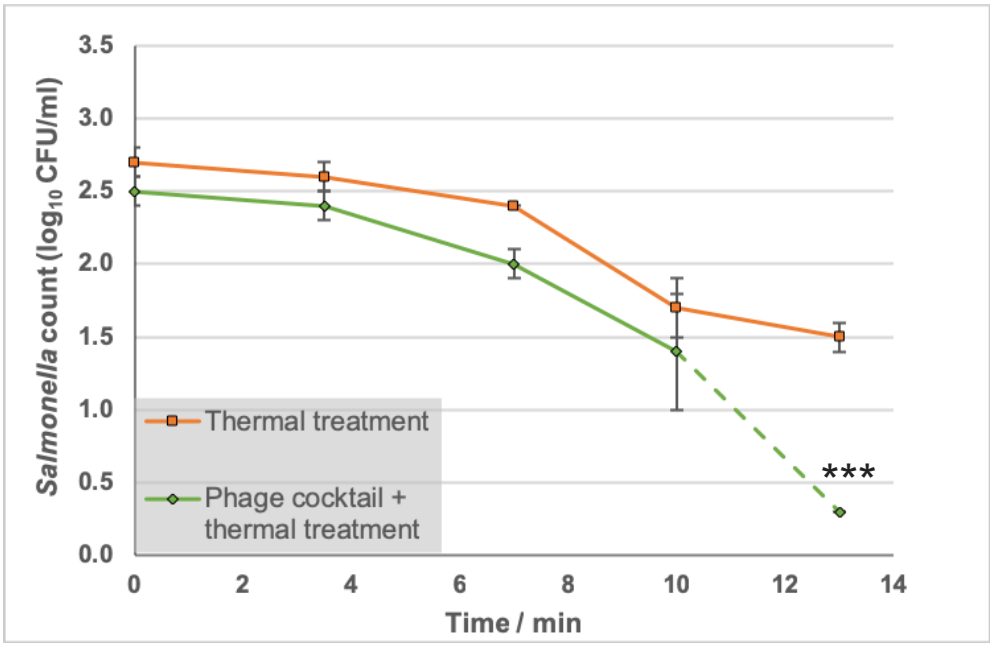
Figure 4.2 Lytic effects of the phage cocktail (OSY-STA+OSY-SHC) against specific *Salmonella* serotypes in liquid whole eggs at held 4 °C. Data reported are means ± standard deviations of three independent trials. Standard deviation of each treatment is indicated by error bars.

4.4.3 Efficiency of phage cocktail combined with heat treatment at 55 °C against *Salmonella* in liquid whole egg

Before thermal treatment, liquid egg samples were inoculated with *Salmonella* and then treated with the phage cocktail or BPW (control) for 24 h at 4°C. Heating temperature was set at 55°C. Data reported in **Figure 4.3** illustrate the *Salmonella* survivors after 3.5, 7, 10 and 13 min of heating.



(a)



(b)

Figure 4.3 Effects of phage cocktail and thermal treatment at 55 °C on *Salmonella* serotypes with different treatment time. (a) *Salmonella* Typhimurium LT2; (b)

Salmonella Enteritidis 99-30581-13. Data reported are means ± standard deviations of three independent trials. Standard deviation of each treatment is indicated by error bars.

All trials with phage-heat treatment showed greater efficiency to reduce *Salmonella* counts compared to heat-only trials. As expected, longer heating time produced greater degree of inactivation. Minor differences in population of *S.* Typhimurium were observed in treatments with heat and phage-heat when the heating was applied for 3.5, 7 and 10 min; the maximum difference was approximately 0.3 $\log_{10}$ CFU/ml. After extended heating for 13 min, *Salmonella* Typhimurium population decreased significantly ($P < 0.05$) by the phage-heat combination, compared to heat-only, with the decease reaching 0.9 $\log_{10}$ CFU/ml. Moreover, *Salmonella* population decreased to undetectable level (no S*almonella* colony was observed on the lowest dilution (10^0) XLT-4 plate; *Salmonella* count < 10 CFU/ml) in 1 2 out of the 3 replicates in this experiment. In addition, Figure 4.3 revealed shows that at least 10-min heat treatment was required to achieve 1 log reduction in samples with or without phage treatments when heating temperature was 55 °C.

Higher log reduction in *Salmonella* Enteritidis population was observed at each thermal treatment time point (treatment time > 3.5 min) than that in *Salmonella* Typhimurium population, thus, we assumed that *S.* Enteritidis displayed greater susceptibility to subsequent heat treatment after applying phage cocktail application than did *S.* Typhimurium. Average 0.3 log reduction in *S.* Enteritidis counts was achieved with combined treatment within 10 min in contrast to heat treatment only for same time

period. The bacterial counts in samples treated with phage cocktail first, followed by heating process for 13 min significantly decreased to undetectable levels ($P < 0.001$; *Salmonella* count < 10 CFU/ml), while 1.5 $\log_{10}$ CFU/ml of bacteria was still presented in samples treated with 55 °C heating only.

4.5 Discussion

Use of phage cocktail to control *Salmonella* contamination in liquid whole egg was evaluated in this study. Population of the pathogen in samples *S.* Enteritidis-contaminated liquid egg that was treated with phage cocktail were significantly lower than those in control samples by 0.4 $\log_{10}$ CFU/ml after incubation at 4 °C for 24 h, whereas no significant reduction was observed in *S.* Typhimurium-contaminated liquid egg samples treated under same conditions. Opposite results were obtained by Hong et al. (2016). They reported that under refrigeration, the concentrations of *S.* Typhimurium in phage-treated liquid eggs were lower than control samples by 0.61 $\log_{10}$ CFU/ml, and there was no difference in the population of *S.* Enteritidis between phage-treated and untreated samples at 4 °C after 24 h. Considering different host strains and phages used in their study from present study, it is possible that phage applications exhibited different efficiency against same *Salmonella* serovar. Efficacy of a same phage was tested on different food matrices including turkey deli meat, chocolate milk, hot dogs, seafood and egg yolk (Guenther et al., 2012). These researchers used high MOI (10^5 PFU/g?) for a one-day treatment at 15 °C. They observed an overall reduction by at least 2 logs in *Salmonella* population in all tested foods except egg yolk which produced less than 1 log reduction. This showed that phage application was not very effective in liquid egg

compared to that in other food matrices. This observation may be an outcome of the differences in phage accessibility to the cells of targeted pathogen; easier accessibility is expected on solid than in liquid foods. Viscosity of liquid egg may also have played a factor in limiting this accessibility. Therefore, it is necessary to ensure the even distribution and sufficient diffusion of virus particles in order to achieve ideal pathogen decontamination efficiency (Guenther et al., 2009).

Although the ability of phage applications in liquid eggs is restricted, bacterial cells could be stressed during phage treatments.

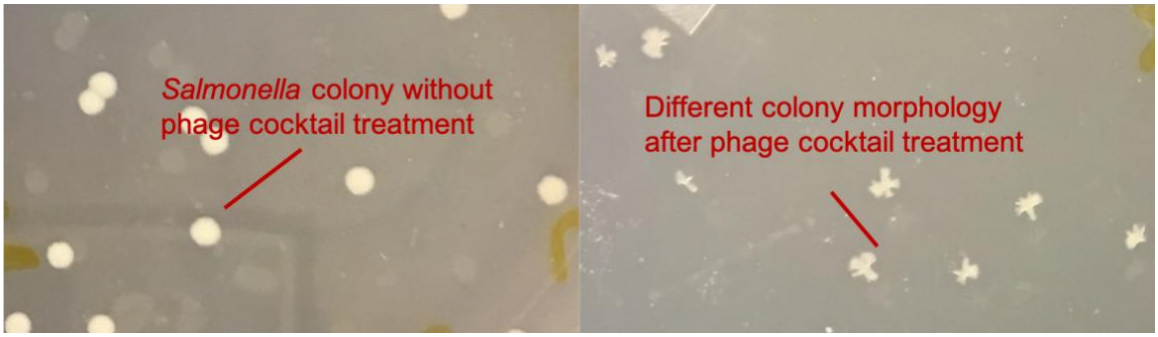


Figure 4.4 Morphology of *Salmonella* Typhimurium colonies. Left: non-phage treated *Salmonella* sample after incubation at 4 °C for 24 h. Right: *Salmonella* was treated with phage cocktail and incubated at 4 °C for 24 h.

As shown in **Figure 4.4**, after phage cocktail treatment, most colonies of *S.* Typhimurium appeared to be damaged and presented as irregular shape. On contrary,

typical flat, round shape colonies were identified in non-phage treated *Salmonella* samples.

Injured bacterial cells are still able to resuscitate and repair their functionalities under favorable environments. These cells, however, may lose some distinctive qualities under sublethal stress, which makes them more susceptible to other treatments such as heat (Wu, 2018).

In the present study, we treated *Salmonella*-contaminated liquid eggs with phage cocktail at 4°C for 24 h, followed by heat treatment at 55°C to assess the combined efficiency of the multiple treatments. Liquid egg samples were heated for at least 3.5 min according to the minimal liquid egg pasteurization requirements of USDA (60 °C for 3.5 min). Significant differences in *Salmonella* survivors between the combination treatments and heat treatment alone were observed until samples were heated for up to 13 min. In addition, population of *S.* Enteritidis were reduced from 2.7 $\log_{10}$ CFU/ml to undetectable level when the phage cocktail treatment was followed by 13-min of heating at 55°C. The come-up period required for liquid egg samples to reach 55 °C may affect the total heating time needed for eliminating Salmonella.

This work demonstrates the efficacy of a lytic phage cocktail and its combination with heat treatment process to control *S.* Typhimurium and *S.* Enteritidis in liquid whole egg. Our findings show the combination treatments could considerably reduce *Salmonella* concentration in foods. Further studies should focus on the optimization of both heat treatment timing and phage cocktail concentration.

Reference

Berghaus, R.D., Thayer, S.G., Maurer, J.J., Hofacre, C.L., 2011. Effect of vaccinating breeder chickens with a killed *Salmonella* vaccine on *Salmonella* prevalences and loads in breeder and broiler chicken flocks. J. Food Prot. 74, 727–734. https://doi.org/10.4315/0362-028x.jfp-10-542

Moreno Switt, A.I., den Bakker, H.C., Vongkamjan, K., Hoelzer, K., Warnick, L.D., Cummings, K.J., Wiedmann, M., 2013. *Salmonella* bacteriophage diversity reflects host diversity on dairy farms. Food Microbiol. 36, 275–285. https://doi.org/10.1016/j.fm.2013.06.014

Davies, R., Breslin, M., 2004. Observations on *Salmonella* contamination of eggs from infected commercial laying flocks where vaccination for *Salmonella enterica* serovar Enteritidis had been used. Avian Pathol. 33, 133–144. https://doi.org/10.1080/03079450310001652040

Diarra, M.S., Delaquis, P., Rempel, H., Bach, S., Harlton, C., Aslam, M., PRITCHARD, J., Topp, E., 2014. Antibiotic Resistance and diversity of *Salmonella enterica* Serovars associated with broiler chickens. J. Food Prot. 77, 40–49. https://doi.org/10.4315/0362-028.JFP-13-251

Fiorentin, L., Vieira, N.D., Barioni, W., 2005. Oral treatment with bacteriophages reduces the concentration of *Salmonella* Enteritidis PT4 in caecal contents of broilers. Avian Pathol. 34, 258–263. https://doi.org/10.1080/01445340500112157

Fsis, U., 2009. Egg Products and Food Safety 1–4.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol. 154, 66–72. https://doi.org/10.1016/j.ijfoodmicro.2011.12.023

Guenther, S., Huwyler, D., Richard, S., Loessner, M.J., 2009. Virulent bacteriophage for efficient biocontrol of *listeria monocytogenes* in ready-to-eat foods. Appl. Environ. Microbiol. 75, 93–100. https://doi.org/10.1128/AEM.01711-08

Hutchison, M.L., Gittins, J., Walker, A., Sparks, N., Humphrey, T.J., Burton, C., Moore, A., 2016. An A assessment of the microbiological risks involved with egg washing under commercial conditions. J. Food Prot. 67, 4–11. https://doi.org/10.4315/0362-028x-67.1.4

Jamshidi, A., Kalidari, G.A., Hedayati, M., 2010. Isolation and identification of *Ssalmonella* e Enteritidis and *Salmonella* Typhimurium from the eggs of retail stores

in Mashhad, Iran using conventional culture method and multiplex pcr assay. J. Food Saf. 30, 558–568. https://doi.org/10.1111/j.1745-4565.2010.00225.x

Long, M., Lai, H., Deng, W., Zhou, K., Li, B., Liu, S., Fan, L., Wang, H., Zou, L., 2016. Disinfectant susceptibility of different *Salmonella* serotypes isolated from chicken and egg production chains. J. Appl. Microbiol. 121, 672–681. https://doi.org/10.1111/jam.13184

Mogollon-Vergara, D.C., Rodriguez-Gutierrez, V.E., Verjan-Garcia, N., 2016. Prevalence and molecular identification of *Salmonella* spp. isolated from commercialized eggs at Ibague, Colombia. Rev. Salud Anim. 38, 1–10.

Namata, H., Méroc, E., Aerts, M., Faes, C., Abrahantes, J.C., Imberechts, H., Mintiens, K., 2008. Salmonella in Belgian laying hens: An identification of risk factors. Prev. Vet. Med. 83, 323–336. https://doi.org/10.1016/j.prevetmed.2007.09.002

Service, I., 1970. Risk Assessments for *Salmonella* Enteritidis in Shell Eggs and *Salmonella* spp. in Egg Products Executive Summary 2–5.

Spricigo, D.A., Bardina, C., Cortés, P., Llagostera, M., 2013. Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry. Int. J. Food Microbiol. 165, 169–174. https://doi.org/10.1016/j.ijfoodmicro.2013.05.009

Wales, A., Breslin, M., Carter, B., Sayers, R., Davies, R., 2007. A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. Avian Pathol. 36. https://doi.org/10.1080/03079450701338755

Wang, H., Slavik, M.E., 2016. Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. J. Food Prot. 61, 276–279. https://doi.org/10.4315/0362-028x-61.3.276

Whiley, H., Ross, K., 2015. *Salmonella* and eggs: From production to plate. Int. J. Environ. Res. Public Health 12, 2543–2556. https://doi.org/10.3390/ijerph120302543

Wu, V.C.H., 2008. A review of microbial injury and recovery methods in food. Food Microbiol. 25, 735–744. https://doi.org/10.1016/j.fm.2008.04.011

List of reference

Abedon, S.T., Hyman, P., Thomas, C., 2003. Experimental examination of bacteriophage latent-period evolution as a response to bacterial availability. Appl. Environ. Microbiol. 69, 7499–506. https://doi.org/10.1128/AEM.69.12.7499

Abuladze, T., Li, M., Menetrez, M.Y., Dean, T., Senecal, A., Sulakvelidze, A., 2008. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 74, 6230–8. https://doi.org/10.1128/AEM.01465-08

Ackermann H.W. (2009) Phage Classification and Characterization. P127-140. In: Clokie M.R., Kropinski A.M. (eds) Bacteriophages. Methods and protocols, vol 1: Isolatin, Characterization, and Interation. Humana Press. https://doi.org/10.1007/978-1-60327-164-6_13

Amarillas, L., Rubí-Rangel, L., Chaidez, C., González-Robles, A., Lightbourn-Rojas, L., León-Félix, J., 2017. Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrug-resistant *Escherichia coli*. Front. Microbiol. 8, 1–18. https://doi.org/10.3389/fmicb.2017.01355

Andreatti Filho, R.L., Higgins, J.P., Higgins, S.E., Gaona, G., Wolfenden, A.D., Tellez, G., Hargis, B.M., 2007. Ability of bacteriophages isolated from different sources to reduce *Salmonella enterica* serovar Enteritidis *in vitro* and in vivo. Poult. Sci. 86 (9), 1904-1909. https://doi.org/10.1093/ps/86.9.1904

Atterbury, R.J., Van Bergen, M.A.P., Ortiz, F., Lovell, M.A., Harris, J.A., De Boer, A., Wagenaar, J.A., Allen, V.M., Barrow, P.A., 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl. Environ. Microbiol. 73, 4543–4549. https://doi.org/10.1128/AEM.00049-07

Badr, H.M., 2006. Effect of gamma radiation and cold storage on chemical and organoleptic properties and microbiological status of liquid egg white and yolk. Food Chem. 97, 285–293. https://doi.org/10.1016/j.foodchem.2005.05.004

Bai, J., Jeon, B., Ryu, S., 2019. Effective inhibition of *Salmonella* Typhimurium in fresh produce by a phage cocktail targeting multiple host receptors. Food Microbiol. 77, 52–60. https://doi.org/10.1016/j.fm.2018.08.011

Bailly-Bechet, M., Vergassola, M., Rocha, E., 2007. Causes for the intriguing presence of tRNAs in phages. Genome Res. 17, 1486–1495. https://doi.org/10.1101/gr.6649807

Bao, H., Zhang, P., Zhang, H., Zhou, Y., Zhang, L., Wang, R., 2015. Bio-control of s*almonella* Enteritidis in foods using bacteriophages. Viruses 7, 4836–4853. https://doi.org/10.3390/v7082847

Bardina, C., Spricigo, D.A., Cortés, P., Llagostera, M., 2012. Significance of the bacteriophage treatment schedule in eeducing *Salmonella* colonization of Poultry. Appl. Environ. Microbiol. 78, 6600–6607. https://doi.org/10.1128/aem.01257-12

Berghaus, R.D., Thayer, S.G., Maurer, J.J., Hofacre, C.L., 2011. Effect of vaccinating breeder chickens with a killed *Salmonella* vaccine on *Salmonella* prevalences and loads in breeder and broiler chicken flocks. J. Food Prot. 74, 727–734. https://doi.org/10.4315/0362-028x.jfp-10-542

Bertani, G. 1973. Lysogenic Versus Lytic Cycle Phage Multiplication. Cold Spring Harb Symp Quant Biol 1953. 18: 65-70. https:// doi:10.1101/SQB.1953.018.01.014

Bielke, L., Higgins, S., Donoghue, A., Donoghue, D., Hargis, B.M., 2007. *Salmonella* host range of bacteriophages that infect multiple genera. Poult. Sci. 86, 2536–2540. https://doi.org/10.3382/ps.2007-00250

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G. V., Heinemann, J.A., 2008. Phage inactivation of foodborne pathogens on cooked and raw meat. Food Microbiol. 25, 400–406. https://doi.org/10.1016/j.fm.2007.11.003

Brown, T. (2012). Introduction to Genetics: AMolecular Approach. New York, NY: Garland Science.

Cao, W., Zhu, Z.W., Shi, Z.X., Wang, C.Y., Li, B.M., 2009. Efficiency of slightly acidic electrolyzed water for inactivation of *Salmonella* enteritidis and its contaminated shell eggs. Int. J. Food Microbiol. 130, 88–93. https://doi.org/10.1016/j.ijfoodmicro.2008.12.021

Carey-Smith, G. V., Billington, C., Cornelius, A.J., Hudson, J.A., Heinemann, J.A., 2006. Isolation and characterization of bacteriophages infecting *Salmonella* spp. FEMS Microbiol. Lett. 258, 182–186. https://doi.org/10.1111/j.1574-6968.2006.00217.x

Carter, C.D., Parks, A., Abuladze, T., Li, M., Woolston, J., Magnone, J., Senecal, A., Kropinski, A.M., Sulakvelidze, A., 2012. Bacteriophage cocktail significantly reduces *Escherichia coli* O157. Bacteriophage 2, 178–185. https://doi.org/10.4161/bact.22825

CDC, 2012. *Salmonella* Bareilly and Nchanga Infections Associated with a Raw Scraped Ground Tuna Product - *Salmonella* [WWW Document], n.d. URL https://www.cdc.gov/salmonella/bareilly-04-12/index.html (accessed 7.29.19).

CDC, 2017. Pathogens Transmitted by Food Contaminated by Infected Persons Who Handle Food, and Modes of Transmission of Such Pathogens.

CDC, 2018. Reports of *Salmonella* Outbreak Investigations from 2018. URL https://www.cdc.gov/salmonella/outbreaks-2018.html (accessed 5.18.19).

CDC, 2018. Salmonellosis (Nontyphoidal) - Chapter 3, 2018 Yellow Book -Travelers' Health | CDC [WWW Document], n.d. URL https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/salmonellosis-nontyphoidal (accessed 6.6.19).

CDC, 2019. Outbreak of *Salmonella* Infections Linked to Frozen Raw Tuna - Outbreak of *Salmonella* Infections Linked to Frozen Raw Tuna. URL https://www.cdc.gov/salmonella/newport-04-19/index.html (accessed 7.29.19).

Chen, Y., Sun, E., Song, J., Tong, Y., Wu, B., 2018. Three *Salmonella enterica* serovar Enteritidis bacteriophages from the *Siphoviridae* family are promising candidates for phage therapy. Can. J. Microbiol. 64, 865–875. https://doi.org/10.1139/cjm-2017-0740

Clark, S., 2014. Food Processing: Principles and Applications. John Wiley & Sons, Ltd, Hoboken, NJ.

Clavijo, V., Baquero, D., Hernandez, S., Farfan, J.C., Arias, J., Arévalo, A., Donado-Godoy, P., Vives-Flores, M., 2019. Phage cocktail SalmoFREE® reduces Salmonella on a commercial broiler farm. Poult. Sci. https://doi.org/10.3382/ps/pez251

Clokie, M.R.J., Millard, A.D., Letarov, A. V., Heaphy, S., 2011. Phages in nature. Bacteriophage 1, 31–45. https://doi.org/10.4161/bact.1.1.14942

Cumby, N., Reimer, K., Mengin-Lecreulx, D., Davidson, A.R., Maxwell, K.L., 2015. The phage tail tape measure protein, an inner membrane protein and a periplasmic chaperone play connected roles in the genome injection process of *E.coli* phage HK97. Mol. Microbiol. 96, 437–447. https://doi.org/10.1111/mmi.12918

Davies, R., Breslin, M., 2004. Observations on *Salmonella* contamination of eggs from infected commercial laying flocks where vaccination for *Salmonella enterica* serovar Enteritidis had been used. Avian Pathol. 33, 133–144. https://doi.org/10.1080/03079450310001652040

Delesalle, V.A., Tanke, N.T., Vill, A.C., Krukonis, G.P., 2016. Testing hypotheses for the presence of tRNA genes in mycobacteriophage genomes. Bacteriophage 6, e1219441. https://doi.org/10.1080/21597081.2016.1219441

Diarra, M.S., Delaquis, P., Rempel, H., Bach, S., Harlton, C., Aslam, M., PRITCHARD, J., Topp, E., 2014. Antibiotic Resistance and diversity of *Salmonella enterica* Serovars associated with broiler chickens. J. Food Prot. 77, 40–49. https://doi.org/10.4315/0362-028.JFP-13-251

Duc, H.M., Son, H.M., Honjoh, K. ichi, Miyamoto, T., 2018. Isolation and application of bacteriophages to reduce *Salmonella* contamination in raw chicken meat. LWT - Food Sci. Technol. 91, 353–360. https://doi.org/10.1016/j.lwt.2018.01.072

El-Dougdoug, N.K., Cucic, S., Abdelhamid, A.G., Brovko, L., Kropinski, A.M., Griffiths, M.W., Anany, H., 2019. Control of *Salmonella* Newport on cherry tomato using a cocktail of lytic bacteriophages. Int. J. Food Microbiol. 293, 60–71. https://doi.org/10.1016/j.ijfoodmicro.2019.01.003

Ellis E.L, Delbruck M. (1939) The growth of bacteriophage. J Gen Physiol. 22:365–84.

Eng, S.K., Pusparajah, P., Ab Mutalib, N.S., Ser, H.L., Chan, K.G., Lee, L.H., 2015. *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. Front. Life Sci. 8, 284–293. https://doi.org/10.1080/21553769.2015.1051243

European Food Safety Authority. 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16. https://doi.org/10.2903/j.efsa.2018.5500

Fiorentin, L., Vieira, N.D., Barioni, W., 2005. Oral treatment with bacteriophages reduces the concentration of *Salmonella* Enteritidis PT4 in caecal contents of broilers. Avian Pathol. 34, 258–263. https://doi.org/10.1080/01445340500112157

Friesema, I.H., de Jong, A.E., Fitz James, I.A., Heck, M.E., van den Kerkhof, J.H., Notermans, D.W., van Pelt, W., Hofhuis, A., 2012. Outbreak of *Salmonella* Thompson in the Netherlands since July 2012. Eurosurveillance 17, 1–4.

Fsis, U., 2009. Egg Products and Food Safety 1–4.

Galarce, N.E., Bravo, J.L., Robeson, J.P., Borie, C.F., 2015. Bacteriophage cocktail reduces *Salmonella enterica* serovar Enteritidis counts in raw and smoked salmon tissues. Rev. Argent. Microbiol. 46, 333–337. https://doi.org/10.1016/s0325-7541(14)70092-6

Gantois, I., Eeckhaut, V., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2008. A comparative study on the pathogenesis of egg contamination by different serotypes of *Salmonella*. Avian Pathol. 37, 399–406. https://doi.org/10.1080/03079450802216611

Garcia-Gonzalez, L., Geeraerd, A.H., Elst, K., Van Ginneken, L., Van Impe, J.F., Devlieghere, F., 2009. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. J. Supercrit. Fluids 51, 74–82. https://doi.org/10.1016/j.supflu.2009.06.020

Grant, A., Hashem, F., Parveen, S., 2016. *Salmonella* and *Campylobacter*: Antimicrobial resistance and bacteriophage control in poultry. Food Microbiol. 53, 104–109. https://doi.org/10.1016/j.fm.2015.09.008

Grant, A., Parveen, S., Schwarz, J., Hashem, F., Vimini, B., 2017. Reduction of Salmonella in ground chicken using a bacteriophage. Poult. Sci. 96, 2845–2852. https://doi.org/10.3382/ps/pex062

Gu, L., Zhou, S., Zhu, L., Liang, C., Chen, X., 2015. Small-Molecule inhibitors of the type III secretion system. Molecules 20, 17659–17674. https://doi.org/10.3390/molecules200917659

Guard-Petter, J., 2001. Guard-Petter J: The chicken, the egg and *Salmonella* enteritidis. Environ. Microbiol. 3, 421–430.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol. 154, 66–72. https://doi.org/10.1016/j.ijfoodmicro.2011.12.023

Guenther, S., Huwyler, D., Richard, S., Loessner, M.J., 2009. Virulent bacteriophage for efficient biocontrol of *listeria monocytogenes* in ready-to-eat foods. Appl. Environ. Microbiol. 75, 93–100. https://doi.org/10.1128/AEM.01711-08

Hanlon, G.W., 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int. J. Antimicrob. Agents 30, 118–128. https://doi.org/10.1016/j.ijantimicag.2007.04.006

Hatfult, G.F., Sarkis, G.J., 1993. DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol. Microbiol. 7, 395–405. https://doi.org/10.1111/j.1365-2958.1993.tb01131.x

Hendrix RW., 2002. Bacteriophages: evolution of the majority. Theor Popul Biol. 61:471–480.

Heyse, S., Hanna, L.F., Woolston, J., Sulakvelidze, A., Charbonneau, D., 2015. Bacteriophage Cocktail for Biocontrol of *Salmonella* in Dried Pet Food. J. Food Prot. 78, 97–103. https://doi.org/10.4315/0362-028x.jfp-14-041

Hong, Y., Schmidt, K., Marks, D., Hatter, S., Marshall, A., Albino, L., Ebner, P., 2016. Treatment of *Salmonella* -contaminated eggs and pork with a broad-spectrum, single bacteriophage: assessment of efficacy and resistance development. Foodborne Pathog. Dis. 13, 679–688. https://doi.org/10.1089/fpd.2016.2172

Huang, C., Shi, J., Ma, W., Li, Z., Wang, J., Li, J., Wang, X., 2018. Isolation, characterization, and application of a novel specific *Salmonella* bacteriophage in different food matrices. Food Res. Int. 111, 631–641. https://doi.org/10.1016/j.foodres.2018.05.071

Huang, C., Virk, S.M., Shi, J., Zhou, Y., Willias, S.P., Morsy, M.K., Abdelnabby, H.E., Liu, J., Wang, X., Li, J., 2018. Isolation, characterization, and application of bacteriophage LPSE1 against *Salmonella enterica* in Ready to Eat (RTE) foods. Front. Microbiol. 9, 1–11. https://doi.org/10.3389/fmicb.2018.01046

Hudson, J.A., Billington, C., Cornelius, A.J., Wilson, T., On, S.L.W., Premaratne, A., King, N.J., 2013. Use of a bacteriophage to inactivate *Escherichia coli* O157: H7 on beef. Food Microbiol. 36, 14–21. https://doi.org/10.1016/j.fm.2013.03.006

Humphrey T.J., 1994. Contamination of egg shell and contents with *Salmonella* enteritidis: A review. Int J Food Microbiol 21:31–40.

Hutchison, M.L., Gittins, J., Walker, A., Sparks, N., Humphrey, T.J., Burton, C., Moore, A., 2016. An A assessment of the microbiological risks involved with egg washing under commercial conditions. J. Food Prot. 67, 4–11. https://doi.org/10.4315/0362-028x-67.1.4

Imamovic, L., Muniesa, M., 2011. Quantification and evaluation of infectivity of shiga toxin-encoding bacteriophages in beef and salad. Appl. Environ. Microbiol. 77, 3536–3540. https://doi.org/10.1128/aem.02703-10

Jamshidi, A., Kalidari, G.A., Hedayati, M., 2010. Isolation and identification of *Ssalmonella* e Enteritidis and *Salmonella* Typhimurium from the eggs of retail stores in Mashhad, Iran using conventional culture method and multiplex pcr assay. J. Food Saf. 30, 558–568. https://doi.org/10.1111/j.1745-4565.2010.00225.x

Jończyk, E., Kłak, M., Międzybrodzki, R., Górski, A., 2011. The influence of external factors on bacteriophages-review. Folia Microbiol. (Praha). 56, 191–200. https://doi.org/10.1007/s12223-011-0039-8

Kang, H.-W., Kim, J.-W., Jung, T.-S., Woo, G.-J., 2013. wksl3, a new biocontrol agent for *Salmonella enterica* serovars Enteritidis and Typhimurium in foods: characterization, application, sequence analysis, and oral acute toxicity study. Appl. Environ. Microbiol. 79, 1956–1968. https://doi.org/10.1128/aem.02793-12

Kropinski, A.M., Anany, H., Kuhn, J.H., Tolstoy, I., Kutter, E., Adriaenssens, E.M., 2017. To create a new bacteriophage family, *Ackermannviridae*, containing two (2) new subfamilies including four (4) genera. ICTV Rep. 1–12. https://doi.org/10.13140/RG.2.2.29173.88800

Labrie, S.J., Samson, J.E., Moineau, S., 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. 8, 317.

Leader, B.T., Frye, J.G., Hu, J., Fedorka-Cray, P.J., Boyle, D.S., 2009. High-throughput molecular determination of *Salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. J. Clin. Microbiol. 47, 1290–1299. https://doi.org/10.1128/JCM.02095-08

Lechevalier, V., Guérin-Dubiard, C., Anton, M., Beaumal, V., David Briand, E., Gillard, A., Le Gouar, Y., Musikaphun, N., Tanguy, G., Pasco, M., Dupont, D., Nau, F., 2017. Pasteurisation of liquid whole egg: Optimal heat treatments in relation to its functional, nutritional and allergenic properties. J. Food Eng. 195, 137–149. https://doi.org/10.1016/j.jfoodeng.2016.10.007

Lenski R.E. (1988) Dynamics of interactions between bacteria and virulent bacteriophage. Adv Microb Ecol. 10:1–44.

Lettini, A.A., Saccardin, C., Ramon, E., Longo, A., Cortini, E., Dalla Pozza, M.C., Barco, L., Guerra, B., Luzzi, I., Ricci, A., 2014. Characterization of an unusual Salmonella phage type DT7a and report of a foodborne outbreak of salmonellosis. Int. J. Food Microbiol. 189, 11–17. https://doi.org/10.1016/j.ijfoodmicro.2014.07.021

Leverentz, B., Conway, W.S., Alavidze, Z., JanisiewicZ, W.J., Fuchs, Y., Camp, M.J., Chighladze, E., Sulakvelidze, A., 2016. Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: A model study. J. Food Prot. 64, 1116–1121. https://doi.org/10.4315/0362-028x-64.8.1116

Li, Meng, Li, Mengzhe, Lin, H., Wang, J., Jin, Y., Han, F., 2016. Characterization of the novel T4-like *Salmonella enterica* bacteriophage STP4-a and its endolysin. Arch. Virol. 161, 377–384. https://doi.org/10.1007/s00705-015-2647-0

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., Griffiths, M.W., 2016. Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. Int. J. Food Microbiol. 217, 49–58. https://doi.org/10.1016/j.ijfoodmicro.2015.10.011

Long, M., Lai, H., Deng, W., Zhou, K., Li, B., Liu, S., Fan, L., Wang, H., Zou, L., 2016. Disinfectant susceptibility of different *Salmonella* serotypes isolated from chicken

and egg production chains. J. Appl. Microbiol. 121, 672–681. https://doi.org/10.1111/jam.13184

López-Cuevas, O., Castro-del Campo, N., León-Félix, J., González-Robles, A., Chaidez, C., 2011. Characterization of bacteriophages with a lytic effect on various *Salmonella* serotypes and *Escherichia coli* O157:H7. Can. J. Microbiol. 57, 1042–1051. https://doi.org/10.1139/w11-099

M. Donoghue, A., Bielke, L.R., E. Higgins, S., J. Donoghue, D., M. Hargis, B., Tellez, G., 2009. Use of wide-host-range bacteriophages to reduce *Salmonella* on poultry products. Int. J. Poult. Sci. 6, 754–757. https://doi.org/10.3923/ijps.2007.754.757

Magnone, J.P., Marek, P.J., Sulakvelidze, A., Senecal, A.G., 2013. Additive Approach for Inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* spp. on contaminated fresh fruits and vegetables using bacteriophage cocktail and produce wash. J. Food Prot. 76, 1336–1341. https://doi.org/10.4315/0362-028x.jfp-12-517

Mahony, J., Alqarni, M., Stockdale, S., Spinelli, S., Feyereisen, M., Cambillau, C., Van Sinderen, D., 2016. Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. Sci. Rep. 6, 1–10. https://doi.org/10.1038/srep36667

Mai, V., Ukhanova, M., Visone, L., Abuladze, T., Sulakvelidze, A., 2010. Bacteriophage administration reduces the concentration of *Listeria monocytogenes* in the gastrointestinal tract and its translocation to spleen and liver in experimentally infectedmice. Int. J. Microbiol. 2010. https://doi.org/10.1155/2010/624234

Mathew, S., 2016. A need for careful consideration of bacteriophage therapy. Indian J. Med. Res. 144, 482–483. https://doi.org/10.4103/0971-5916.198691

Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., Imai, S., 2005. Bacteriophage therapy: A revitalized therapy against bacterial infectious diseases. J. Infect. Chemother. 11, 211–219. https://doi.org/10.1007/s10156-005-0408-9

Modi, R., Hirvi, Y., Hill, A., Griffiths, M.W., 2016. Effect of Phage on Survival of *Salmonella* Enteritidis during manufacture and storage of cheddar cheese made from raw and pasteurized milk. J. Food Prot. 64, 927–933. https://doi.org/10.4315/0362-028x-64.7.927

Mogollon-Vergara, D.C., Rodriguez-Gutierrez, V.E., Verjan-Garcia, N., 2016. Prevalence and molecular identification of *Salmonella* spp. isolated from commercialized eggs at Ibague, Colombia. Rev. Salud Anim. 38, 1–10.

Monfort, S., Mañas, P., Condón, S., Raso, J., Álvarez, I., 2012. Physicochemical and functional properties of liquid whole egg treated by the application of Pulsed Electric Fields followed by heat in the presence of triethyl citrate. Food Res. Int. 48, 484–490. https://doi.org/10.1016/j.foodres.2012.04.015

Moreno Switt, A.I., den Bakker, H.C., Vongkamjan, K., Hoelzer, K., Warnick, L.D., Cummings, K.J., Wiedmann, M., 2013. *Salmonella* bacteriophage diversity reflects host diversity on dairy farms. Food Microbiol. 36, 275–285. https://doi.org/10.1016/j.fm.2013.06.014

Moye, Z.D., Woolston, J., Sulakvelidze, A., 2018. Bacteriophage applications for food production and processing. Viruses 10, 1–22. https://doi.org/10.3390/v10040205

Müller, A.J., Hoffmann, C., Galle, M., Van Den Broeke, A., Heikenwalder, M., Falter, L., Misselwitz, B., Kremer, M., Beyaert, R., Hardt, W.D., 2009. The *S.* Typhimurium effector sopE induces caspase-1 activation in stromal cells to initiate gut inflammation. Cell Host Microbe 6, 125–136. https://doi.org/10.1016/j.chom.2009.07.007

Nabil, N.M., Tawakol, M.M., Hassan, H.M., 2018. Assessing the impact of bacteriophages in the treatment of *Salmonella* in broiler chickens. Infect. Ecol. Epidemiol. 8. https://doi.org/10.1080/20008686.2018.1539056

Namata, H., Méroc, E., Aerts, M., Faes, C., Abrahantes, J.C., Imberechts, H., Mintiens, K., 2008. Salmonella in Belgian laying hens: An identification of risk factors. Prev. Vet. Med. 83, 323–336. https://doi.org/10.1016/j.prevetmed.2007.09.002

Newase, S., Kapadnis, B.P., Shashidhar, R., 2019. Isolation and genome sequence characterization of bacteriophage vB_SalM_PM10, a Cba120virus, concurrently infecting *Salmonella enterica* serovars Typhimurium, Typhi, and Enteritidis. Curr. Microbiol. 76, 86–94. https://doi.org/10.1007/s00284-018-1588-8

Peters, T., Hopkins, K.L., Lane, C., Nair, S., Wain, J., De Pinna, E., 2010. Emergence and characterization of *Salmonella enterica* serovar typhimurium phage type DT191a. J. Clin. Microbiol. 48, 3375–3377. https://doi.org/10.1128/JCM.00109-10

Pinto, P., Ribeiro, R., Sousa, L., Verde, S.C., Lima, M.G., Dinis, M., Santana, A., Botelho, M.L., 2004. Sanitation of chicken eggs by ionizing radiation: Functional and nutritional assessment. Radiat. Phys. Chem. 71, 35–38. https://doi.org/10.1016/j.radphyschem.2004.03.065

Ramirez, K., Cazarez-Montoya, C., Lopez-Moreno, H.S., Castro-del Campo, N., 2018. Bacteriophage cocktail for biocontrol of *Escherichia coli* O157:H7: Stability and

potential allergenicity study. PLoS One 13, 1–19. https://doi.org/10.1371/journal.pone.0195023

Rocha, C., Danchin, A., 2002. Base composition bias might result from competition for. Trends Genet. 18, 291–294.

Roseliza, R., Maswati, M.A., Hasnah, Y., Ramlan, M., 2011. Identification of *Salmonella* serotypes. Malaysian J. Vet. Res. 59–64.

Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchison, C.A., Slocombe, P.M., Smith, M., 1977. Nucleotide sequence of bacteriophage φX174 DNA. Nature 265, 687–695. https://doi.org/10.1038/265687a0

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F., Petersen, G.B., 1982. Nucleotide sequence of bacteriophage λ DNA. J. Mol. Biol. 162, 729–773. https://doi.org/https://doi.org/10.1016/0022-2836(82)90546-0

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.P11101

Scallan, E., Hoekstra, R.M., Mahon, B.E., Jones, T.F., Griffin, P.M., 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. Epidemiol. Infect. 143, 2795–2804. https://doi.org/10.1017/s0950268814003185

Scholl, D., Rogers, S., Adhya, S., Merril, C.R., 2002. Bacteriophage K1-5 encodes Two two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of *Escherichia coli*. J. Virol. 75, 2509–2515. https://doi.org/10.1128/jvi.75.6.2509-2515.2001

Serrano, L.E., Murano, E.A., Shenoy, K., Olson, D.G., 1997. D values of *Salmonella* enteritidis isolates and quality attributes of shell eggs and liquid whole eggs treated with irradiation. Poult. Sci. 76, 202–206. https://doi.org/10.1093/ps/76.1.202

Service, I., 1970. Risk Assessments for *Salmonella* Enteritidis in Shell Eggs and *Salmonella* spp. in Egg Products Executive Summary 2–5.

Sharma, M., Patel, J.R., Conway, W.S., Ferguson, S., Sulakvelidze, A., 2016. Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and Lettuce. J. Food Prot. 72, 1481–1485. https://doi.org/10.4315/0362-028x-72.7.1481

Snyder, A.B., Perry, J.J., Yousef, A.E., 2016. Developing and optimizing bacteriophage treatment to control enterohemorrhagic *Escherichia coli* on fresh produce. Int. J. Food Microbiol. 236, 90–97. https://doi.org/10.1016/j.ijfoodmicro.2016.07.023

Soffer, N., Abuladze, T., Woolston, J., Li, M., Hanna, L.F., Heyse, S., Charbonneau, D., Sulakvelidze, A., 2016. Bacteriophages safely reduce *Salmonella* contamination in pet food and raw pet food ingredients. Bacteriophage 6, e1220347. https://doi.org/10.1080/21597081.2016.1220347

Soni, K.A., Nannapaneni, R., Hagens, S., 2009. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100 . Foodborne Pathog. Dis. 7, 427–434. https://doi.org/10.1089/fpd.2009.0432

Spricigo, D.A., Bardina, C., Cortés, P., Llagostera, M., 2013. Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry. Int. J. Food Microbiol. 165, 169–174. https://doi.org/10.1016/j.ijfoodmicro.2013.05.009

SPX Process Bulletin: Pasteurized Liquid Egg, 2008. URL http;//www.spx.com (assessed 7.29.19).

Sukumaran, A.T., Nannapaneni, R., Kiess, A., Sharma, C.S., 2015. Reduction of *Salmonella* on chicken meat and chicken skin by combined or sequential application of lytic bacteriophage with chemical antimicrobials. Int. J. Food Microbiol. 207, 8–15. https://doi.org/10.1016/j.ijfoodmicro.2015.04.025

Sukumaran, A.T., Nannapaneni, R., Kiess, A., Sharma, C.S., 2016. Reduction of *Salmonella* on chicken breast fillets stored under aerobic or modified atmosphere packaging by the application of lytic bacteriophage preparation SalmoFresh™. Poult. Sci. 95, 668–675. https://doi.org/10.3382/ps/pev332

Sulakvelidze, A., 2013. Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. J. Sci. Food Agric. 93, 3137–3146. https://doi.org/10.1002/jsfa.6222

Sutterwala, F.S., Lara-Tejero, M., Flavell, R.A., Bertin, J., Galán, J.E., Ogura, Y., Coyle, A.J., Grant, E.P., 2006. Role of the caspase-1 inflammasome in *Salmonella* typhimurium pathogenesis. J. Exp. Med. 203, 1407–12. https://doi.org/10.1084/jem.20060206

Thung, T.Y., Krishanthi Jayarukshi Kumari Premarathne, J.M., San Chang, W., Loo, Y.Y., Chin, Y.Z., Kuan, C.H., Tan, C.W., Basri, D.F., Jasimah Wan Mohamed Radzi, C.W., Radu, S., 2017. Use of a lytic bacteriophage to control *Salmonella* Enteritidis in retail food. LWT - Food Sci. Technol. 78, 222–225. https://doi.org/10.1016/j.lwt.2016.12.044

Thung, T.Y., Lee, E., Mahyudin, N.A., Anuradha, K., Mazlan, N., Kuan, C.H., Pui, C.F., Ghazali, F.M., Mahmud Ab Rashid, N.K., Rollon, W.D., Tan, C.W., Radu, S., 2019. Evaluation of a lytic bacteriophage for bio-control of *Salmonella* Typhimurium in different food matrices. Lwt 105, 211–214. https://doi.org/10.1016/j.lwt.2019.02.033

Tiwari, B.R., Kim, S., Kim, J., 2013. A virulent *Salmonella enterica* serovar Enteritidis phage SE2 with a strong bacteriolytic activity of planktonic and biofilmed cells. J. Bacteriol. Virol. 43, 186–194. https://doi.org/10.4167/jbv.2013.43.3.186

Viator, C.L.; Muth, M.K.; Brophy, J.E. Costs ofFood Safety Investments; Report; RTI International: Research Triangle Park, NC, USA, 2015. Retrieved from https://www.fsis.usda.gov/wps/wcm/connect/0cdc568e- f6b1-45dc-88f1-45f343ed0bcd/Food-Safety-Costs.pdf?MOD=AJPERES (accessed 7.29.2019).

Vital, P.G., Caballes, M.B.D., Rivera, W.L., 2017. Antimicrobial resistance in *Eescherichia coli* and salmonella spp. Isolates from fresh produce and the impact to food safety. J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes 52, 683–689. https://doi.org/10.1080/03601234.2017.1331676

Wales, A., Breslin, M., Carter, B., Sayers, R., Davies, R., 2007. A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. Avian Pathol. 36. https://doi.org/10.1080/03079450701338755

Wang, C., Yang, J., Zhu, X., Lu, Y., Xue, Y., Lu, Z., 2017. Effects of *Salmonella* bacteriophage, nisin and potassium sorbate and their combination on safety and shelf life of fresh chilled pork. Food Control 73, 869–877. https://doi.org/10.1016/j.foodcont.2016.09.034

Wang, H., Slavik, M.E., 2016. Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. J. Food Prot. 61, 276–279. https://doi.org/10.4315/0362-028x-61.3.276

Whiley, H., Ross, K., 2015. *Salmonella* and eggs: From production to plate. Int. J. Environ. Res. Public Health 12, 2543–2556. https://doi.org/10.3390/ijerph120302543

Wittebole, X., De Roock, S., Opal, S.M., 2014. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. Virulence 5, 209–218. https://doi.org/10.4161/viru.25991

Wong, C.L., Sieo, C.C., Tan, W.S., Abdullah, N., Hair-Bejo, M., Abu, J., Ho, Y.W., 2014. Evaluation of a lytic bacteriophage, Φ st1, for biocontrol of *Salmonella*

enterica serovar Typhimurium in chickens. Int. J. Food Microbiol. 172, 92–101. https://doi.org/10.1016/j.ijfoodmicro.2013.11.034

Wu, J. (2014). Eggs and Egg Products Processing. In Food Processing (eds S. Clark, S. Jung and B. Lamsal). https://doi.org/10.1002/9781118846315.ch19

Wu, V.C.H., 2008. A review of microbial injury and recovery methods in food. Food Microbiol. 25, 735–744. https://doi.org/10.1016/j.fm.2008.04.011

Xu, D., Jiang, Y., Wang, L., Yao, L., Li, F., Zhai, Y., Zhang, Y., 2018. Biocontrol of *Salmonella* Typhimurium in raw salmon fillets and scallop adductors by using bacteriophage SLMP1. J. Food Prot. 81, 1304–1312. https://doi.org/10.4315/0362-028x.jfp-17-525

Ye, J., Kostrzynska, M., Dunfield, K., Warriner, K., 2016. Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. J. Food Prot. 73, 9–17. https://doi.org/10.4315/0362-028x-73.1

Young, R., Wang, I.N., Roof, W.D., 2000. Phages will out: Strategies of host cell lysis. Trends Microbiol. 8, 120–128. https://doi.org/10.1016/S0966-842X(00)01705-4

Zuber, S., Ngom-Bru, C., Barretto, C., Bruttin, A., Brüssow, H., Denou, E., 2007. Genome analysis of phage JS98 defines a fourth major subgroup of T4-like phages in *Escherichia coli*. J. Bacteriol. 189, 8206–8214. https://doi.org/10.1128/JB.00838-07