MOLECULAR CHARACTERIZATION OF TWO BACTERIOPHAGE STRAINS AND THEIR ROLE IN THE GASTROINTESTINAL TRACT OF MICE

by

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Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

in Microbiology

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July 2018

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by National Natural Science Foundation of China and Jiangsu Agricultural Science and Technology Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Prof. Stefan Schmidt (Supervisor)

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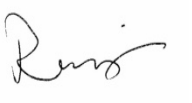

Signed: Prof. Ran Wang (Co-supervisor)

Date: July 2018

DECLARATION

I, Hongduo Bao, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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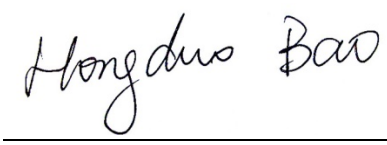

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ABSTRACT

Due to the abuse of antibiotics for chicken's growth promotion and the rapid emergence of multi-drug resistant *Salmonella* infections of animal intestine, there is increasing pressure to use nonantibiotics to promote animal health. Bacteriophage use is therefore a potential alternative. In this study, a lytic gut phage vB_SenM-PA13076 (PA13076) infecting *Salmonella* Enteritidis was isolated from fecal samples of chickens, while a temperate gut phage vB_SpuP-BP96115 (BP96115) was induced by mitomycin C from a *Salmonella* Pullorum gut isolate. Transmission electron microscopy (TEM) indicated that the isolated lytic phage belonged to the family *Myoviridae*, with an oval head (66±4 nm) and a contractile tail (90±5 nm). However, the temperate phage matched the family *Podoviridae* of the C1 morphotype, possessing a small head (diameter, 54±4 nm) and a short tail (10±2 nm) with fibers. One-step growth curves under optimal growth conditions revealed the latent periods of both phages to be 10 min and burst sizes of 21 and 24 PFU per infected cell, for the lytic and temperate phage, respectively. The lytic gut phage PA13076 had a broad host range, infecting 222 out of 311 tested *Salmonella* strains representing different serovars. However, the temperate phage BP96115 had a narrow host range with the lytic effect on only 34.61% (9/26) of tested *Salmonella enterica* subsp. *enterica* strains. The complete linear genome sequences of PA13076 and BP96115 comprised of 52,474 bp and 41,264 bp and contained 69 and 62 ORFs, respectively. Bioinformatic analyses identified 44 and 47 ORFs in the genome of PA13076 and BP96115, coding for DNA packaging and morphogenetic proteins, lysis components, and proteins necessary for DNA recombination, regulation, modification and replication. The temperate phage BP96115 also carried the functional lysogeny modules. Furthermore, comparative genome sequence analysis revealed a high similarity of gut phage PA13076 with the two *Salmonella* phages BP63 and UPF_BP2 (97% sequence identities), while the temperate phage BP96115 showed high similarity to several temperate phages, especially enterobacteria phage ST104 with 99% sequence identity.

In order to elucidate the colonization of the gastrointestinal tract of mice by phages, the lytic gut phage PA13076 and the temperate gut phage BP96115 were fed to mice. Moreover, phage titers in blood and spleen were detected to verify the phage

penetration. Both phages were purified to reduce the endotoxin levels of phage PA13076 and BP96115 solutions to <1 EU/mL. For the animal experiment, 60 female mice were divided into a control group, a lytic group, and a temperate group on the basis of body weight. About 4×10^8 PFU of phages were fed daily to each mouse in the lytic or the temperate group via drinking water over 31 days. Results showed that phage titers were similar at day 16 and day 31 in different segments of the gastrointestinal tract. Both phages survived in the gastrointestinal tract, and highest phage titers were detected in the cecum as well as in feces with 10^4 and 10^6 PFU/g for PA13076 and BP96115. In addition, phages entered the bloodstream and appeared in spleen of mice. The phage titer in blood was at 10^2~10^3 PFU/mL, while in the spleen tissue, the titer of the lytic phage was lower than the temperate phage titer. Oral administration of both phages over 31 days induced a slight but not significant increase of serum IgG and ileal secretory IgA (sIgA) levels.

The overuse of antibiotics in livestock has led to a series of threats to public health, especially the rapid emergence of gut superbugs. Phages, the most abundant species in the mammalian gut, have numerous advantages over antibiotics. In this study, mice were orally treated with the lytic gut phage PA13076 (group B), the temperate phage BP96115 (group C), no phage (group A) or streptomycin (group D) over 31 days. At the end of the experiment, fecal microbiota diversity and composition was determined and compared using high-throughput sequencing of the V3-V4 hyper-variable region of the 16S rRNA gene and virus-like-particles (VLPs) were quantified in feces. There was high diversity and richness of microbiota in the lytic and temperate gut phage treated mice, with the lytic gut phage causing an increased alpha diversity based on the Chao 1 index ($p<0.01$). However, the streptomycin treatment reduced the microbiota diversity and richness ($p=0.0299$). Both phage and streptomycin treatments reduced the abundance of *Bacteroidetes* at the phylum level and increased the abundance of the phylum *Firmicutes*. Interestingly, two beneficial genera, *Lactobacillus* and *Bifidobacterium*, were enhanced by treatment with the lytic and temperate gut phage. The abundance of the genus *Escherichia*/*Shigella* was higher in mice after temperate phage administration than in the control group, but lower than in the streptomycin group. Moreover, streptomycin treatment increased the abundance of the genera *Klebsiella* and *Escherichia*/*Shigella*. In terms of the gut virome, fecal VLPs did not change significantly after phage treatment. This study showed that lytic

and temperate gut phage treatment modulated the composition and diversity of gut microbiota and the lytic gut phage promoted a beneficial gut ecosystem, while the temperate phage may promote conditions enabling diseases to occur.

Several studies have shown the efficacy of phage therapy in reducing intestinal pathogens. However, phage-based probiotic treatment is poorly studied in view of effects on the gut microbiota and intestinal inflammation. In this study, lytic (approximate 4×10^8 PFU per day) or temperate phages (approximate 4×10^8 PFU per day) or a streptomycin solution (approximate 40mg per day) were preadministered to mice via drinking water for 31 days. Subsequently, mice were challenged with *Salmonella* Typhimurium, which was not the host of both phages, and the composition of the gut microbiota and the counts of *Enterobacteriaceae* and *Lactobacillus* spp. in cecum were determined and compared with those of non-treated mice or mice challenged with *Salmonella* Typhimurium. For intestinal inflammation evaluation, mice were given one dose of streptomycin for 24h before the *Salmonella* Typhimurium challenge. High-throughput sequencing analysis revealed that the phylum *Firmicutes* became the most abundant, while the phylum *Actinobacteria* increased and the phylum *Tenericutes* declined in pretreatment with both phages. The alpha diversity of bacterial communities was higher in these two groups compared to other groups. Moreover, pretreatment with the lytic and the temperate phage before bacteria challenge increased two beneficial genera, *Lactobacillus* and *Bifidobacterium*. However, in mice pretreated with the temperate phage, an increased abundance of the genus *Escherichia*/*Shigella* was noted. Counts of *Enterobacteriaceae* and *Lactobacillus* spp. in the mice pretreated with phages were not different from the control mice. According to the pathological analysis of ileum and cecum, the pretreatment using temperate or lytic gut phage markedly reduced intestinal inflammation, which was also confirmed by the lower concentration of LPS and DAO in the serum and lower expression of most of inflammatory cytokines in the jejunum compared to the mice challenged with *Salmonella* Typhimurium and streptomycin treated mice.

ACKNOWLEDGMENTS

It is a significant and unforgettable experience for me to spend such a long time on writing, modifying and polishing this thesis. Many kind-hearted people give me a great amount of help, professional advice and encouragement. Thus, here I would like to express my gratitude to all those who helped me during the PhD study.

Firstly, I would like to extend my sincere gratitude to my supervisor, Professor Stefan Schmidt, for his instructive advice, invaluable guidance, and encouragement through the whole processes. He has walked me through all the stages of the writing of my thesis. Without his detail guidance, helpful comments, and illuminating instruction, this thesis could not have reached its present form.

Secondly, I will also express my sincere gratitude to my co-supervisors, Professor Ademola Olaniran and Professor Ran Wang. They provided me with beneficial help and offered me precious comments during the whole process of my writing. I also own my appreciation to Professor Ran Wang, who had given me a lot of inspiration, instructions, and support for the experiments.

Besides my supervisor and co-supervisors, I have benefited from many colleagues and classmates in Jiangsu Academy of Agricultural Sciences, Prof. Hui Zhang, Dr. Yan Zhou, Dr. Maoda Pang, Dr. Lichang Sun, Dr. Hang Zhao, Dr. Tao He, Fang Ji, Shu Yao and Guangfei Wang. They gave me their moral support and encouragement over the course of this journey.

Finally, very special thanks go to my beloved family for their unfailing support, their patience and all the sacrifices they have made along the way. My heart swells with gratitude to all the people who helped me.

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INTRODUCTION

The rapid emergence of multi-drug-resistant *Salmonella* spp. and the serious damage inflicted upon the bacterial flora in the gut due to antibiotic overuse are an emerging global crisis (Gould and Bal, 2013; Martin et al., 2015; Ventola, 2015), reflecting the worldwide misuse of antimicrobial agents in animal breeding (Bartlett et al., 2013; Park et al., 2017). Most of the antimicrobial agents are used globally as growth supplements in livestock production (Van Boeckel et al., 2015). A study showed that 92,700 tons of antibiotics inclusive of 36 kinds of antibiotics were consumed in China in 2013, of which 52% were consumed by animals (Zhang et al., 2015). The incidence of intestinal *Salmonella* spp. infections has increased dramatically in recent years in poultry and pig farming (Christenson, 2013). There are more than 93 million cases of foodborne salmonellosis annually (Majowicz et al., 2010), at an average cost of more than $1000 per case in each country (FCC, 2011; Scharff, 2012). The European Union began to restrict antibiotics use as growth promoters for farm animals (EC, 2005). Therefore, research has been carried out to identify alternatives to antibiotics such as bacteriophages, probiotics, enzymes, and organic acids (Bourassa et al., 2018; Cresci et al., 2013; Golkar et al., 2014; Koyuncu et al., 2013).

Among the alternatives to antibiotics, bacteriophages (phages), which are the most abundant entities on earth, can attack and kill a target bacterium, including even multi-drug resistant bacteria (Ahmadi et al., 2016; Hong et al., 2016; Latz et al., 2016). For many years, phages have been used to biocontrol infections caused by pathogenic bacteria in animals (Housby and Mann, 2009; Sharma et al., 2017). Phages inevitably encounter diverse enteric pathogens and other microorganisms in the complex gut ecosystem of animals (Olivo et al., 2016). However, little is known about the role of phages in the gut.

Perhaps as many as 10^{15} phage particles exist in the mammalian gut, collectively referred to as the ‘phageome’ (Dalmasso et al., 2014). They are perhaps the most abundant living beings in the gut (Gaidelyte et al., 2005; Ventura et al., 2011). Most phages in the animal gut are likely temperate phages, which often exist in the form of prophages (Held and Sidhu, 2004; Kanji et al., 2017).

Metagenomics have been increasingly employed as an efficient method for exploring

and charting the virome (Dutilh et al., 2014). Metagenomic studies have indicated the existence of a large diversity of phages in the gut. Indeed, the mammalian gut may be the ideal place for phage propagation (Comeau and Krisch, 2005). The vast majority of phages are unknown in the animal gut, mainly because most of their genome sequences are still unknown (Kleber-Janke et al., 2001; Ventura et al., 2011). Dutilh and colleagues (2014) recently discovered a previously unidentified phage referred to as crAssphage, which is present in the majority of mammalian fecal metagenomes. Several kinds of phages, such as *Escherichia coli* phages, *Salmonella* phages and *Bacteroides fragilis* phages, have been isolated from feces, at up to 10^5 PFU/g of phages using cultural methods (Breitbart et al., 2003). Gut-associated phages therefore likely affect the diversity and structure of the bacterial microflora in the intestinal tract (Lepage et al., 2008); however, they have not been extensively studied.

The gastrointestinal tract (GIT) of animals is an incredibly complex ecosystem (John and Mullin, 2016; Korecka and Arulampalam, 2012). It is the most densely populated area of the body, is inhabited by lots of microbes including large amounts of bacteria, viruses, fungi, and contains a plethora of collective interacting genomes (Minot et al., 2011; Ogilvie and Jones, 2015). The intestinal microbiota is thought to outnumber the cells of the animal body by approximately 10 to 1, and is believed to play a crucial role in animal health (Bianconi et al., 2013; Hao and Lee, 2004). In recent years, diverse microorganisms in the animal gut (gut microbiota) have been shown to affect mammalian health and overall well-being, as well as diseases of the gut (Betrapally et al., 2017). The gut microbiota provides some important functions maintaining animal health, such as supporting food digestion, developing immune system, and preventing pathogenic infection (Kinross et al., 2008; Koch, 2015). The relationships between gut microbiota and health have therefore been recognized as a major challenge in the 21st century.

The viral gut community, also known as the gut virome, is a highly complex community (Cadwell, 2015). It contains the eukaryotic virome, phages, the archaeal virome, and viral genes (Virgin et al., 2009). Moreover, phages are the dominant fraction in the gut virome (Dunn et al., 2016). However, it is rarely known that the effect of abundant phages in the GIT on the gut ecosystem, although Mills et al. (2013) demonstrated that the influence of phages on the gut ecosystems is manifold, including the shaping of the mammalian gut microbiota. It is established that

temperate phages confer horizontal gene transfer between the bacterial communities of gut microbiota (Koskella and Brockhurst, 2014; Ley et al., 2006) and are important factors for dissemination of the resistance genes (Brussow et al., 2004; Harrison and Brockhurst, 2017; Reyes et al., 2010). Our current knowledge is still limited and too fragmented to understand the roles of phages in animal health comprehensively, especially in view of shaping the gut microbiota of healthy and sick animals.

The aims of this thesis were to:

1. Isolate and comprehensively characterize a lytic and a temperate *Salmonella* gut phage.
2. Understand the distribution of both phages in the animal GIT using a mouse model.
3. Analyze their impact on the diversity and composition of gut microbiota by metagenomic analysis.
4. Elucidate the roles of these phages in modulating intestinal microbial communities composition for a long-term and resistance to intestinal inflammation caused by *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

This thesis consists of six chapters. The first chapter is the literature review that describes general characteristics, phage application, the animal intestinal ecosystem, the relationship between phages and intestinal ecosystems, and the possible role of phages in gut microflora disorders. The second, third, fourth, and fifth chapter report on the experiments undertaken in this study. Each chapter is an independent research article. The second chapter reports the morphological, physiological, and molecular characterization of lytic gut phage vB_SenM-PA13076 and temperate gut phage vB_SpuP-BP96115 isolated from chicken feces. The third chapter is on the distribution of orally administrated lytic and temperate *Salmonella* gut phages in mice. The fourth chapter reports alterations in the diversity and composition of mice gut microbiota by lytic or temperate *Salmonella* gut phage treatment. The fifth chapter is the impact of preadministration of lytic and temperate *Salmonella* gut phage on gut

microbiota composition and intestinal inflammation in a mouse model. The last chapter, chapter six, summarizes and analyses the main findings and points out the direction of further research.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Bacteriophages

Bacteriophages (simply known as phages) are parasitic bacterial viruses, which amplify using their bacterial host (Twort, 1936). The term “bacteriophage” came from Greek “φαγειν”, “to devour”, which literally means “bacteria eater” (Schultz, 1927). As early as 1896, researchers reported that there was something in the water of Ganges River acted against cholera and can across a very small pore size filter. In fact, phages were formally discovered in the mid to late 20^{th} century with the first publication by Edward Twort in 1915 and the second in 1917 by Felix d’Herelle (Ho, 2001), who devoted his life to phages and their therapeutic uses in humans and animals. Phages are the most common, abundant, and diverse organisms in the biosphere (Hatfull, 2015). They are ubiquitous viruses, which exist anywhere in the presence of bacteria. The number of phages is estimated to reach upwards of 10^{31} PFU on the earth, more than all other organism combined, including bacteria (Suttle, 2005; Weinbauer, 2004). Each phage particle is composed of proteins that encapsulate its nucleic acid genome and may have relatively simple or refined structures. The genetic material of phages can be either DNA or RNA (Weitz et al., 2005). They often contain unusual or modified bases in the nucleic acids of phages. These bases prevent phage nucleic acids from nucleases splicing during infection (Court et al., 2007). They attach themselves to susceptible bacteria and infect host cells by replicating in the bacterium after injection of their genome into the intracellular. Following infection, the phage prevents the bacterium from replicating of bacteria and instead uses the bacterial cell machinery to produce their own components. Eventually, new offspring phages break through the bacterial cell membrane in a process called lysis (Mavrich and Hatfull, 2017).

1.2 General characteristics of phages

1.2.1 Life cycles

Phages have different life cycles relating with the interacted physical environment (Hargreaves et al., 2014). However, phages primarily exhibit two classic lifestyles: virulent or temperate (Aitken, 2009) (Fig.1.1).

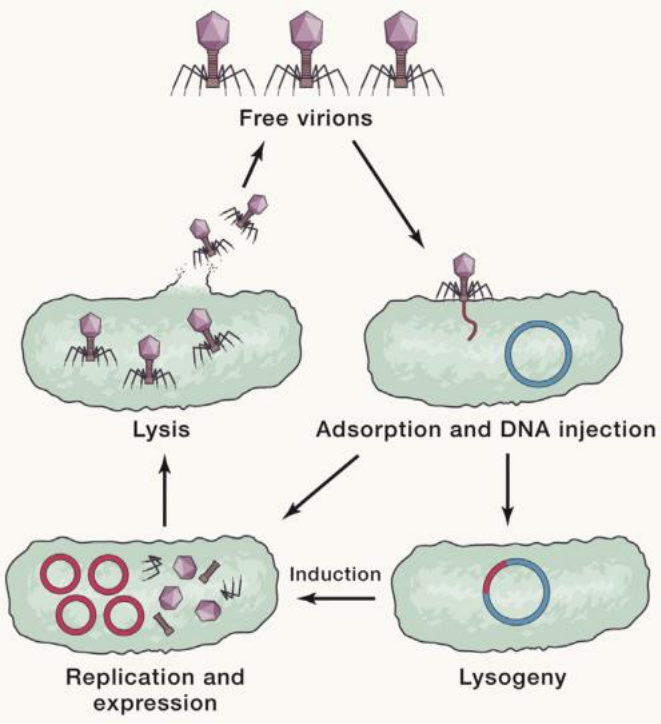

Fig. 1.1 Phage life cycles (Ofir and Sorek, 2018)**.**

Lytic bacteriophages reproduce themselves based on host bacterial cell and produce their progeny at the end of their life cycles. Lytic phage can govern the number of bacterial population in various environments by destroying bacteria and occasionally assist bacterial long-term evolution via generalized transduction (Weinbauer and Rassoulzadegan, 2004). Phages encounter their bacterial hosts randomly and attach to the host cell via specific receptors, including proteins, oligosaccharides, teichoic acids, peptidoglycans and lipopolysaccharides (Park et al., 2012). The second step is that of phage synthesis and assembly, which is also called the eclipse phase. Phage DNA is transcribed into mRNA, although this step is not required for RNA viruses. The third step, depending on the energy and reproduction elements of its bacterial cell, the phage produces a lot of its own nucleic acids and proteins in the bacterial cell using its genome as a template, and then assembles into thousands of phage particles. After a given period of time, the bacteria begin to be lysed due to accumulation of endolysin and offspring phages are released from the bacteria into the surrounding environment. Although the burst size is typically in a range from 50 to 100 phage particles, there are some special phages, the burst size of which is as high as 1000 progeny phages particles (Santos et al., 2015).

Lysogenic or temperate phages enter a state of equilibrium in which the phage

becomes part of the bacterial cell (Santillan and Mackey, 2004). In this quiescent state, the phage genome becomes circular and latent, persisting in an integrated form within the bacterial genome or independently, in the cytoplasm as a circular 'plasmid' (Abedon, 2017). The genome of phages, which is integrated into its host genome, is called a prophage. The prophage is not a real phage, but can be converted to a phage by induction of its external environment (Zhao et al., 2010). The presence of the prophage will not adversely affect its host and this state will not change until the link between phage and host breaks down, either spontaneously or under the action of physical or chemical agents. The host bacterial cell containing one or more prophages is termed a lysogenic bacteria and the process that produces novel phages in the presence of specific agents is called "induction" (Broudy et al., 2001).

1.2.2 Classification

As early as 1962, the LHT system was proposed to be a classification method, which is based on the type of nucleic acid, the shape of the capsid, and numbers of envelope and capsomers (Lwoff, 1962). Until 1965, the Provisional Committee on Nomenclature of Viruses (PCNV) adopted the LHT scheme for viruses' classification. In 1966, this organization was renamed the International Committee for classification of viruses (ICNV), and in 1973, International Committee on Taxonomy of Viruses (ICTV) became the professional organization for viruses classification (Matthews, 1983). The first report of the ICTV on the classification of viruses included six phage genera and their descriptions: T4 and relatives, λ, lipid-containing phage PM2, ψ174-like phages, filamentous phages, and the 'ribophage' group (Fenner, 1971). This was followed by several ICTV reports published at irregular intervals. Phages are extremely multifarious in their genomic and proteins' characteristics. The genetic materials are double-stranded (ds) or single-stranded (ss) DNA or RNA. Phage particles are isometric, polyhedral, pleomorphic, filamentous, tailed or no tail (Fig.1.2).

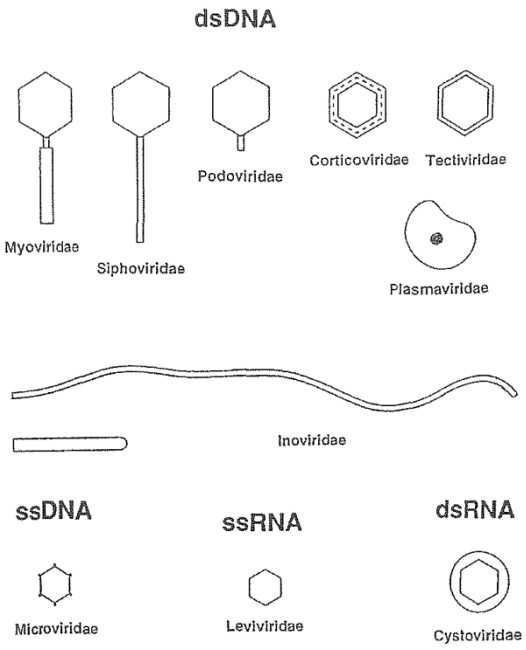

Fig. 1.2 Basic phage morphologies (Borysowski, 2014)

The majority of phages are dsDNA, while ssDNA, ssRNA, and dsRNA are only small part. In 2010, phages comprised of three orders, the *Caudovirales*, *Ligamenvirales*, and the unassigned, and 19 families and 348 genera were recognized that infect bacteria and archaea (Onodera, 2010). Among them only two viral families contain RNA. Of the families with DNA genomes, only two are ssDNA, eight are circular while nine are linear. Studying on more than 5568 strains of phage by the electron microscopy, the results indicated that most of phages belong to *Caudovirales* (Ackermann, 2007). Although up to 40 classification criteria are used, there are still no effective standard for phage classification (Kutter and Sulakvelidze, 2005). As of 2017, 9 orders, 131 families, 46 subfamilies, 803 genera, and 4,853 species of viruses have been defined by the ICTV. However, the number of phage genera and species is in constant expansion and can only be estimated.

1.2.3 Host range

Host range is an important biological characteristic of the phage (Kutter, 2009). Host ranges are different from non-productive infections (i.e., restrictive, abortive, or even lysogenic) to productive infections (Hyman and Abedon, 2010). Thus, the selected method of measuring the host range of a specific phage is very important. Many other factors affect host ranges such as receptor alteration, epitopes inhibition of DNA

injection, superinfection immunity by resident prophages, restriction endonucleases destroying, or the clustered regularly interspaced palindromic repeat (CRISPR) system (Barrangou et al., 2007). The most common method for detecting host range of phage is the spotting test. In this method, a drop of phage suspension is dripped on to a lawn of tested bacteria. However, spotting test tends to overestimate the real host range (Khan Mirzaei and Nilsson, 2015). Thus, the efficiency of plating (EOP) using the double agar layer method is more efficient for identification and selection of good phages, which are broad range.

Host recognition is an important factor that determines the host range of a phage. This is determined by interaction between specific receptors of bacterial host's surface and the receptor binding proteins (RBPs) of phage (Mahony and van Sinderen, 2012; Simpson et al., 2016). The first step of phage infection is reversibly. However, during the infection step, phages attach to the same receptor as in the adsorption step or to another receptor nonreversible (Dowah and Clokie, 2018). The receptors can be lipopolysaccharides (LPS), membrane proteins or teichoic acid (Bertozzi Silva et al., 2016). For the phage of Gram-negative bacteria, LPS is the most common receptor for phage targeting (Hyman and van Raaij, 2018). For example, there are two distinct ways that the phage T4 recognizes *E. coli* strains, OmpC-dependent and OmpC-independent (Washizaki et al., 2016). Similarly, membrane protein OmpC is the receptor of *Salmonella* phage Gifsy phage (Ho and Slauch, 2001), while T5-like phages uses vitamin B12 protein BtuB as its receptor (Kim and Ryu, 2011). The RBPs of *Salmonella* phage SP6 determines the host range and this phage exhibits double host adsorption systems (Gebhart et al., 2017). Understanding the phage recognition process and specificity is therefore very important and may facilitate the implementation of successful phage therapy.

1.2.4 Genome

Phage genomic sequences are as rich as the number of phages on earth (Rohwer, 2003). So far, genomes of a number of selected phage strains have been sequenced including coliphages, mycobacteriophages, bacteroid phages, and dairy phages (Chibani- Chennoufi et al., 2004). Since the first complete genome of phage ΦX174 was published in 1977 (Sanger et al., 1977), the number of sequenced phage genomes have been steadily increased. Nevertheless, the known genomes of phage are still

small compared to the bacterial sequenced genomes (Hatfull, 2008; Weinbauer, 2004). Recent metagenomic phage studies have highlighted the extraordinarily high phage genome diversity. The size of sequenced phage genomes varies dramatically, from 2,435 base pairs [bp] (*Leuconostoc* phage L5) to 497,513bp (*Bacillus* phage G) (Deschavanne et al., 2010). The majority of phages belong to *Siphoviridae*, according to their genomic information, which account for approximately 55%, while small genomes ranging from 5 kb to 20 kb are the second most abundant group and account for approximately 27% (Deschavanne et al., 2010). Double stranded DNA phages are predominant and are an enormous source of unknown phage genes. Phage genomes are critical for the expression of proteins ensuring replication, the assembly of newly formed progeny virions, and lysis of host cells (Klumpp et al., 2013). Generally, genes coding for regulatory proteins or transfer RNAs (tRNAs), which can replace host functions under specific conditions of bacterial cell growth, are absent in small phage genomes (Henry et al., 2010). However, *Pseudomonas* phage 201φ2-1 has a large phage genome of 316,674bp, which includes one tRNA-encoding gene. Conversely, there are 41 tRNAs encoded in the genome of the phage Myrna, which has a size of 164,602 bp.

1.3 Application of phages in reducing pathogens

Phages have been employed for more than 100 years. Phage therapy mainly uses for treating pathogenic infections in humans and animals (Zelasko et al., 2017). The emergences of multi-resistant bacteria continue to threaten standard therapies against bacterial infections. However, the developments of new antimicrobial compounds are not sufficient to replace old and obsolete drugs (Hughes and Karlen, 2014). This is the main driver for searching alternatives to treat antibiotic-resistant bacterial infections (Mattila et al., 2015; Periasamy and Sundaram, 2013). Phages have therefore received renewed attention and are promising products to replace antibiotics in the control of bacterial infections (Pirisi, 2000; Speck and Smithyman, 2016).

1.3.1 Purification

Because bacterial lysis is involved in phage stock preparation and has the potential to release bacterial toxins, including endotoxins from Gram-negative bacteria (Gill and Hyman, 2010), an ongoing concern with respect to phage therapy is the potential for injection of bacterial toxins into patients over the course of phage application. A

standard method for phages preparation and application is still lacking, but is necessary to ensure that toxins are not introduced into patients. Thus, phage preparations must be purified. To date, various techniques including phase separation (Aida and Pabst, 1990), anion exchange and affinity chromatography (Liu et al., 1997; Petsch et al., 1997) have been investigated to remove toxins from recombinant protein solutions. In a recent study, Khan Mirzaei et al. (2016) used polyethylene glycol (PEG 8000) for precipitation and followed by using cesium chloride (CsCl) gradient ultracentrifugation, achieving LPS levels in the phage preparation below 1.0 endotoxin units/mL. Dufour et al. (2016) subsequently contradicted that this protocol is not efficient to remove endotoxin from the phage solution. His team used a different protocol, which was composed of concentration, ultrafiltration and two ultracentrifugation steps (one CsCl gradient and one isopycnic gradient). Following dialysis, commercial affinity chromatography columns were used to remove endotoxins, with the last step repeated for three to five times. For most phage solutions, this protocol can guarantee below 0.5 endotoxin units/mL (Henry et al., 2013). Another efficient method for endotoxin removal is a combination of deoxycholate treatment and ultrafiltration from T7 phage preparations (Hashemi et al., 2013). The advantage is that deoxycholate's small molecular weight enables it easy removal by ultrafiltration. The results indicated that a single round of deoxycholate treatment and ultrafiltration was able to reduce LPS concentration by 42% without any significant loss of the phage recovery. After three cycles of deoxycholate treatment and ultrafiltration, the concentration of LPS was reduced to 0.83 EU/ml. Using this method for the removal of LPS from other phage particles such as λ and T4 or virus-like particles (VLPs) may be beneficial and convenient for the development of quality phage for animal health and food safety (Hashemi et al., 2013). Hence, the combination of deoxycholate treatment and ultrafiltration appears to be a worthy method for removing LPS from phage solutions.

1.3.2 Clinical experience

Phage therapy against bacterial infections in humans has a long tradition. Clinical phage therapy was frequently employed in the former Soviet Union, the Republic of Georgia, as well as in Russia, and Poland (Waldor et al., 2005). Most of the phage products were used for the military during the Second World War and much of the

literatures mainly descripted phage therapy of the major problems such as gangrene and dysentery in wartime (Hanlon, 2007). Currently, studies of phage therapy focus on three main fields: to combat antibiotic-resistant bacteria infections; to combat the untreatable infections in patients (e.g., due to poor circulation, biofilm formation, diabetic ulcers or osteomyelitis); and to combat targeting bacteria, for example, due to patient allergy to beta-lactams, intractable bowel problems or *Clostridium difficile*. In addition, concerns with excess exposure of human and environment to antibiotics in food and agricultural applications (Abedon et al., 2011; Periasamy and Sundaram, 2013). A recent study showed that phage therapy could cure patients with recalcitrant chronic respiratory tract infections caused by multidrug-resistant *Pseudomonas aeruginosa* strains, which indicates a new treatment method for those multi-resistant bacterial infections (Waters et al., 2017). Phage BioDerm, a kind of phage product, has been licensed and approved in Georgia. It is a mixture of non-toxic polymer with phages as well as the antibiotics ciprofloxacin and benzocaine (Markoishvili et al., 2002). In Poland, phage therapy was also performed successfully for treating patients with antimicrobial-resistant bacterial infections (Miedzybrodzki et al., 2012). So far, a trial of phage therapy in children using a commercial coliphage product demonstrated a safe gut transit and improved diarrhea outcome, but failed phage amplification in the gut (Sarker et al., 2016). The emergence of this phenomenon may be caused by low concentrations of pathogenic *E. coli* or the insufficient phage coverage. Therefore, more research should be studying the phage-bacterium interaction *in vivo*. Despite some of the potential obstacles of phage treatment, the current knowledge regarding phages indicates that phage application has scientific merit and deserves attention.

1.3.3 Use of phage against intestinal pathogens

Smith's group published a series of successful phage studies demonstrating the efficient use of phages in treating *E. coli* infections in many animals (Smith and Huggins, 1982, 1983; Smith et al., 1987). Phages have also been proved to protect mice from systemic infections (Pouillot et al., 2012; Soothill, 1992). A number of studies demonstrated that phages are good alternatives to control animal diseases (Callaway et al., 2011; Cisek et al., 2017; Gorski et al., 2016; Kaur et al., 2016). Because of the lesser efficacy of antibiotics for cleaning out pathogens of intestinal carriage, phage therapy is now a method to be considered for the treatment of the

intestinal pathogens. However, the treatment of enteric and zoonotic pathogens using phages might fail because of the possible development of resistance (Barrow, 2001; Sabouri et al., 2017). Furthermore, the study of Maura et al. (2012) indicated that the entero-aggregative *E. coli* O104:H4 phages were not uniformly distributed throughout the gut in mice, which might be another reason for less efficiently reducing pathogens in the animal gut. Three strains of virulent gut phages had been proved to be useful for reducing *E. coli* O157:H7 via oral administration (Tanji et al., 2005), but they did not completely eliminate the pathogens from the animal gut. In fact, the number of pathogens was simply reduced to too low level to cause clinical diseases. Thus, bacterial phage resistance is a concern when controlling bacterial infections (Sanchez, 2011). Even an obligate lytic phage may be unsuitable for therapeutic application when bacteria can acquire resistance to this phage (Orquera et al., 2015). However, different from the bacterial resistance to static antibiotics, phages and bacteria coevolve (Betts et al., 2016). To overcome bacterial phage resistance, a therapeutic phage cocktail, which is composed of more than one phage, can be used (Gill and Hyman, 2010) and this cocktail was successful in treating *Salmonella enterica* subsp. *enterica* serovar Typhimurium U288 contamination (Hooton et al., 2011). Several factors such as environmental conditions and animal organs will affect the effectiveness of phage therapy. The biggest problem with oral administration of phages is the destruction caused by the stomach's acids and the damage caused by proteolytic activity (Ryan et al., 2011). A possible solution is the use of microencapsulation of phage particles. The microencapsulated *Salmonella* phage FelixO1 using the method of Chitosan-Alginate survived well in the pig GIT (Radford et al., 2017; Whichard et al., 2003).

Unfavorable environmental factors, such as salinity, gastrointestinal conditions, pH, and temperature, affect the vitality and survival of phages (Lobocka et al., 2018). In a previous study, when microencapsulated phages were stored at -80°C for one month, there was only a minor reduction of phage titers (Ramirez et al., 2018). In addition, the stability of microencapsulated phages decreased slightly after three and six months (Colom et al., 2017). Therefore, studying the influence of environmental factors on the stability and sensitivity of phage is important and challenges the development and application of phage products.

1.3.4 Biocontrol using phages

Phages are a sustainable and well established alternative to antimicrobial agents that improve food safety (McCallin et al., 2013) and can be used therapeutically or for disinfection in foods (Hudson et al., 2005; Mahony et al., 2011). Phage biocontrol in food mainly includes two types of application: (i) decontaminating pathogen cross-contaminated surfaces of equipment and tables in food-processing and other food establishments; and (ii) biocontrol of the harvested food using direct applications of phages (Endersen et al., 2014; Sulakvelidze, 2013). In 2006, a phage product, which was composed of six kinds of purified phages, was approved to be used against *Listeria monocytogenes* on the surface of ready-to-eat food by the US Food and Drug Administration (FDA) (Lang, 2006). In addition, *E. coli* phage product (EcoShield ECP-100, Intralytix), a cocktail of three *E. coli* O157:H7 phages was approved by FDA and USDA food safety and Inspection Service for food application in 2011 (Bai et al., 2016). Recently, *Salmonella* phage product (SalmoFresh, Intralytix), which is a cocktail of six *Salmonella* phages, was designated "generally recognized as safe" by the FDA in 2013 (Sharma, 2013). Currently, research is focused on the phage therapy in foods to control a broad range of pathogens such as *Listeria* spp. (Bigot et al., 2011; Guenther et al., 2009; Oliveira et al., 2014), *Salmonella* spp. (Augustine and Bhat, 2015; Guenther et al., 2012; Woolston et al., 2013), pathogenic *E. coli* (*E. coli* O157:H7, in particular) (Abdulamir et al., 2014; Abuladze et al., 2008; Kudva et al., 1999), *Campylobacter jejuni* (Goode et al., 2003; Loc Carrillo et al., 2005), and *Staphylococcus aureus* (Garcia et al., 2007; Garcia et al., 2009; O'Flaherty et al., 2005). These studies have indicated that phages might be efficient alternative products to control specific food-borne pathogens and are considered safe for human consumption.

1.4 Gut microbiota

The animal GIT is composed of trillions of intestinal microorganisms. These microorganisms are referred to as gut microbiota (Walter, 2008), the amount of which is approximately 10 times bigger than the amount of human body cells (Eckburg et al., 2005). The stomach typically contains 10^3-10^4 cfu of bacteria, the jejunum 10^5-10^6 cfu, the terminal ileum 10^8-10^9 cfu, while the largest number of bacterial cells is found in the cecum and colon, with 10^{11}-10^{12} cfu/g of bacteria (Tlaskalova-Hogenova et al.,

2011). The complex microbial community of the gut contains bacteria, eukaryotes, viruses, and archaea (Ferrario et al., 2017). The gut microbiota is extremely diverse and contains approximately 1000 different kinds of bacterial species (Brestoff and Artis, 2013). Although there are more than 50 known bacterial phyla, gut-associated microbiota in animals are dominated by four bacterial phyla, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* (Zhang et al., 2016), even though only a minority of these microbial taxa can be cultivated by current microbiological methods (Shendure and Ji, 2008). Nevertheless, recent work has increased the number of species that can be recovered from the gut by improving cultural isolating techniques (Lagier et al., 2016). The virome, which is an important part of the microbiome, comprises bacteriophages, eukaryotic and prokaryotic viruses, and endogenous retroviruses (Pfeiffer and Virgin, 2016). More recently, the remarkable diversity of the gut virome, which is composed of viruses that infect prokaryotic and eukaryotic cells, has increasingly attracted interest (Virgin, 2014), with highly diverse and abundant phages present in the mammalian gut (Mills et al., 2013). The commensal bacteria play a very important regulatory role for health and disease; thus, gut microbiota can be considered as a metabolic organ (O'Hara and Shanahan, 2006), which is critical for overall health of animals. Of particular interest is its ability to resist the colonization of pathogens, so called colonization resistance, which is particularly important in young animals.

1.5 Roles of phages in the gut ecosystem

Phages are ubiquitous in the world, and their potential impact on the ecology of the environment has been recognized (Parmar et al., 2017). Phages are also the most abundant biological entities in the animal gut (Virgin, 2014).

1.5.1 Abundance and diversity of phages in the gut

Some studies have described the presence of phages in human and animal feces (Calci et al., 1998; Dhillon et al., 1976; Dutilh et al., 2014; Hartard et al., 2015; Sazinas et al., 2016). There are at least 10^9 virus like particles (VLPs) per gram in the feces (Kim et al., 2011; Rohwer, 2003). VLPs in the feces appear to be dominated by temperate phages, but the situation are exactly opposite in aquatic environments where mainly occupying by lytic phage (Reyes et al., 2010). A previous study estimated that there are up to 10^{15} individual phage particles in the mammalian gut (Lepage et al., 2008).

However, the viral particles, which referred to gut virome, are extremely different between individualities, even when comparing a mother and her daughter (Minot et al., 2011; Reyes et al., 2010). A high frequency of specific *E. coli* phages in feces (about 90% of fecal samples) was reported, while the percentages of other phages were relatively low (Cornax et al., 1994). Gantzer et al. (2002) also showed that up to 70% of human fecal samples contained up to 4×10^3 plaque forming units (PFU/g) of coliphages. The first metagenomic analyses of the gut virome of human feces showed that the recognizable viruses were primarily *Siphophages* (Breitbart et al., 2003). Therefore, it appears that the species and concentration of phages differs among individuals and shows no relationship with sex or age (Rohwer, 2003).

1.5.2 Impact of phages in the healthy animal gut

It has been well established that phages are important vectors for horizontal gene transfer in complex environmental conditions, and in particular, in the gut ecosystem (Modi et al., 2013; Ogilvie et al., 2013; Reyes et al., 2010). This process is called transduction, which means that DNA is moved from one bacterium to another via phages (Goh, 2016). Phages may impact the gut bacterial community function through gene transfer, or may themselves encode some functions benefit to the host (Reyes et al., 2013). For example, *E. coli* prophages are essential to their bacterial hosts, enhancing their host's tolerance to adverse circumstances such as oxidative, osmotic, and acid; as well as bacterial biofilm, thereby increasing host survival (Wang et al., 2010). Intestinal phages are important for controlling local bacterial populations, according to the principal model of phage therapy (Atterbury et al., 2005). Barr et al., (2013) demonstrated phages enriched within mucosal samples suggesting that phage depended on their immunoglobulin-like protein domains adherence to mucus through a non-host-derived immunity. Thus, phages can limit and control mucosal bacteria and interact more frequently with bacteria and animal hosts (Barr et al., 2013). These results indicate that phages do a certain affect human health. However, the ecological impacts of phages on the microbial community structure in the animal gut and how do they affect animal health are unknown,

1.5.3 Phage and host immunity

Oral or intravenous administration of phages may result in rapid elimination from the circulatory system (Bruttin and Brussow, 2005; Speck and Smithyman, 2016), with

the capsid protein of phages responsible (Leiman et al., 2003). Indeed, foreign phages can directly act on the immune system and give rise to humoral immunity. For example, high titers of staphylococcal phage antibody could be detected in 10% of healthy persons and 44% of staphylococcus infections (Hedstrom and Kamme, 1973; Kamme, 1973), while two studies showed that orally administered phages triggered both innate and adaptive immune responses (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008). Moreover, the humoral immune response was induced by foreign phages (Gorski et al., 2012; Majewska et al., 2015). Phages can additionally inhibit immune cells to produce reactive oxygen species when challenged by endotoxins (Miedzybrodzki et al., 2008; Przerwa et al., 2006). This indicates phages play a potential protective role in oxidative stress (Miedzybrodzki et al., 2008; Przerwa et al., 2006). However, the induced humoral immune response does not follow by a simple scheme, instead appearing to depend on characteristics of the phage and the route of phage therapy (Gorski et al., 2006). The influence of phages on the immune system also depends on the dosage and application schedule, and may depend on other undetermined characteristics (Dabrowska et al., 2014; Gorski et al., 2012). However, little is known on the mechanism of phages eliciting innate immune responses. In the mammalian body, immunological reaction caused by foreign phages is strictly regulated by the cytokines secreted by immune cells. Endotoxin-free phage particles stimulate macrophages to produce tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) (Eriksson et al., 2009). The immune cells recognize foreign antigens or the molecular patterns (Virgin, 2014). Toll-like receptors (TLRs), such as TLR2, TLR3, TLR7, TLR8 and TLR9 are innate immune sensors. It was reported that they are involved in the identification of viral structure (Foca et al., 2015). Further, the activation of Toll-like receptors triggers activation of the nuclear transcription factors such as nuclear factor-κB (NF-κB), interferon regulatory factor (IRF) 3, and IRF7. Finally, these effectors promote the expression of the next level of antiviral effectors such as type I interferon, pro-inflammatory cytokines such as Interleukin (IL)-1β and IL-6, and chemokines such as IL-8 and C-X-C motif chemokine 10 (Yan and Chen, 2012). The interactions of phages and the immune system are a fascinating and novel field that is worth to study in-depth because these interactions can synergistically affect bacterial pathogen elimination. Further deep-going studies are needed to elucidate the interactions of phage with the immune system, and the

contribution of phage to homeostasis based on this relationship.

1.5.4 Phages and gut microflora disorders

Due to its relevance for health, in recent years, researches have focused on the bacterial dysbiosis of diseases characterized by alterations in the composition of intestinal microbiota (Clemente et al., 2012; Sobhani et al., 2011). For example, differences in the number of VLPs are apparent between healthy people and Crohn's disease (CD) patients; the titers of VLPs in the intestinal mucosa in CD patients (2.9×10^9 VLPs/biopsy) was 24 times as large as that of healthy individuals (1.2×10^8 VLPs/biopsy) (Lepage et al., 2008), and the increase of gut virome abundance and reduction of bacterial flora diversity was significant in CD patients and ulcerative colitis patients (Perez-Brocal et al., 2013). Gut phages may cause ecological disorders, leading to the transition from health to disease, which is defined as the imbalance between symbiotic bacteria (De Paepe et al., 2014). Phages may give rise to the realization of non-host derived immunity. It is possible to prevent and/or treat intestinal diseases by regulating mucosal phage complements (Barr et al., 2013). Such a micro tuned modulation of phage on the immune responses of intestinal may allow foreign pathogens to invade intestines through the epithelial cell layer (Fig. 1.3). The filamentous phage M13 has been applied as vector for treating Alzheimer's disease and Parkinson's diseases, because it can combine the typical β-amyloid and α-synuclein plaques causing plaque depolymerization in the brain (Ksendzovsky et al., 2012). Phages have been verified to successfully inhibit tumor formation and promote tumor regression (Dabrowska et al., 2004; Pajtasz-Piasecka et al., 2008). The inflammatory cells were recruited and cytokines were induced after phage therapy for cancer, so that the life span of mice is prolonged. Genetically engineered phages can also be used as delivery vectors of anticancer drugs (Kia et al., 2013), as well as anticancer proteins, which induce the apoptotic death of cancer cells resulting in breast tumor regression (Shoae-Hassani et al., 2013).

Phages can affect the ecosystem of the intestinal community through several mechanisms (De Paepe et al., 2014). First, the gut ecosystems is affected by the predation and propagation on sensitive bacteria using the "kill the winner" model (Allen et al., 2011; Golomidova et al., 2007; Weinbauer, 2004). Phages also can interact with the bacterial ecosystems in the gut via the clustered regularly interspaced

short palindromic repeat (CRISPR) systems of the bacteria. CRISPR systems of bacteria can identify and silence foreign genes such as phages, thus providing an acquired immunity (Duerkop and Hooper, 2013). The third mechanism, defined as the "biological weapon" model, describes that the symbiotic bacteria can use phages to kill their competitor present in the gut ecosystem (Bossi et al., 2003; Brown et al., 2006). Another model, which was called the "community shuffling" model, is used by temperate phages. These phages can lyse their former lysogenic host upon induction (Zhang et al., 2000). Moreover, temperate phages also use the "emergence of new bacterial strains" model to impact the ecosystem. They carry genes which modify bacterial phenotypes but without killing bacteria (Martinsohn et al., 2008).

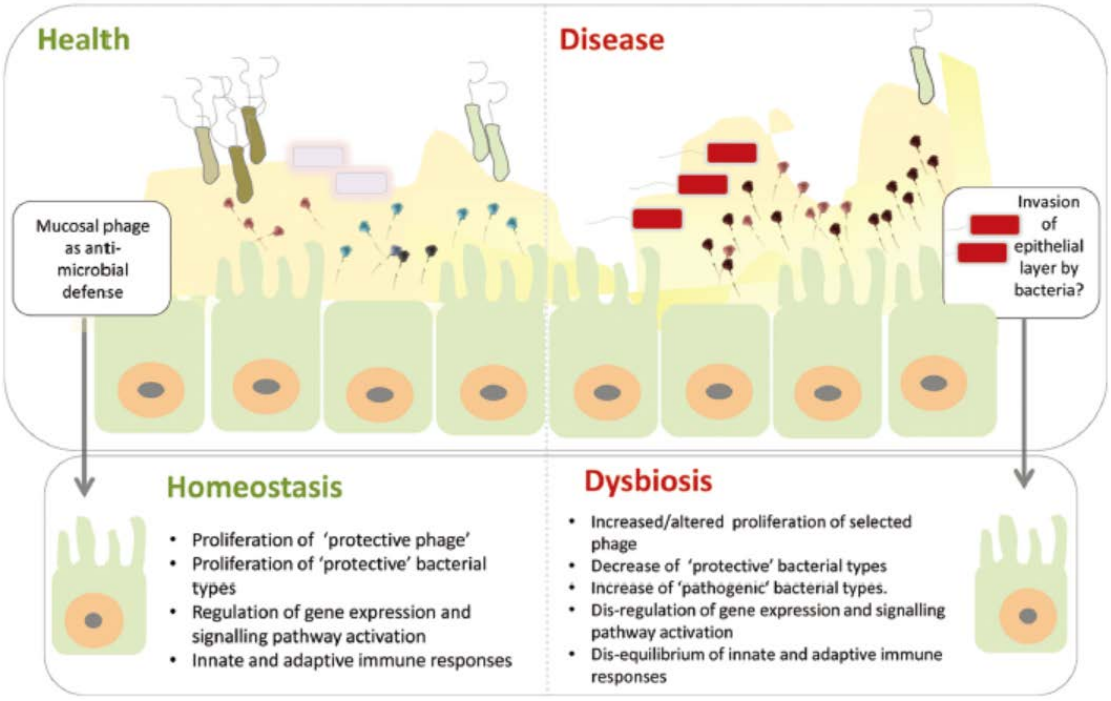

Fig. 1.3 Phage effects on homoeostasis and dysbiosis (Ogilvie and Jones, 2015).

The phage adherence to intestinal mucosa is modified by genetic factors and environmental stressors. Compared to healthy individuals, the properties of the mucosal phages are altered in the diseased status, which leads to intestinal flora dysbiosis.

1.6 Detection methods for gut microbiota

The study on gut microbiota is mainly limited by the available qualitative and quantitative detection methods for microorganisms, including viruses (Foca et al.,

2015). In fact, in order to characterize the composition of the bacterial community in the intestine, research approaches have shifted from traditional culture and microscopy-based observation to sequencing technologies for studying the potential diversity at genomic level (Ogilvie and Jones, 2015). The traditional culture method is used to isolate phages from environmental and intestinal samples using a bacterial strain as the decoy. It has been used to characterize and understand phages since the first discovery of phages, and will continue to be an important tool for the analysis and study of the gut virome. These techniques are essential, inexpensive tools and ongoing efforts have increased the numbers of culturable microorganism (Rajilic-Stojanovic and de Vos, 2014). However, because of the inherent limitation of the current culture technologies, it is impossible to cultivate the majority of the microbial species. Another similarly important technique for phage analysis is transmission electron microscopy (TEM), which is effective in studying phage morphologies and classification. In fact, TEM studies have revealed that tailed phages (Order *Caudovirales*) are most prevalent in the animal gut (Flewett et al., 1974; Letarov and Kulikov, 2009). The combination of sequencing technologies and related bioinformatics tools enable us to better comprehend the composition, structure, and function of gut microbiota. Although some of the entities present in the animal gut have been characterized benefiting from the development of metagenomic sequencing techniques, in-depth genomic studies of gut microbiota using NGS sequencing technology mainly depend on how to efficiently assemble sequence fragments of genes into a large genome.

The virus-like-particles (VLPs) in fecal samples are generally isolated using filtration with 0.22 μm pore size and ultra-centrifugation employing a CsCl-density gradient (Thurber et al., 2009). The matching metagenomic sequences of viral genomes can be gained via the following extraction of nucleic acids (Thurber et al., 2009). The detection of VLPs provides for a better understanding of the gut virome, and enables a deeper analysis of the inventory, composition and function of gut viral particles (Breitbart et al., 2003; Kim et al., 2011; Minot et al., 2011). However, RNA viruses are often ignored using metagenomic sequencing analyses and the current VLP isolation method may not efficiently detect all viruses in fecal samples (Virgin, 2014). It has been estimated that only 0.0002% of viral genes have been sequenced (Rohwer, 2003). Moreover, VLPs from environmental and intestinal samples using

metagenomic sequencing yield large quantities of unidentified sequences (Breitbart et al., 2003; Minot et al., 2011; Reyes et al., 2010). Therefore, studying intestinal VLPs using metagenomic technologies can be an efficient way to discover new phage genomes. However, to further study the function of phages still requires cultivating the phage and its host based on the traditional culture method. Moreover, research approaches need to optimize the combination of high-throughput “omics” analyses and biological data and should further develop comparative volunteer studies to enable a better understanding of the roles of phages in intestinal disorders.

1.7 Conclusions

Phages are the most abundant biological entities on the earth, thriving and coexisting with their bacterial hosts. Lytic phages are potential means to reduce contaminations due to pathogenic bacteria in foods, or to tackle bacterial infections in animal breeding. Presumably, most of the phages in the intestinal tract are lysogenic, which is a particularly advantageous status in the gut. In addition, studies on the mammalian gut virome have provided an understanding of the extreme diversity of the phage community. It can be expected that phages often play an essential role in regulating the composition of the mammalian gut microbiota. However, as research on the effects of phages in the gut ecosystem is still in its infancy, particularly regarding the manipulation of the gut microbiota in intestinal diseases, addressing these research questions will ultimately lead to innovative applications of phages in animal health. These could enable the reliable identification and eradication of harmful imbalances in the gut microbiome, and by employing appropriate experimental strategies pinpoint the best possible treatment for intestinal diseases.

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CHAPTER TWO

MORPHOLOGICAL, PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF LYTIC GUT PHAGE vB_SenM-PA13076 AND TEMPERATE GUT PHAGE vB_SpuP-BP96115 ISOLATED FROM CHICKEN FECES

2.1 Introduction

Salmonella enterica subsp. *enterica* serovar Entertidis and Pullorum are important reported bacterial pathogens, not only related to the prevalence of salmonellosis in farm animals but also related to foodborne disease outbreaks and public health (Christenson, 2013; Ebel et al., 2016). *Salmonella* infections remain a cause for global concern and cause significant economic losses due to morbidity (Taylor et al., 2015). Moreover, *Salmonella* can colonize the intestinal tract and cause systemic infections, gastroenteritis and septicaemia, depending on the animal species and the infecting bacterial strain (Schultz et al., 2017; Szmolka et al., 2015). *Salmonella* infects animals mainly through the oral route and the pathogenesis of *Salmonella* is extremely complex (Bello et al., 2016; Christenson, 2013).

Prophylactic use of nonantibiotic products to promote gut health of animals is important for sustaining animal production. Although these alternative products are contradictory to antibiotics, they have generated financial benefits to producers. They improved feed conversion, developed innate immunity, stimulated immune response, increased vitality, and decreased mortality (Francois et al., 2016).

Phages are ubiquitous in nature and the most abundant living entities on earth (Abedon et al., 2017; Rohwer, 2003), and show host specificity. Phages can exhibit one of two life cycle types, lytic or temperate (Hobbs and Abedon, 2016). The ability of lytic phages to lyse host bacterial cells rapidly forms the basis for the development of phage therapy for controlling bacterial pathogen contamination or infection, whereas the DNA of temperate phages is often integrated into the host's DNA (Sharma et al., 2017). Furthermore, temperate phages go into the prophage state and multiply by the reproduction of the host, and producing independent phages under adverse circumstances (Hendrix et al., 1999). Prophage DNA is replicated with the replication of the cell genome and the DNA is inherited to offspring (Obregon et al.,

2003). Phages are present in all environments, including oceans, soil, and gastrointestinal tracts (Gross, 2014). In the gastrointestinal tract, total phage densities have been estimated as 10^{15} phage particles, dominated by temperate phages (Dabrowska et al., 2005; Dalmasso et al., 2014). Temperate phages have potential functions in regulating the microbial balance in the gut ecosystem by horizontal gene transfer to exchange genetic material between bacteria (Bakhshinejad and Ghiasvand, 2017; Holmes, 2011). Since *Salmonella* spp. infections of the intestinal system are an emerging problem in animal farming (Schulte and Hensel, 2016), phages isolated from animal feces, known as gut phages, were suggested as an alternative to treat gastrointestinal disease (McCarville et al., 2016). While a temperate phage, which integrates its DNA into its host genome without lysis action is not suitable for phage therapy, lytic phages bring about rapid lysis of bacteria, and are therefore good agents for phage therapy (Jin et al., 2014). In fact, orally administrated encapsulated lytic phages are efficacious in protecting broiler intestines against *Salmonella* colonization (Colom et al., 2015). Similarly, oral administration of lytic phage mixtures significantly reduced gastrointestinal *Escherichia coli* O157:H7 (Tanji et al., 2005), *Shigella* spp. (Volker, 2015) and *Listeria monocytogenes* (Mai et al., 2010) in experimentally infected mice.

So far, there are more than 6600 complete genomes of phages in GenBank of the National Center for Biotechnology Information (NCBI) (June 2018). Typical *Salmonella* lytic and temperate phages, including FelixO1 (Whichard et al., 2010), SP6 (Dobbins et al., 2004), Gifsy-2 (GenBank accession No: NC_010393.1), and P22 (Vander Byl and Kropinski, 2000) have been reported and their genomes sequenced. Comparative genome analysis is an important method for understanding the evolutionary genetics of phage genomes and the functions of all kinds of coding genes (Al-Jarbou, 2012). In despite of lots of sequenced phage in the GenBank, the information on the genomics of gut-associated phages is still limited. Moreover, characterization of phages' genome is a requirement before practical application. The objectives of this study were to characterize the properties of a lytic and temperate *Salmonella* phage isolate from chicken feces, as well as to sequence and analyze the phage genomes.

2.2 Materials and Methods

2.2.1 Bacterial strains and media

In total, 311 strains of *Salmonella* spp. were used in this study (Table S2.1, supplementary material). All of the strains were isolated and characterized from diseased chicken and foods in accordance with the National Standard of China (GB/T 4789.4-2010). Among the 311 tested strains, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) ATCC13076 was used as a reference strain and *Salmonella enterica* subsp. *enterica* serovar Pullorum (*Salmonella* Pullorum) SPu-115 was an isolated strain from diseased chicken gut. All strains were cultivated at 37℃ in liquid TSB or solid TSB.

2.2.2 Isolation of lytic gut phage vB_SenM-PA13076

Several fecal samples of chicken obtained from different parts of Jiangsu Province were used for isolating lytic gut phages. Five gram of chicken feces were suspended in 50 mL of SM buffer (50 mM Tris-HCl [pH 7.5], 0.10 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$ and 0.01% gelatin) and left overnight on a shaker (HY-8A, Jingda instruments manufacturing Co., Ltd, Jintan, China) at room temperature (25℃) and at 100 rpm/min. Twenty-five milliliters of the sample solution was centrifuged (15,000×*g*, 15 min, 4℃) and the supernatants were filtered through a 0.22 μm pore-size filter (Merck Millipore, MA, USA). Filtered solution (10 mL) was mixed with 500 μL of 12 h-old *Salmonella* Enteritidis ATCC13076 cultures grown in 20 mL TSB broth at 37℃. After incubation for 18 h, the mixture was centrifuged using the same method as above, filtered again and the filtrate was tested for the presence of lytic phages by inoculating 10 μL of the filtrate on lawns of *Salmonella* Enteritidis ATCC13076 prepared on TSB agar. The plates were cultured for 18 h and inspected for clear zones. The filtrate causing lysis was inoculated at 500 μL per 10 mL fresh TSB with *Salmonella* Enteritidis ATCC13076 for phage amplification. Lytic phages were purified according to the method of Adams (Adams, 1959). 200 μL of phage filtrate was mix with 200 μL of *Salmonella* Enteritidis ATCC13076 culture (1×10^9 cfu/mL) and the mixture was immobile at room temperature for 20 min. 4 mL of liqiud LB agar (0.7%) was added and paved on the prepared LB agar. After solidification, the plates were incubated

overnight at 37℃. Clear plaques were selected for the following experiments. For amplification, the mixture of the phage and the host strain at a multiplicity of infection (MOI) 0.1 was added to 10 mL of TSB medium at 37°C for 6 h. The phage lysate was prepared by centrifugation at 12000×g for 10 min at 4℃ and the supernatant was filtered through a 0.22 μm pore size membrane (Merck Millipore Ltd., Ireland). The phage titers were determined using the double-layer agar method.

2.2.3 Induction of temperate gut phage vB_SpuP-BP96115

First, exponential cultures of selected *Salmonella* strains (10 putative lysogenic strains containing the prophage) which were isolated from the guts of diseased chickens, were prepared by growing cultures until an OD_{600nm} of 0.3 was reached. Mitomycin C (Sigma, USA) was then added at a final concentration of 0.5 μg/mL and the suspension was incubated at 37℃ for 4-5 h under vigorous shaking (Garcia et al., 2007). The treated bacterial cultures were centrifuged (11,000×*g*, 10min, 4℃) and filtered by 0.22 μm. The presence of temperate phages was determined by adding a 10 μL volume of filtrate onto lawns of 10 *Salmonella* strains. Finally, the temperate gut phage was successfully induced from the gut-associated strain *Salmonella* Pullorum SPu-115, and its host was *Salmonella* Pullorum SPu-109. The obscure plaques formed were picked up for further propagation and purification.

2.2.4 Purification, propagation of phage and precipitation of phage particles

Single plaques was picked and dissolved in 1 mL SM buffer at 4℃ overnight. As 10^5-10^6 PFU/mL of phage per plaque, 10^3 and 10^4 dilutions of this suspension were used for the next cycle of purification, with at least 5 cycles carried out. Before amplifying the phage, the optimal multiplicity of infection (MOI) were determined using the different ratio of phage to its host among 0.001~1000. For inoculation, exponentially growing host strains were infected with the phage at 1 of optimal MOI in 100 mL TSB at 37℃ for 6 h. Then, crude phage lysate was obtained after centrifugation and filtration. After appropriate dilution with SM buffer, phage titers were detected.

Phage particles were precipitated with 1M NaCl and 10% (w/v) PEG 8000 (Amersco, Ohio, USA). After decanting the supernatant, sediments were dissolved with 2 mL SM buffer. The resulting mixture was extracted with chloroform, followed by

centrifugation (3000×*g*, 15 min, 4℃). Phage particles were drawn using pipette and stored at 4℃. And then, they were further ultracentrifuged by the method of cesium chloride gradient at 100,000×*g* (Beckman L-80XP, CA, USA) for 24 h at 4℃ (Ozkan et al., 2016).

2.2.5 Electron microscopy

The precipitated phage particles were diluted with SM buffer without gelatin. Phages particles (~10^8 PFU/mL) were negatively stained with phosphotungstic acid (1% w/v, pH 7.2). The morphologies of phages were examined with a transmission electron microscope (TEM) (H-7650, Hitachi High-Technologies Corporation, Japan) at different magnifications. The phage sizes were calculated.

2.2.6 Optimization of multiplicity of infection (MOI)

The optimization of phage replication was determined in mixtures of phage and host at different MOI ratios (0.001-1000) according to the titers (Wong et al., 2014). The host strain culture containing 1×10^8 CFU of cells was added into phage solutions and multiplicities of infection are 0.01, 0.1, 1, 10, 100 and 1000. After incubation for 8 h, the phage titers were established using double-layer agar plates after preparing dilutions of the filtrate in SM buffer.

2.2.7 Host range determination

The host ranges of the phages were detected using the spotting test as described by Atterbury et al. (2007). 311 strains of epidemic *Salmonella* spp. were used for host range detection of lytic phage PA13076 in this study. Because temperate phage BP96115 was not suitable for phage therapy, only twenty-six strains of *Salmonella*, the majority of which were serovar Pullorum, were used for detecting the host range of BP96115. Ten microliters of phage solution (~10^8 PFU/mL) was spotted on the lawn of the bacterial test strains. Plaque was observed next day.

2.2.8 Thermal and pH susceptibility tests

Phage solutions were cultured ranging from 30 to 90℃ for 30 min or 60 min to determine thermal resistance. Phage solutions were mixed with buffered peptone water (BPW) at different pHs (adjusted using 1 mol/L of NaOH or HCl) to detect the

pH resistance. The mixed solutions were incubated at 37℃ for 2 h. Phage titers at the different temperature and pH value were determined.

2.2.9 One-step growth curve

One-step growth curves of phages were established using a 10-min interval method as previously described (Bao et al., 2011). Host cells (10^8 cfu/mL) and the specific phage were mixed at a MOI of 10~100 without shaking at 37℃ for 20 min. After centrifugation at 12,000×*g* for 60 s, the sediments of infected host cells were re-suspended in 10 mL of pre-warmed TSB and were incubated at 37℃. After samples was centrifuged, the phage titer of supernatant was determined every 10 min. Assays were repeated for three times. Latent period and burst time were determined according to the one-step growth curve. Burst sizes were defined as the final titers of phage divided by the concentration of initial host cells.

2.2.10 Phage adsorption

Adsorption kinetics of phages was determined as described by Tanji et al. (2004). Exponentially growing (OD_{600nm}=0.5) cells of host strains in LB or TSB broth were centrifuged and diluted to 1×10^8 CFU/mL with 10 mL of fresh sterile LB/TSB broth. Phage inoculum was added (MOI=0.1) to be 1×10^7 PFU/mL for the final concentration and the mixtures were incubated at 37℃. 200 μL samples were taken and centrifuged. Then, the titers of free phages in the supernatant were determined. The adsorbed rate was 0 at the beginning.

2.2.11 Phage DNA extraction, sequencing and genomic analysis

A high titer suspension of phage particles (>10^9 PFU/mL) in SM buffer was prepared. The phage genome was extracted according to Kang et al. (2013). Briefly, 1 μL DNase I (10 mg/mL; Sigma-Aldrich, UK) and 1 μL RNase A (10 mg/mL; Sigma-Aldrich, UK) were added in 750 μL of phage suspension and they were cultured at 37℃ for 30 min. Then, 75 μL 5% sodium dodecyl sulfate (SDS) and 75 μL 500 μg/mL proteinase K were added and the mixture was incubated at 56℃ for 1 h. Then, using equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol, Sigma-Aldrich) to extract the phage genome. The phage genomes were precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol and centrifuged (15,000×*g*, 30 min, 4℃). The pellets were rinsed twice with

70% ethanol, dried in air, and then dissolved in 100 μL sterilized deionized distilled water containing 50 μg/mL RNase A.

The phage whole genome sequencing was done by Guangzhou Gene de-novo Biotechnology Co. Ltd (Guangzhou, China). The genomic library was prepared using an Illumina® TruSeq® Nano DNA Library Prep kit (Illumina, CA). Phage DNA was sequenced on an Illumina HiSeq X-Ten platform to 100-fold coverage. The poor quality data in the original sequences were removed, and assembled to a genome by SeqMan II sequence analysis software (DNASTAR Inc.).

The open reading frames (ORF) were analyzed using ORF Finder and verified by Glimmer 3.02 (Bardina et al., 2016). Homologs of nucleotide sequences and predicted protein sequences were scanned using search tools (BLASTP and BLASTN) available at the NCBI database. The annotations were compared to published genomes of other phages.

2.2.12 SDS-PAGE analysis

A high titer (1×10^{11} PFU/mL) of phage was used for SDS -PAGE analysis. 80 μL of phage particle solution was mixed with 20 μL 5×SDS loading buffer (Solarbio, China) and heat denatured at 95℃ for 5 min according to Niu et al. (2012). Then, the phage samples were loaded onto a standard ready to use 12% SDS-PAGE gel (Yeasen, China). The gels were run at a constant voltage of 120V. After staining with coomassie brilliant blue G-250 colloidal protein stain (EZBlue G1041, Sigma-Aldrich, UK), protein bands were visualized using a transilluminator (Bio-Rad, Hercules, CA).

2.2.13 Accession number of genomes

The complete genomes of PA13076 and BP96115 have been deposited in GenBank under the accession numbers MF740800 and MG407615.

2.3 Results

2.3.1 Lytic and temperate gut phage isolation

One lytic gut phage, named vB_SenM-PA13076 (subsequently referred to as PA13076), was successfully isolated from the fecal samples collected from a commercial chicken farm in Haimen city, Jiangsu Province, China. After successive

purification steps by picking plaques, this lytic gut phage formed the same size of plaques on lawns of its host strain *Salmonella* Enteritidis ATCC13076. The gut phage PA13076 formed clear plaques using the host strain *Salmonella* Enteritidis ATCC13076 with a diameter of 0.8±0.05 mm (Fig.2.1A). Furthermore, a lysogenic prophage (temperate), named vB_SpuP-BP96115 (subsequently referred to as BP96115), was released from the gut pathogen *Salmonella* Pullorum SPu-115, induced by mitomycin C (0.5 μg/mL). Gut phage BP96115 was temperate, and formed turbid plaques on *Salmonella* Pullorum SPu-109 (its host strain) (Fig. 2.1B). The diameter of plaques formed by temperate gut phage BP96115 was 4.0±0.5 mm.

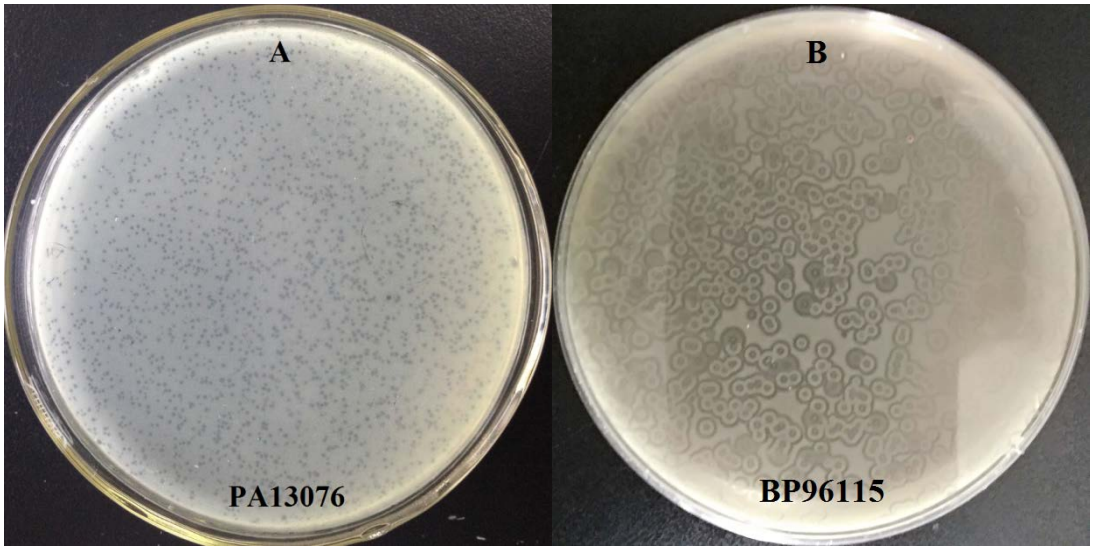

Fig. 2.1 The plaques of the isolated phages PA13076 (A) and BP96115 (B).

2.3.2 Morphological characterization of isolated phages

According to the TEM analysis, phage PA13076 was characterized by an oval head with a length of 66±4 nm and a contractile tail of 90±5 nm in length (Fig. 2.2A), characteristic of the family *Myoviridae* (Maniloff and Ackermann, 1998). However, BP96115 matched the *Podoviridae* family C1 morphotype, with a small head (diameter, 54±4nm) and a short tail with tail fibers (10±2 nm in length) (Fig. 2.2B).

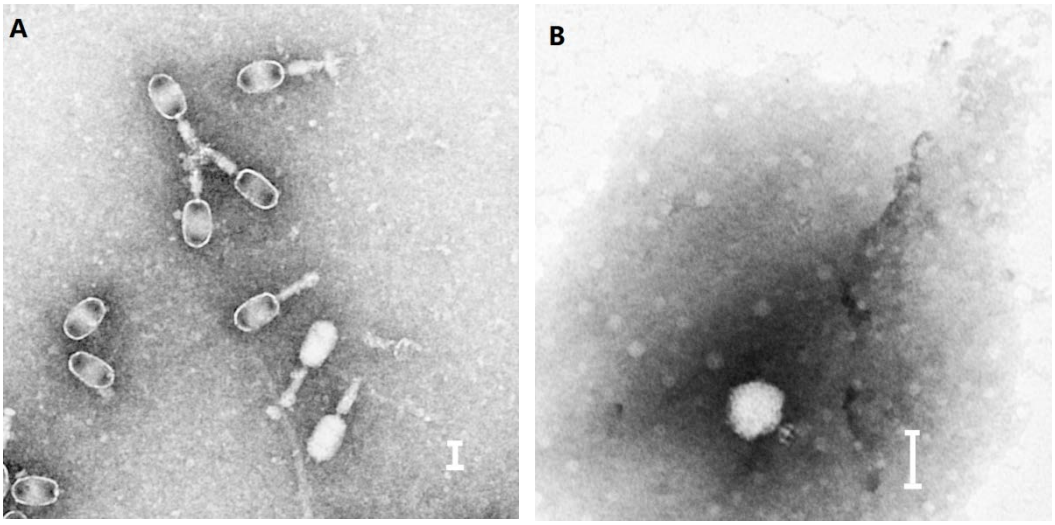

Fig. 2.2 TEM images of lytic phage PA13076 (A) and temperate phage BP96115 (B). Phages were negatively stained with 1% phosphotungstic acid. Bar=50 nm.

2.3.3 The optimal MOI of lytic phage PA13076 and temperate phage BP96115

The highest propagation titers for phage PA13076 and BP96115 were observed at the lowest (MOI=0.01) and the highest MOI ratio (MOI=1000), respectively (Table 2.1). Thus, the optimal MOI is 0.01 for phage PA13076 and 1000 for BP96115.

Table 2.1 The MOI of lytic phage PA13076 and temperate phage BP96115.

MOI[a]	Number of host bacteria (CFU)	Number of phages (PFU)	Propagating titers of PA13076 (PFU/mL)	Propagating titers of BP96115 (PFU/mL)
0.01	1×10^{8}	1×10^{6}	1.1×10^{10}	7.3×10^{9}
0.1	1×10^{8}	1×10^{7}	3.4×10^{9}	4.2×10^{9}
1	1×10^{8}	1×10^{8}	8.6×10^{9}	3.6×10^{9}
10	1×10^{8}	1×10^{9}	7.5×10^{8}	9.0×10^{8}
100	1×10^{8}	1×10^{10}	1.4×10^{8}	1.1×10^{10}
1000	1×10^{8}	1×10^{11}	2.7×10^{9}	5.9×10^{10}

[a]phage titer/ bacterial cfu

2.3.4 One-step growth curve of lytic phage PA13076 and temperate phage BP96115

The latent period for both phages was about 10 min (Fig. 2.3), while the average burst size of phage PA13076 and BP96115 was calculated to be 21 and 24 phage particles per infected cell, respectively.

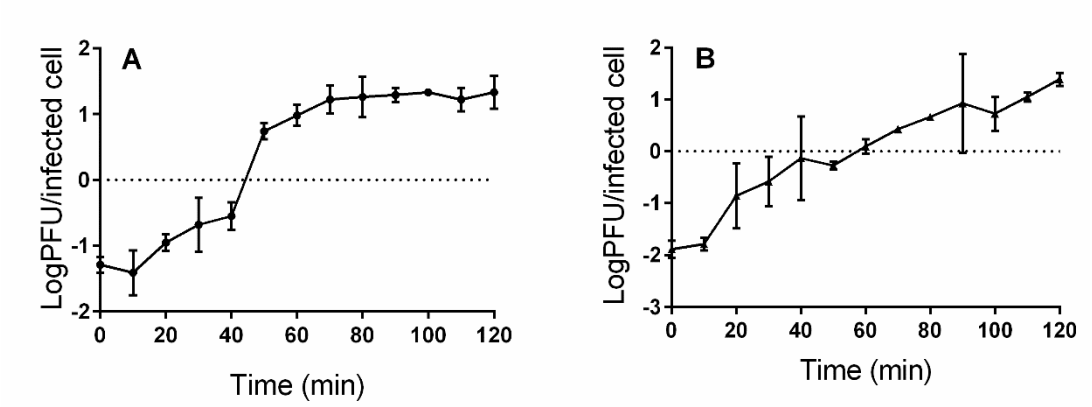

Fig. 2.3 One-step growth curves of lytic phage PA13076 (A) in *Salmonella* Enteritidis ATCC13076 and temperate phage BP96115 (B) in *Salmonella* Pullorum SPu-109 in TSB broth at 37℃. Values correspond to the numbers of PFU per infected cell.

2.3.5 Phage adsorption

The adsorption of PA13076 to its host *Salmonella* Enteritidis ATCC13076 is shown in Fig. 2.4A. Phage particles adsorbed to host cells indicated that approximately 40% within 2 min, 75% in 4 min, and nearly 98% in 10 min. For the adsorption of temperate phage BP96115 onto its host strain *Salmonella* Pullorum SPu-109, approximately 80% at 2 min, more than 83% within the first 5 min, and 99% were adsorbed at 10 min post-infection.

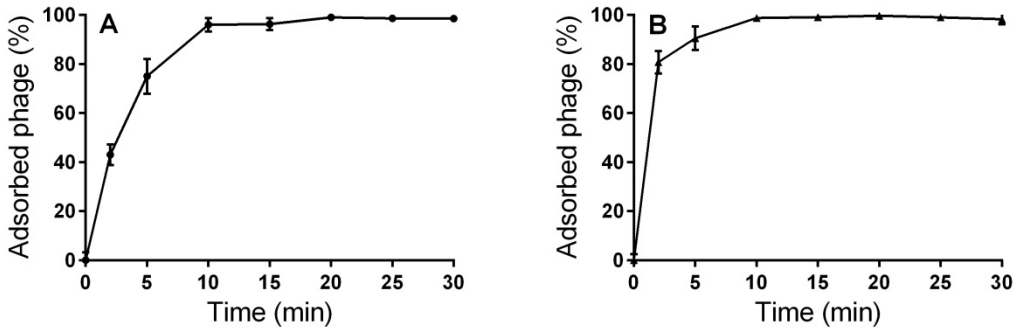

Fig. 2.4 Adsorption of lytic phage PA13076 (A) and temperate phage BP96115 (B) to their host cells. Each data point is the mean ± standard deviation from three independent experiments.

2.3.6 Host range of phage PA13076 and BP96115

The lytic phage PA13076 possessed a wide host range. The spotting test results indicated that phage PA13076 lysed 71.4% (222/311) of epidemic *Salmonella* isolates tested (Table S2.1, supplementary materials). However, the temperate phage BP96115 had a narrow host range with lytic effect on only 34.61% (9/26) of tested strains. Phage BP96115 can only infect specific strains of the serotypes Pullorum, Enteritidis, and Typhimurium of *Salmonella enterica* subsp. *enterica* (Table S2.2, supplementary materials).

2.3.7 Thermal and pH susceptibilities of PA13076 and BP96115

PA13076 was most stable at pH 5-9 (Fig. 2.5A), while BP96115 was most stable at pH 5-7 (Fig. 2.5B), with decreased stability observed at pH values <5 and >7. Both phages were stable between 30℃ to 60℃ for 30 min and 60 min, with a sharp decline in the phage titer above 60℃, decreasing to 0 PFU/mL at 70℃ for PA13076 for 30 min and 60 min (Fig. 2.5C). However, 2.59% viability of BP96115 was retained at 70℃, relative to the observed phage titer at 60℃, with a total viability loss at 80℃ (Fig. 2.5D).

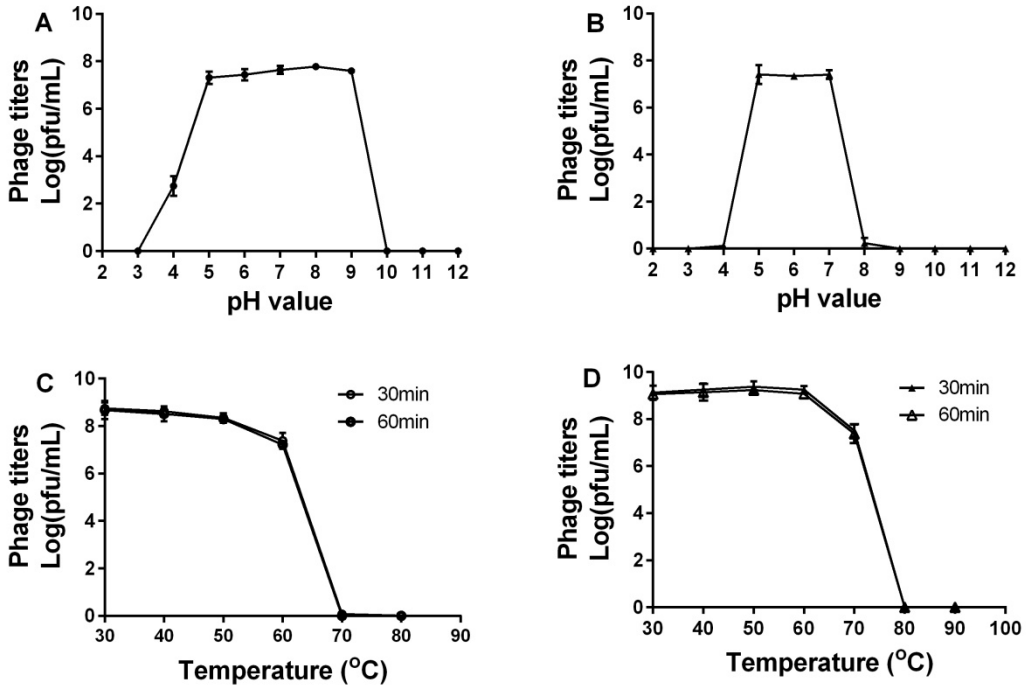

Fig. 2.5 pH stability of lytic phage PA13076 (A) and temperate phage BP96115 (B) and thermo-stability of lytic phage PA13076 (C) and temperate phage BP96115 (D).

2.3.8 The lytic gut phage PA13076 genome sequence

The lytic gut phage PA13076 contained double-stranded DNA with 52,474 bp and a G+C content of 46.12 mol%. Genome analysis of the phage revealed 69 ORFs, but no tRNA genes. However, most of the genes (44 of 69 ORFs) were hypothetical proteins (Table S2.3, supplementary material).

The genome of PA13076 is comprised of four main functional protein clusters: (1) DNA packaging (ORF18: terminase large subunits), (2) structural assembly proteins (ORF8, ORF9, ORF64: tail protein, ORF62, ORF63: tail fiber assembly protein, ORF15: major capsid protein, ORF16: scaffold protein, ORF17: portal protein), (3) host lysis proteins (ORF60: lysozyme), and (4) DNA replication, modification, regulation proteins (ORF22: thymidylate kinase, ORF32: TPR repeat: kinesin light chain, ORF36: primase, ORF39: putative DNA binding protein, ORF43: PAS domain S-box, ORF46: nucleotide-binding protein, ORF48 deoxycytidylate deaminase, ORF

50: DEAD box helicase, ORF54 thymidylate synthase, ORF55, DNA polymerase beta subunit, ORF56: DNA polymerase (Fig. 2.6).

A progressive MAUVE multiple alignment revealed considerable sequence similarity between the genomes of phage PA13076 and *Salmonella* phage BP63 (GenBank accession no. KM366099.1) and *Salmonella* phage UPF_BP2 (GenBank accession no. KX826077.1) (Fig. 2.7). Phage PA13076 showed 97% DNA sequence similarity to phage BP63 and 97% DNA sequence similarity to phage UPF_BP2 (Fig. 2.7).

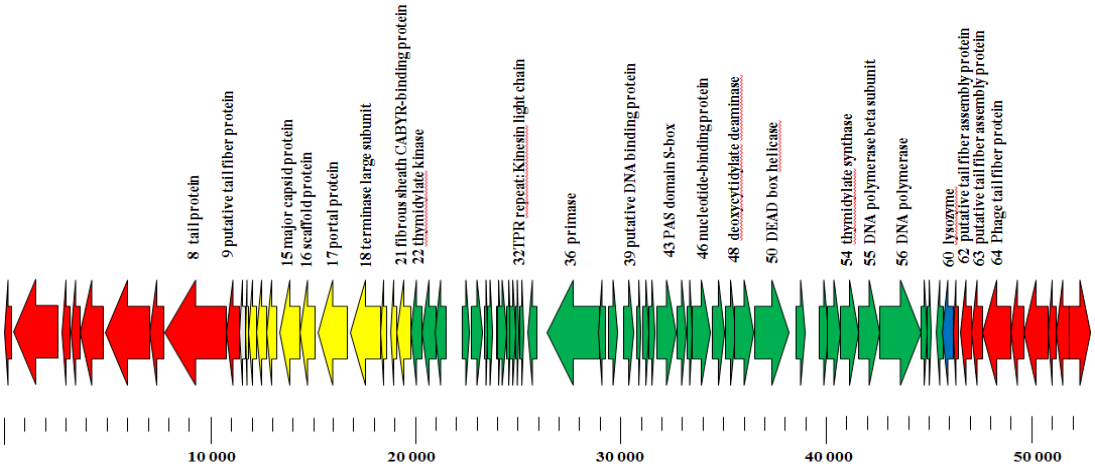

Fig. 2.6 Genome map of PA13076. Genes are numbered according to annotation. The lysozyme gene is shown in blue, DNA packaging, replication and modification genes are shown in green, structural genes are depicted in yellow, while the tail proteins are shown in red.

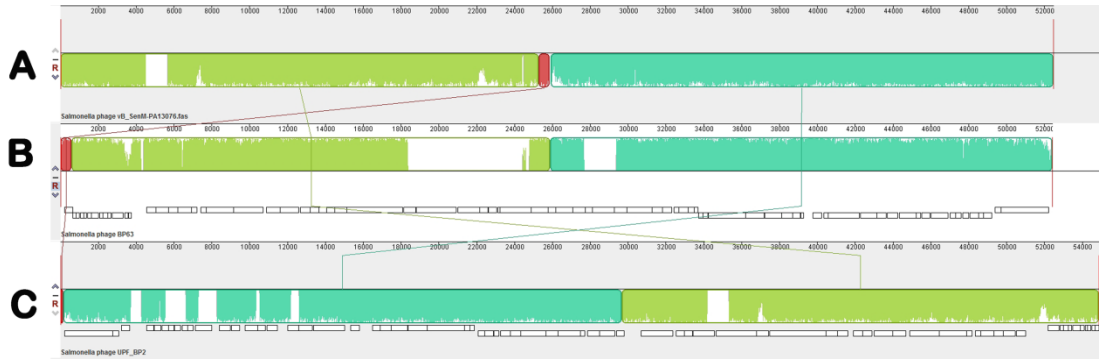

Fig. 2.7 Genome comparison of PA13076 (A) with *Salmonella* phage BP63 (B) and UPF_BP2 (C) at DNA level.

2.3.9 The genome analysis of temperate phage BP96115

The genome of temperate phage BP96115 consists of a double-stranded linear DNA molecule of 41,264 bp, containing 62 ORFs ranging from 81 to 2178 bp in length, possessing an overall GC content of 48.71%. Of the 62 ORFs, 47 predicted proteins with good coding potential. A complete list of ORFs is shown in table S2.4 of supplementary material. The nucleotide sequence of temperate phage BP96115 shared high similarity with that of other temperate phages, enterobacteria phage ST104 (GenBank accession no. AB102868.1) (coverage 100%, identity 99%), *Salmonella* phage SE1 (GenBank accession no. DQ003260.1) (coverage 74%, identity 98%), *Salmonella* phage vB_SalP_PM43 (GenBank accession no. MF188997.1) (coverage 63%, identity 97%), *Salmonella* phage ST64T (GenBank accession no. AY052766.1) (coverage 63%, identity 97%), phage P22 (GenBank accession no. AF217253.1) (coverage 62%, identity 98%) and *Salmonella* phage epsilon34 (GenBank accession no. EU570103.1) (coverage 43%, identity 97%).

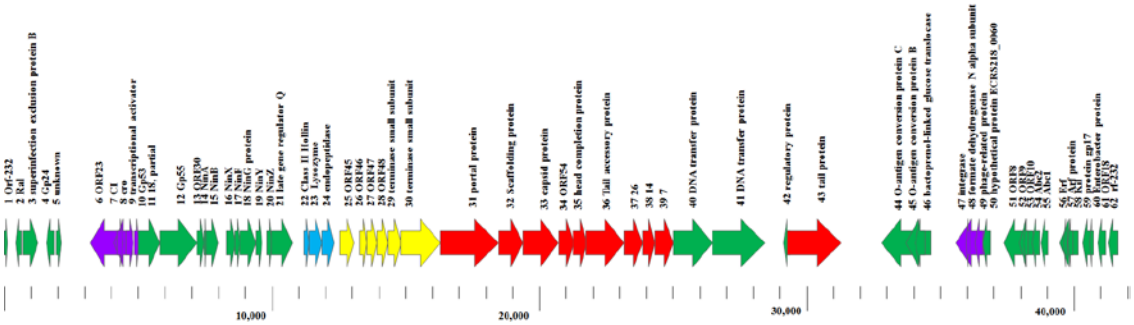

Fig. 2.8 Schematic representation of the linear dsDNA genome of temperate phage BP96115. Positions and predicted functions of ORFs are represented by an arrow. The lysis genes are shown in blue. DNA packaging genes are shown in yellow. Structural genes are marked in red. Genes involved in DNA replication/modification/ regulations are shown in green, while the lysogeny genes are marked in purple.

Genomic analysis indicated that temperate phage BP96115 carries DNA replication, conversion cassette, integrase, superinfection exclusion, antitermination, endopeptidase, probable regulatory protein N, packaging head, lysis, and tail proteins, while it also contained hypothetical genes (Fig. 2.8). ORF5 (1902-2123, unknown) was present in BP96115 but absent in other similar temperate phages. Comparative genomic analysis indicated that BP96115 has a different genomic organization compared to enterobacteria phage ST104, and *Salmonella* phages P22, epsilon34, SE1,

vB_SalP_PM43 and ST64T (Fig. 2.9). Most of the protein sequences of temperate phage BP96115 were closely related to those of other temperate phages.

Fig. 2.9 Alignment of the genome of temperate phage BP96115 with other phages using software MAUVE V2.3.1. Names of the phages are mentioned under their maps line, from top to bottom; (A) BP96115, (B) ST104, (C) P22, (D) epsilon34, (E) SE1, (F) vB_SalP_PM43 and (G) ST64T. Colored blocks indicate regions of nucleotide similarity while colorless blocks correspond to dissimilar regions.

2.3.10 SDS-PAGE analysis

SDS-PAGE analysis was conducted on both PA13076 and BP96115. For phage PA13076, six protein bands were observed, with the most abundant polypeptide having a molecular mass of about 35 kDa. Two main bands with molecular masses of about 58 and 70 kDa were observed in BP96115 samples (Fig. 2.10). These bands, which were easily detected, were most likely the major head and tail proteins.

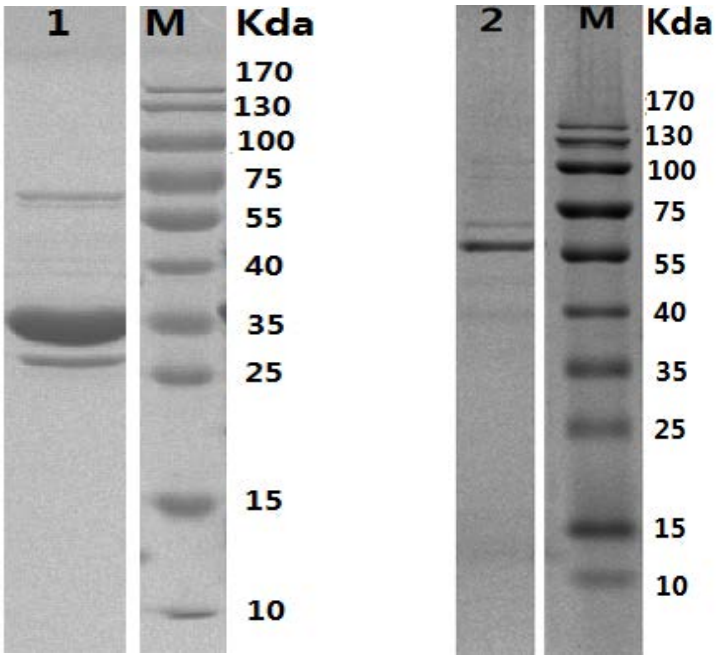

Fig. 2.10 SDS-PAGE analysis of proteins of lytic phage PA13076 (1) and temperate phage BP96115 (2). Lane M are the protein molecular size markers (kDa).

2.4 Discussion

Salmonella spp. are Gram-negative rods with more than 2,600 serovars (Wu et al., 2016). They can infect animals and humans, cause inflammation of the intestine and generally produce a chronic asymptomatic carrier state (Kurtz et al., 2017). The control strategies adopted for treating *Salmonella* infections, including preventive measures and antibiotic treatments, have not been sufficient to eradicate salmonellosis. Moreover, several studies reported the emergence of multidrug-resistant *Salmonella* strains (Pan et al., 2009; Venza Colon et al., 2004). Phages are the most numerous and ancient biological entities in microbial ecosystems of water, soil, and the animal gut (Skurnik and Strauch, 2006). Phage abundance exceeds host populations by 10-100-fold (Manrique et al., 2017). However, the majority of phages in the animal gut is lysogenic, and therefore spend most of the time as integrated prophages (Cadwell, 2015).

In this study, one lytic gut phage and one temperate prophage were isolated and named as vB_SenM-PA13076 (PA13076) and vB_SpuP-BP96115 (BP96115), based on the naming rules for phages (Kropinski et al., 2009). The tailed phages comprise of three families (*Myoviridae*, *Siphoviridae* and *Podoviridae*) (Ackermann, 1998), and about 96% of investigated phages belonged to the tailed phages (Ackermann, 2007).

The lytic gut phage PA13076 matched the *Myoviridae* based on the contractile tail. Although this phage is unique, its head is oval in shape, which is very different from other reported *Salmonella* phages belonging to the *Myoviridae* (Santos et al., 2011; Whichard et al., 2010), it is a classical virulent *Myoviridae* phage. The temperate phage BP96115 with short, noncontractile tails was morphologically similar to members of family *Podoviridae* (Ackermann, 1998). The size of this phage was close to that reported for the phage P22 with a capsid length of 64 nm and the tailspike length of 6.4 nm (Venza Colon et al., 2004).

Determination of the host range of phages is important in order to properly characterize them. The lytic phage PA13076 demonstrated a wide host range, similar to *Salmonella* phage PVP-SE1 (Santos et al., 2011) and T-even phage S16 (Marti et al., 2013). The previous studies on these wide host range phages indicated that they were suitable and good candidates of phage therapy. However, the host range of the temperate phage BP96115 was relatively narrow. The host range of BP96115 was almost the same as that of temperate phage P22, which was reported to inhibit about 12 isolates of *Salmonella* Typhimurium as well as *Salmonella* Derby and *Salmonella* Enteritidis but not strains of serotypes Newport, Muenchen and Muenster (Zinno et al., 2014). Therefore, phage BP96115 may also be used as a tool of molecular biology like phage P22. The tail fibers and the base plate determined the host cell recognition (Garcia-Doval and van Raaij, 2012; Rego et al., 2012). Phage P22 tail fiber proteins bind to the O-antigen moiety of the LPS of its *Salmonella* host (Andres et al., 2010). Therefore, due to the high similarity of the tail fiber proteins between phage P22 and the isolated phage BP96115, the receptor molecules of BP96115 may also be O-antigens of the lipopolysaccharide (LPS). The burst sizes of both phages were somewhat smaller than that of previously reported *Myoviridae* phages (50-100 PFU/cell) (Chang et al., 2005; Park et al., 2012; Raya et al., 2006) and smaller than that of the previously characterized *Salmonella* Pullorum phage PSPu-95 (78 particles per cell) and PSPu-4-116 (86 particles per cell) (Bao et al., 2011). Similar small burst sizes have been found in other phages such as *Staphylococcus* phages phiIPLA-RODI and phiIPLA-C1C (25 and 15 particles per cell) (Gutierrez et al., 2015), *Salmonella* Typhimurium lytic phage Φst1 (22 particles per cell) (Wong et al., 2014), and *Leuconostoc mesenteroides* phage 1-A4 (24 particles per cell) (Mudgal et al., 2006). The latent period was shorter compared with phage Φst1, whose latent period was 40

min (Wong et al., 2014), but similar to the *Salmonella* lytic phages ST4 and SG3 (10min) (Hong et al., 2013). In this study, 98% of lytic phage PA13076 and 99% of temperate phage BP96115 particles adsorbed to their host cell within the first 10min. Similarly, all of the *E. coli* phage JS09 particles adsorbed to their host cells within 9 min (Zhou et al., 2015). However, the adsorption was approximately 30% of phages ΦPSA1 and ΦPSA2 in 5 min, increasing very slowly to 40-50% after 30 min (Di Lallo et al., 2014). The adsorption rate is a specific property of each phage-host pair and may vary depending on phage/host ratio, pH, temperature, or the media composition (Lau et al., 2012; Rakhuba et al., 2010). Multiplicity of infection (MOI)– often defined as the "ratio of phage particles and host cell"– is the key factor governing the phage titers. Depending on the phage–host interactions, MOI should be adjusted to an appropriate value to beneficial phage amplification, so that high phage titers are obtained in the liquid amplification culture (Hyman, 2009). This characteristic also seems to be an important factor to the successful in phage therapy (Sharma et al., 2017). As demonstrated in previous studies, the MOI ranging from 10 to 10000 in *Salmonella* phage PC1 administration reduced *Salmonella* Typhimurium U288 cells significantly (Hooton et al., 2011). Both phages analyzed in the present study showed resistance to heat (up to 60℃) and pH (ranging from 5-9 for PA13076 and 5-7 for BP96115), similar to the *Salmonella* phages ΦSG-JL2 (Kwon et al., 2008). The resistance to heat and pH can influence the efficacy of phage therapy for preventing *Salmonella* infections.

Genomic studies of gut phages remain rather limited. Temperate phages are related to the virulence, species diversity and evolution of species (Dalmasso et al., 2016; Moreno Switt et al., 2013), with most of the temperate phages able to transfer horizontal gene by transduction (Schicklmaier and Schmieger, 1995). Up to 80% of phage-encoded proteins from virus-like-particle derived metagenomics in the gut have been reported not to match known proteins (Mills et al., 2013; Reyes et al., 2013). The genomic information indicated that PA13076 are an unique lytic phage. Furthermore, only very small fractions of abundant lytic *Salmonella* phages have been characterized, including: FelixO1 (Whichard et al., 2010), vB_SenM-S16 (Marti et al., 2013), SPN1S (Lim et al., 2012), SP6 (Dobbins et al., 2004), and ΦSG-JL2 (Kwon et al., 2008). Thus, it was not surprising that many proteins of the newly sequenced phage PA13076 carried numerous genes of unknown function. However, there are no

integrase, repressor or transposase genes in its genome, which shares the same characteristics with lytic phages (Whichard et al., 2010; Marti et al., 2013; Lim et al., 2012). However, temperate phage BP96115 was highly similar with other temperate phages such as the well-characterized phage P22 (Vander Byl and Kropinski, 2000), and enterobacteria phage ST104 (Tanaka et al., 2004). Because of the well-characterized genes and proteins of phage P22, most of the ORFs of temperate phage BP96115 matched known proteins. The high similarities of phage BP96115 proteins with other temperate phages' indicated that it is a real temperate phage (Zinno et al., 2014).

The instinct of a phage is the lytic ability for bacteria. Therefore, research on the endolysin gene is very import for understanding phage evolution and the impact of phages on the bacterial flora in animal intestine. In fact, the majority of tailed phages use a lysis cassette containing endolysin and holin components to puncture the cytoplasmic membrane and lyse the peptidoglycan layer of the bacterial cell wall (Young, 2014). The holin genes are usually in the upstream of the endolysin, occasionally overlapping it (Santos et al., 2011). However, the position of the holin gene is far from the putative endolysin (ORF60) in phage PA13076. In the temperate phage BP96115, ORF22 encodes the holin protein, which belongs to the Class II holin family. In addition, the lysozyme of phage BP96115 is equal to the Rz/Rz1 accessory lysis element, which is ubiquitous in Gram-negative bacteria phages (Summer et al., 2007).

In this study, the phage structural proteins were not identified using mass spectrometry. However, the most abundant protein may likely be the major head protein, according to the study of the structural proteins of *Salmonella* phage PVP-SE1 by ESI-MS/MS (Santos et al., 2011). The precursor of major head was 32.6 kDa, and it was smaller than its predicted size (50.6 kDa) (Niu et al., 2012). According to Niu et al. (2012), the predicted protein molecular weights did not agree with the observed protein molecular weights, likely because of proteolytic cleavage.

Phage PA13076 is a classical lytic phage and Phage Bp96115 is a temperate phage. The above data of characteristics are the basis for studying of gut phages in regulating gut microbiota (Lusiak-Szelachowska et al., 2017).

2.5 Supplementary material

Supplementary material: table S2.1, S2.2, S2.3 and S2.4.

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CHAPTER THREE

DISTRIBUTION OF ORALLY ADMINISTRATED LYTIC AND TEMPERATE *SALMONELLA* GUT PHAGES IN MICE

3.1 Introduction

Phages infect bacterial cells and disrupt bacterial metabolism. In case of lytic cycle, phages cause the bacterium to lyse (Cisek et al., 2017; Sabouri Ghannad and Mohammadi, 2012). Phages are considered to be the most numerous biological entities in the world (Sharma et al., 2017) and are common in the gastrointestinal tract (GIT) of mammals (Mills et al., 2013). Especially temperate phages are the important components of the mammalian intestinal flora (Ashelford et al., 2000; Gorski et al., 2003; Merril, 1974). Studying on gut virome using metagenomic analysis has revealed that the potential functions of phages in the gut ecosystem (Breitbart et al., 2003; Foca et al., 2015).

Pathogen infections of the GIT are one of the major causes of morbidity and mortality in economically important animals (Oliveira et al., 2014). Following oral ingestion of pathogenic bacteria, proportions of intestinal microorganism survive in the stomach, some of them reach the ileum and the cecum, and disrupt the already established commensal microbial flora and induce inflammation (Mastroeni and Sheppard, 2004). They are carried from the intestine to the bloodstream. One of the effective way for treating GIT infections were oral administration of phages (Ahmed et al., 2016; Bradley et al., 1991; Hannu et al., 2002; Schattner et al., 2005). The role of intestinal phages is the potential capability to regulate the commensal bacteria populations (Lopetuso et al., 2016). Thus, gut phages may be responsible for defensing harmful bacteria and restricting their widespread transmission and repairing the resulting pathology (Gorski and Weber-Dabrowska, 2005), highlighting that phages play a key role in the gut. However, phage survival as well as gut colonization and distribution in the GIT is still poorly understood. In addition, most phage studies do not report if phages can penetrate the tissue of other organs.

Phage preparations contain large amounts of bacterial endotoxin due to host cell lysis (Gu et al., 2011). It has been known for more than 100 years that Gram-negative bacteria contain a heat-stable toxin, the so-called endotoxin, which chemically

belongs to the lipopolysaccharides (LPS) (Yuan et al., 2012). Even small amounts of LPS from protein preparations (1ng/mL) can induce cytokine production, pyrogenic reactions and septic shock in humans and experimental animals (Abaev et al., 2013; Romanovsky et al., 1996). Therefore, efficient removal of LPS from phage preparations is an ongoing challenge in the application of phages. So far, various techniques including cesium chloride (CsCl) ultracentrifugation (Dufour et al., 2016), anion exchange and affinity chromatography (Boratynski et al., 2004), and ultrafiltration (Hashemi et al., 2013) have been investigated to remove LPS from recombinant protein solutions with varying degrees of success.

This study intended to elucidate the abundance and distribution of phages in the GIT when mice were orally administrated the phage for 31 days. At the same time, the capabilities of phages to translocate to blood and spleen and the levels of IgG in sera and sIgA in ileum samples were determined.

3.2 Materials and Methods

3.2.1 Large scale preparation of phage suspensions

Lytic phage PA13076 and temperate phage BP96115 were used in this study. Phage PA13076, originally isolated from chicken feces, was propagated by using 10^6 CFU/mL of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) ATCC 13076 and 10^8 PFU/mL of PA13076 in 1000 mL TSB broth at 37℃ without shaking for 6 h. The temperate phage BP96115, originally induced from the gut pathogen *Salmonella enterica* subsp. *enterica* serovar Pullorum (*Salmonella* Pullorum), was amplified using its host strain *Salmonella* Pullorum SPu-109 (1×10^8 CFU/mL) and BP96115 (1×10^9 PFU/mL) in 1000 mL of TSB broth for 6 h at 37℃ without shaking. Crude phage lysates were obtained by centrifugation at 15,000×*g* for 10 min at 4℃. After filtrating, phage particles were precipitated using NaCl and PEG 8000 as described in chapter two.

3.2.2 LPS removal

Phage particles (10^{10} PFU/mL) in SM buffer were added on top of discontinuous cesium chloride gradients (CsCl) and centrifuged at 40,000×*g* for 4 h at 4℃ (Watanabe et al., 2007). The phage band was collected with a sterile syringe, dialyzed against sterile SM buffer overnight at 4℃ and changed one time during dialysis.

The CsCl-purified phages were treated with 1% deoxycholate detergent with shaking at 37℃ for 60 min. This was followed by ultrafiltration using Amicon Ultra-15 Centrifugal Filter Devices with a 100 KDa nominal molecular weight cut-off membrane (Merck Millipore Ltd., Ireland) with centrifugation at 4,000×*g* for 5 min at 4℃ (Jun et al., 2013), which was done five times with washing using SM buffer. Finally, the phage suspension obtained from ultrafiltration was filtered again.

3.2.3 Endotoxin quantitation in phage solutions

The endotoxin contents of the raw phage lysate and the purified phage suspensions were measured using a *Limulus* Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation kit (Thermo Fisher, Sweden), according to the accessary protocol.

3.2.4 Animals experimental treatments

C57BL/6 female mice (6-8 week-old) were purchased from the Comparative Medical Centre of Yangzhou University (Yangzhou, China) and maintained under specific pathogen-free conditions in the experimental animal center in Jiangsu Academy of Agricultural Sciences (JAAS). The animal experiment was approved by the JAAS in accordance with the guidelines provided by the Animal Care and Ethics Committee and followed the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines (Kilkenny et al., 2010). Mice were fed ad libitum with a commercial grain formulation (SHOOBREE Rat and Mouse Maintenance Diet, Jiangsu XieTong Organism Co., Ltd., Nanjing) and sterilized mineral water (Evian; pH 7.2; HCO_3^-, 489 mg/L).

After two weeks acclimation, mice were randomly assigned to three experimental treatments on the basis of body weight (~18g), the Control group (20 mice) was separated from phage-treated mice and received no phage in their drinking water, the Lytic group (20 mice) received purified lytic gut phage PA13076 in their drinking water at a concentration of ca. 1×10^8 PFU/mL (approx. 4×10^8 PFU) and the Temperate group (20 mice) were treated with 1×10^8 PFU/mL (approx. 4×10^8 PFU per mouse daily) of purified temperate phage BP96115 in sterilized mineral water daily. The trial was conducted for 31 days.

3.2.5 Sample collection

After being weighed again, four mice in each group were “humanely” euthanized on day 16 and 31 after their first ingestion of phage. For each mouse, 0.2 mL of blood was taken from puncturing the orbital plexus with a plastic tube containing 20 μL heparin (1000 U/mL, Sigma), while simultaneously 0.2 mL of blood was added into a tube without any anti-agglutination additive to produce serum. Six different segments of the GIT with their contents including stomach, duodenum, jejunum, ileum, cecum, and colon, and, additionally spleen, were isolated from mice and weighed prior to phage enumeration. All samples were collected aseptically. In addition, fecal samples were obtained on day 16 and 31 from each mouse before being euthanized.

3.2.6 Phage detection

Freshly collected feces were weighted and homogenized in a sterile plastic tube after addition of sterile 10-fold SM buffer. Each segment of the GIT with its contents and spleen was homogenized in SM buffer with a tissue homogenizer (Shanghai Chemistry and Scientific Co. Ltd., Shanghai, China) making 10-fold diluted suspensions. The un-agglutinated blood (0.2 mL) was homogenized with SM buffer in a final volume of 1 mL. After centrifugation at 12,000×*g* for 10min at 4℃, these solutions were subjected to serial decimal dilution. The phage titers were determined using *Salmonella* Enteritidis ATCC 13076 or *Salmonella* Pullorum SPu-109 as host cells. The detection limits were 50 PFU/g.

3.2.7 Determination of IgG in sera and secretory IgA in the ileum

While IgG levels were tested in sera, secretory IgA (sIgA) levels were tested in ileum homogenates. Samples from day 31 were analyzed using a mouse IgG ELISA (88-50400) and a mouse IgA ELISA (88-50450) kit (eBioscience, San Diego, CA), according to the manufacturer’s protocol. The optical density (OD) was measured at 450 nm with a plate reader (TECAN SUNRISE, Switzerland) and phage-treated samples were compared to the control group using one-way ANOVA. Significant differences were determined using Duncan’s test with significance set at $p<0.01$. All data were analyzed using SPSS 16.0 (SPSS Inc.).

3.3 Results

3.3.1 The endotoxin concentration of purified phages

The endotoxin levels in the crude phage lysate exceeded 700 EU/mL for both phages (Table 3.1). After the phage suspensions were dialyzed following CsCl-ultracentrifugation, still 16.72 and 20.25 EU/mL were detected. After detergent treatment and ultrafiltration, sufficiently low endotoxin levels were reached. Thus, the concentration of endotoxin was reduced to <1 EU/mL, which is an acceptable range for *in vivo* applications (Table 3.1).

Table 3.1 Endotoxin levels after each purification (unit: EU/mL)

Purification Step	The phage solution	PA13076	BP96115
1	Crude phage lysate	748.54	912.20
2	CsCl-ultracentrifugation	16.72	20.25
3	Ultrafiltrated	0.86	0.91
	Phage titer (PFU/mL)	1×10^{10}	1×10^{10}

3.3.2 Distribution of the phages in the GIT

The average body weight of the mice did not differ significantly between the three groups at the times of sampling. Mice were sacrificed for phage enumeration in the gastrointestinal tracts in the middle (day 16) and at the end (day 31) of the trial, indicating that a constant titer was reached at day 16 (Fig. 3.1). In comparison to the control group, significantly increased phage titers were detected in all of the examined segments of the GIT of treated mice for the lytic gut phage PA13076 (Fig. 3.1A) and the temperate phage BP96115 (Fig. 3.1B), indicating that both phages colonized the gastrointestinal tract. Phages were also detected in the ileum, colon, cecum and feces samples of control mice using *Salmonella* Pullorum SPu-109 as the host. More than 10^2 PFU/g were detected in stomach samples of mice treated with phage PA13076 and BP96115 through drinking water.

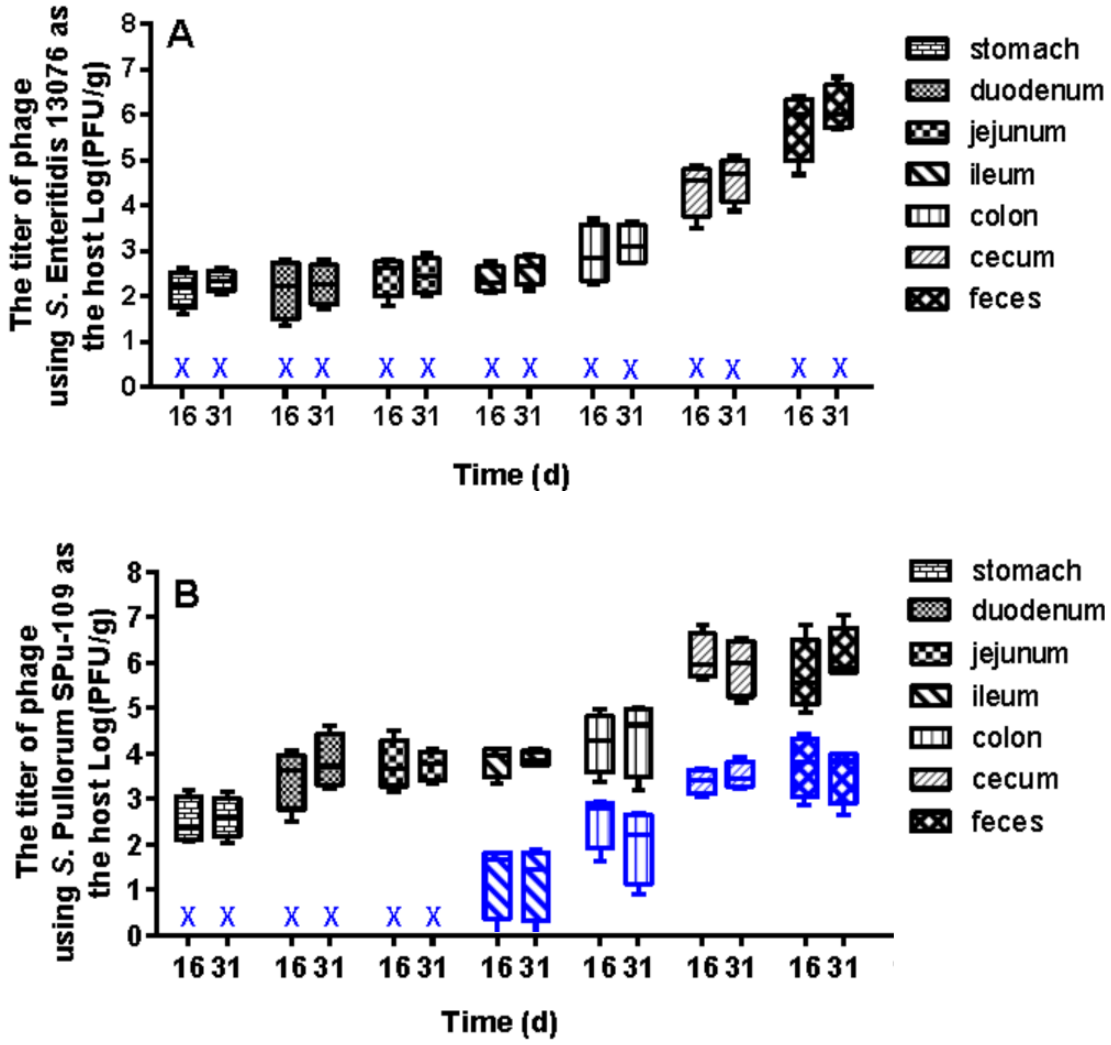

Fig. 3.1 Phage titers in the GIT of mice. The phage titer is expressed as Log PFU/g in form of min to max with the mean for the specified gut segment (stomach, duodenum, jejunum, ileum, colon, cecum and feces) on the 16th and 31st day. Water and food were withdrawn 2 h before killing. A. The titer of phage PA13076 using *Salmonella* Enteritidis ATCC13076 as the host. B. The titer of phage BP96115 using *Salmonella* Pullorum SPu-109 as the host. Black and blue color represents the lytic (A) and lysogenic (B) and control group, respectively. X Represents phage titer values below the detection limit.

3.3.3 Phage recovery from blood and spleen samples

Phages were detected in blood and spleen samples on day 16 and day 31 (Fig. 3.2), and phage titers in the spleen were similar between PA13076 treated and BP96115 treated mice (Fig. 3.2 A and B). The phage titers in whole blood samples ranged from

10^2 to 10^3 PFU/mL, thus lower than those detected in spleen samples wherein up to 10^4 PFU/g were detected.

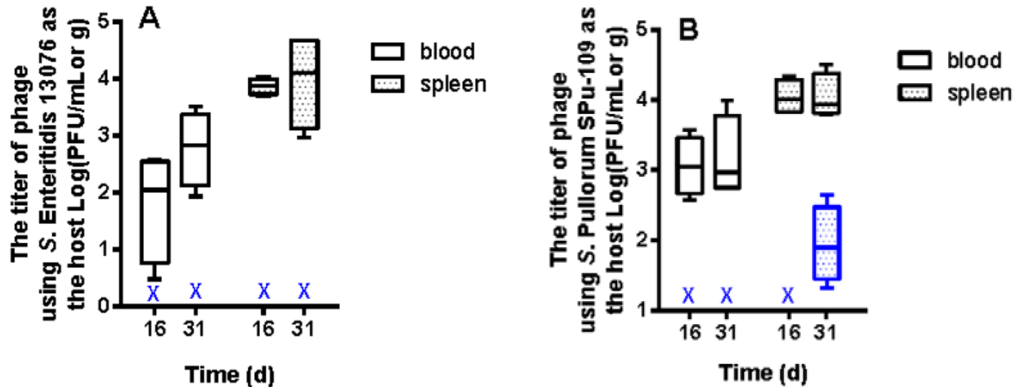

Fig. 3.2 Quantification of phages in blood and spleen. Phage PA13076 and BP96115 (about 4×10^8PFU) were fed to mice via drinking water every day for the lytic and the temperate group. The phage titers were detected on day 16 and 31. A. The titer of phage PA13076 using *Salmonella* Enteritidis ATCC13076 as the host. B. The titer of phage BP96115 using *Salmonella* Pullorum SPu-109 as the host. Black and blue color represents the lytic (A) and lysogenic (B) and control group, respectively. X Represents phage titer values below the detection limit.

3.3.4 The levels of IgG in sera and sIgA in ileum

At day 31 of the phage treatment, slightly increased IgG (p=0.118) and sIgA (p=0.88) levels were detected in comparison to the control mice. However, no significant effect on the levels of sera IgG and ileal sIgA was observed, although IgG and sIgA levels were somewhat higher in the lytic than the temperate group.

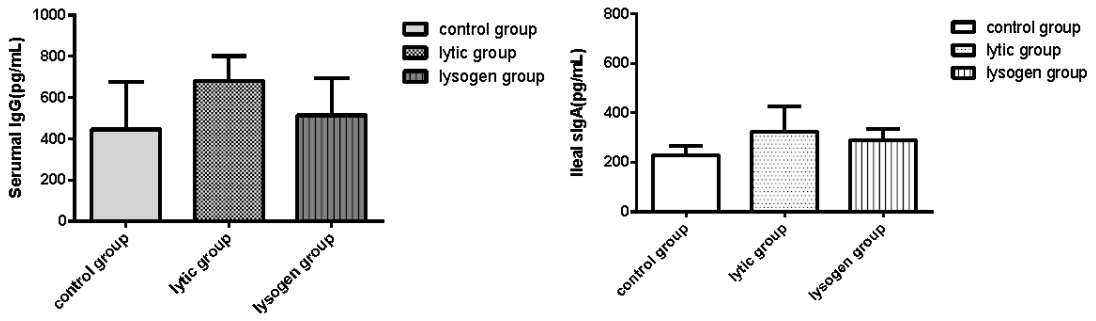

Fig. 3.3 IgG serum and sIgA ileum levels in samples at the end of the experiment (day 31). Results are shown as mean values of pg/mL. The standard deviation of the mean is indicated by error bars (n=4).

3.4 Discussion

In recent years, researchers have increasingly focused on the regulatory role of phages in the gut (Abeles and Pride, 2014; Foca et al., 2015; Lusiak-Szelachowska et al., 2017). However, poor understanding of the preferred distribution of phages in the gut remains a major problem. This study therefore analyzed the abundance and distribution of phages in the GIT when mice were exposed to phages for 31 days.

Oral administration of phages may be a potential route for controlling gastrointestinal infections and systemic infections (Ryan et al., 2011). However, the environment of the stomach is a major challenge for phage stability (Atterbury et al., 2007; Ma et al., 2008; Maura et al., 2012). The current study showed that the viability of phages was rapidly lost upon exposure to gastric acid, as the titers of phages in stomach were always $\leq 10^2$ PFU/g, although the mice were administrated 4×10^8 PFU/day continuously for 31 days in the lytic and temperate groups.

It has been confirmed that phages can migrate to peripheral blood and organs from the gut (Abeles and Pride, 2014; Gorski et al., 2006). Phages, such as *E. coli* phages, can translocate to the blood much more effectively from the intestinal tract than from the stomach (Dabrowska et al., 2005). Keller and Engley (1958) have shown that the oral

inoculation of *Bacillus megaterium* phages could be detected in mice blood in 5 min. Schubbert et al. (1994) demonstrated that orally ingested foreign DNA, phage M13 and plasmid DNA, was able to persist in fragmented form in the GIT of mice, penetrated the intestinal wall and reached the nuclei of various cells. In this study, the presence of phages in spleen and whole blood samples also suggested that phages can cross the epithelial barrier and enter extraintestinal sites even when applied via the oral route. Highest phage titers were detected in the cecum as well as in feces with 10^4 and 10^6 PFU/g in cecum for PA13076 and BP96115 treatment. Similar results showed that phage T3 preferentially absorbed in the higher or deeper sections of the intestine (Hoffmann, 1965). In the current study, the lysogenic phage using *Salmonella* Pullorum SPu-109 as the host was detected at up to 10^4 PFU/g in the cecum in the control mice, while low abundances were detected for the virulent phage using *Salmonella* Enteritidis ATCC13076 as the host in the mice gut. This matches a report by Dhillon et al. (1976), showing that temperate phages predominate in mammalian feces.

However, despite exposing mice to phage over 31 days, the titer of phages in GIT and blood samples did not increase gradually. Phages are foreign invaders, so that they can induce immune response. Moreover, they can be rapidly eliminated by reticulo-endothelial system (Dabrowska et al., 2005). The production of anti-phage antibodies and the inhibitory effect due to adaptive immunity responses are key factors controlling phage colonization in the animal body. A long-term study of antibody induction in mice with T4 phage applied *per os* over 100 days showed that high levels of anti-phage antibodies (IgM, IgG, and secretory IgA), decreased the phage concentrations in the blood of mice (Majewska et al., 2015). In the present study, the numbers of both phages in blood did not exceed 10^3 PFU/mL, indicating that the mice immune response was active.

The residual quantity of bacterial LPS is restricted during the administration of biotherapeutics and vaccines (Kabanov and Prokhorenko, 2010; Wang and Quinn, 2010). Recent studies showed that high phage concentrations can induce

pro-inflammatory responses, and long-term exposure to phages induced an antibody response in spleen (Hodyra-Stefaniak et al., 2015; Majewska et al., 2015). These data highlight the need for an appropriate purification protocol in terms of acceptable endotoxin levels. In order to satisfy the requirements for phage clinical application (Simoliunas et al., 2015), it is necessary to quantify endotoxin levels in the final product prior to application (Merabishvili et al., 2009). An acceptable level of endotoxin, usually below 0.5 EU/mL of endotoxin in the phage solution, was achieved when using a protocol including concentration/washing by ultrafiltration and two CsCl ultracentrifugation steps (a step gradient followed by an isopycnic gradient) (Henry et al., 2013). A combination of ultracentrifugation and ultrafiltration was successfully used in the present study to remove bacterial LPS from prepared phage solutions before these solutions were used for animal experiments, thereby matching previous studies (Hudson et al., 2015; Williamson and Paul, 2006).

The two phages, PA13076 and BP96115, were purified using ultracentrifugation and ultrafiltration methods. The endotoxin levels were reduced to <1 EU/mL. Overall, the current study demonstrated that the gut lytic and gut temperate phage would distribute and locate in the intestinal tract of mice by oral intake, preferentially colonizing the posterior of the gut. Moreover, the temperate phage was more numerous than the lytic phage in mammalian feces. This study therefore provides basis information for studying the impact of gut phage on the gut microbiota.

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CHAPTER FOUR

ALTERATIONS IN THE DIVERSITY AND COMPOSITION OF MICE GUT MICROBIOTA BY LYTIC OR TEMPERATE *SALMONELLA* GUT PHAGE TREATMENT

4.1 Introduction

The global overuse of antibiotics has led to the rapid emergence of multi-drug resistant bacteria, potentially endangering the efficacy of antibiotics and threatening public health (Ventola, 2015; WHO, 2014; Zaman et al., 2017; Zhang et al., 2006). Moreover, antibiotics alter the normal composition of mammalian gut microbiota resulting in short-term or even persistent dysbiosis (Iizumi et al., 2017; Lewis et al., 2017; Namasivayam et al., 2017). Hence, there is increasing demand to find alternatives to antibiotics to tackle bacterial infections.

As the gut contributes to animal health in many ways (Blaut and Clavel, 2007; Marchesi et al., 2016), the term ‘gut health’ of mammalia is increasingly popular (Bischoff, 2011). The mammalian gut is colonized by diverse microorganisms, including bacteria, phages and eukaryotic viruses and other microorganisms (fungi, protozoa and archaea) (Robinson et al., 2016; Rosshart et al., 2017). The intestinal commensal flora plays multifaceted roles in maintaining animal health, such as promoting metabolism, defensing pathogens, developing the immune system and maturation (Belkaid and Hand, 2014; Rampelli et al., 2015; Rosshart et al., 2017).

Compared with traditional sequencing methods, high-throughput sequencing techniques such as Illumina sequencing are considered to be timesaving and more cost-effective and are thus now routinely used for microbial metagenome analysis (Heck et al., 2016). Comparative metagenomic analyses help us understand the composition and structure of gut microbiota in the complex gut ecosystem (Grossart et al., 2013; Norman et al., 2014). Moreover, the development of fecal transplantation is a promising method to treat dysbiosis diseases.

Phages are the dominating species of the gut microbiota, with estimates of 10^{15} particles in the gut ecosystem (Dalmasso et al., 2014), outnumbering bacteria at a 10:1 ratio (Dabrowska et al., 2005). Further, the phages in the intestinal tract are dominated by temperate phages (Reyes et al., 2010). Also, gut phages may have an important

role in disease, such as chronic periodontitis (Gorski and Weber-Dabrowska, 2005) and inflammatory bowel disease (IBD) (Lepage et al., 2008). Moreover, oral prophylactic “phagebiotics” can kill specifically targeted bacterial pathogens in the gastrointestinal tract (GIT)(Volker, 2015). The influence of gut phages on animal health is manifold (De Paepe et al., 2014; Lusiak-Szelachowska et al., 2017; Mills et al., 2013) and phages may have the potential to exert selective pressure and regulate selected members of the mammalian intestinal microbiota. However, there are only few studies showing the impact of phages or phage cocktails on gut microbiota in healthy animals. The aim of this study was therefore to understand the regulating roles of lytic or temperate gut phage treatment on the microbial community and diversity in healthy mice. This information may guide further research on the evaluation of the prophylactic use of phages in livestock rearing.

4.2 Materials and Methods

4.2.1 Phage and antibiotic solutions

The lytic gut phage PA13076 and the temperate gut phage BP96115 were prepared and purified as reported in Chapter Two and Three. The titer of each phage was adjusted to 1×10^8 PFU/mL in drinking water (sterilized mineral water, Evian, pH 7.2; HCO_3^-, 350 mg/L). The concentration of streptomycin sulfate (Solarbio Technology Co., Ltd., Shanghai, China; >98%) was adjusted to 10 mg/mL using the same drinking water. Mice without phage and streptomycin treatment were used as the control group. The phage or streptomycin solutions were prepared every day.

4.2.2 Animals

C57BL/6 female mice (6-8 week-old) were used in this study and they were reared under the same condition with Chapter Three and following the ARRIVE guideline (Kilkenny et al., 2010). Mice were fed ad libitum with a commercial grain formulation (SHOOBREE Rat and Mouse Maintenance Diet, Jiangsu Xietong Organism Co., Ltd., Nanjing, China) and sterilized mineral water. Groups: group A (10 mice) received no phage and no streptomycin in their drinking water and were separated from the other groups, group B (10 mice) received purified lytic gut phage PA13076 in drinking water at a concentration of 1×10^8 PFU/mL (approx. 4×10^8 PFU), group C (10 mice) were treated with 1×10^8 PFU/mL (approx. 4×10^8 PFU per mouse

daily) of purified temperate phage BP96115 in drinking water daily and group D (10 mice) received streptomycin at a concentration of 10mg/mL (approx. 40mg per mouse daily). The experiment was conducted for 31 days.

4.2.3 Feces collection

At 31 days post-inoculation, feces (fresh weight: 0.5 g) were aseptically collected from all 40 individual mice using separate sterile tubes.

4.2.4 16S rRNA gene amplicon sequencing

A total of 15 fecal samples were randomly chosen, containing 4 samples from each of the control, the lytic and the temperate gut phage treated groups, and 3 samples from the streptomycin treated group. The total DNA of each fresh fecal sample was isolated using QIAamp® DNA stool Mini kit (MP Biomedicals, CA). The DNA quality was determined using 2% agarose gel electrophoresis and a Qubit® 3.0 fluorometer (Life, Madison, WI). The V3-V4 regions of the bacterial 16S ribosomal RNA genes were amplified using primers 341F 5'-(barcode) CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth et al., 2013). However, there are eight-base barcode added to the primer 341F. The cycling was performed in triplicate using 15 μL of 2×Taq master Mix, 1 μL of Barcode-PCR primer 341F (10 μM), 1 μL of Primer 805R (10 μM) and 10ng of genomic DNA in a final volume of 20 μL using the following PCR cycling conditions (Klindworth et al., 2013).

The second PCR based amplification using Ilumina bridge type primers was carried out. The PCR products were purified using the MagicPure Size Selection DNA Beads (Transgen Biosciences, Beijing, China), and quantified using the Qubit®3.0 DNA detection kit (Life, Madison, WI). The purified amplicon samples were analyzed by Sangong Biotechnology Co. Ltd. (Shanghai, China) using high-throughput sequencing (2×300 bp, paired-end sequence, equimolar amounts) on the Illumina Miseq platform (Illumina, CA, USA).

4.2.5 Sequence analysis

The data were arranged by changing files, demultiplexing and qualifying of acquired Illumina reads using QIIME (version 1.17) (Kuczynski et al., 2012). The data were further merged using FLASH software (v1.2.3) (Magoc and Salzberg, 2011), and

assigned to each sample according to the unique barcodes. Sequence analysis was performed by UPARSE software (v7.1, http://drive5.com/uparse/) using the UPARSE-OTU and UPARSE-OTUref algorithms. The Operational taxonomic units (OTUs) were aggregated at a 97% similarity level. Chao 1, which indicates the species abundance and Shannon, which tests the diversity indexes were the parameter to evaluate the α-diversity (Lemos et al., 2011). Rarefaction curves were generated and unweighted unifrac distance for Principal Coordinate Analysis (PCoA) were calculated. The accession no. is SRP142620, which was deposit in NCBI Sequence Read Archive.

4.2.6 Statistical analysis

To analysis differences in the abundances of individual taxonomic groups between the four groups, Metastats software (http://metastats.cbcb.umd.edu/) was used (Hess et al., 2011). The differences of bacterial communities between the four groups, ANOSIM (Zerzucha et al., 2012) and MRPP (multi-response permutation procedure) (Anderson and Santana-Garcon, 2015) were performed. Significant differences were set at $p<0.01$.

4.2.7 Extraction and quantitation of virus-like-particles (VLPs) in feces

After the animal experiment, four fecal samples from each group were characterized for the presence of VLPs. VLPs were isolated and counted according to the previous method (Thurber et al., 2009). In brief, 0.5 g fresh fecal sample was dissolved with 10 mL of sterilized SM buffer with vigorous shaking (300 rpm) for 4h in 4℃. The suspension was centrifuged and the supernatant was filtered. Then, the suspension was further separated using CsCl gradients (1.3, 1.5, and 1.7 g/cm^3) with centrifugation at 40,000×g for 4 h at 4℃ according to Chapter three described. The VLPs samples were diluted, and VLPs were stained with 10×SYBR Gold (Thermo fisher, CA) for DNA viruses and 10×SYBR Green II (Solarbio, Shanghai, China) for RNA viruses for 15 min, washed once using Amicon® Ultra-15 Filter (100 KDa nominal molecular weight cut-off (NMWCO) membrane, Merck Millipore Ltd., Ireland), and visualized using a UltraVIEW VoX confocal microscope with NIS-elements (Nikon, Tokyo, Japan). If the background appeared milky or grainy, then samples were diluted further and another slide was created until individual virions became visible. VLPs were quantified from 20 to 30 slides. All fluorescence images were taken at 600×magnification with excitation at 488 nm. Stained particles

<0.22 μm in diameter were regarded as VLPs.

4.3 Results

4.3.1 Metagenomic sequence data summary

In this study, 15 fecal samples were collected from 15 individual mice after a treatment experiment running for 31 days. A total of 672,406 16S rRNA gene comprising 310,655,582 bp were generated from the raw data, and 613,930 valid sequences remained after chimeras were filtered out and low-quality sequences were removed, which were mostly between 400 and 440 bp. A total of 2470 OTUs were authenticated from all samples. Rarefaction analysis was employed (Fig 4.1) to compare the taxon richness between samples and to determine if the diversity of fecal samples was sufficiently sampled. The numbers of OTUs of samples in group D is the lowest (around 200 OTUs at 4000 sequences), however, samples in the other three groups are higher than it (between 360 to 650 OTUs around 4000 sequences). Therefore, the result of rarefaction analysis show higher species richness of samples of group A, B, C than samples in group D. Furthermore, it also shows the amount of sequencing data in each group is reasonable for analysis.

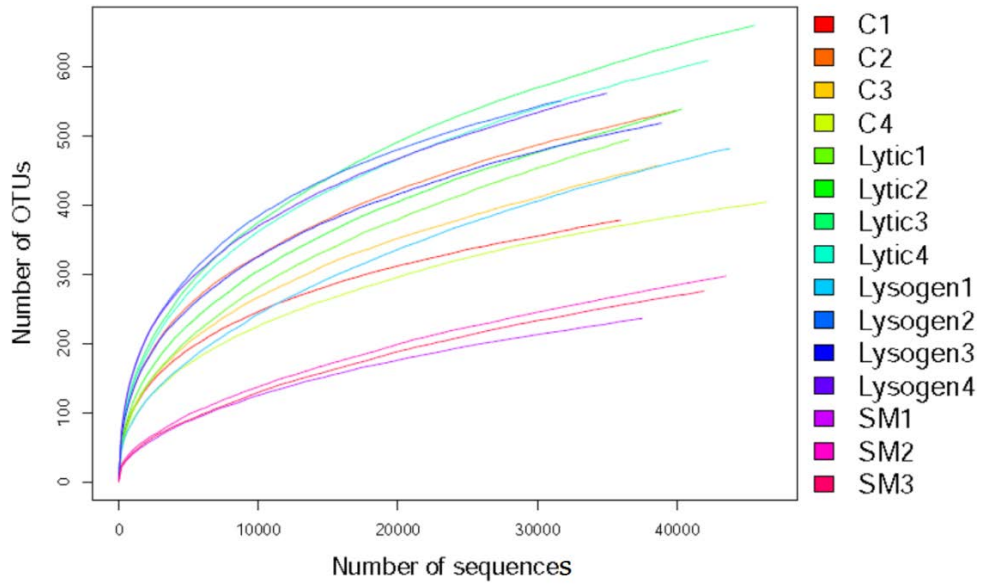

Fig. 4.1 Rarefaction curves for 15 metagenomic data sets established for the four treatment groups comparing phylotype richness at 97% similarity and the number of sequences analyzed. C1–C4, Lytic1–Lytic4, Lysogen1–Lysogen4 and SM1–SM3 indicate the 15 samples of group A (control), group B (lytic gut phage treatment), group C (temperate phage treatment) and group D (streptomycin

treatment).

4.3.2 Alpha diversity of the gut microbiota

The calculated alpha diversities for the analyzed samples based on Chao 1 and Shannon are shown in Fig.4.2. A significant difference was evident between group A (control) and group B (the lytic gut phage treatment) in terms of community richness using the Chao 1 index ($p<0.01$) (Fig.4.2). The temperate phage treatment (group C) apparently enhanced the diversity, but not to a statistically significant level ($P=0.1135$) as did the lytic phage treatment. However, the diversity of the gut microbiota was clearly reduced after streptomycin treatment in group D ($p=0.0299$) (Fig.4.2). The Shannon index indicated that group B (the lytic gut phage treatment) and group C (the temperate phage treatment) were not significantly different in comparison to groups A and D. Both groups had a higher bacterial diversity than the control group A, while the streptomycin treated group D showed the lowest community diversity of the analyzed samples ($p=0.0122$) (Fig. 4.2).

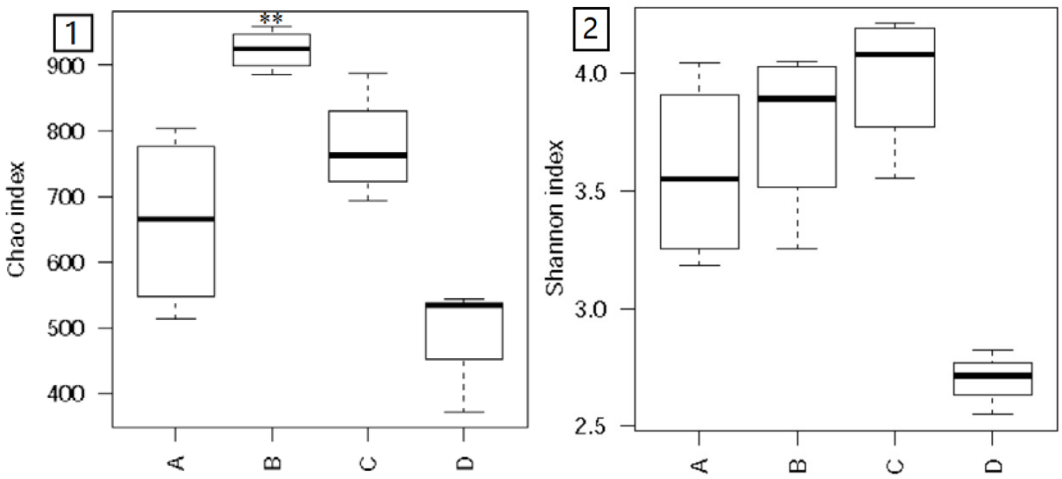

Fig. 4.2 The alpha diversity of the gut microbiota based on the Chao 1 index (1) and the Shannon index (2) for the control group (A), the lytic group (B), the temperate group (C) and the streptomycin group (D) at the end of the experiment (31 days). ** Significant differed by $p<0.01$.

4.3.3 Taxonomic diversity of the gut microbiota

The taxonomic diversity of the gut microbiota at phylum level is shown in Table 4.1. *Bacteroidetes* was the most abundant phylum, accounting for 62.85% of the total reads, and the gut microbiota of all fecal samples were dominated by the phyla

Bacteroidetes, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. However, while the abundance of the phylum *Bacteroidetes* declined after lytic or temperate gut phage and streptomycin treatment from 80.47% in the control group to 49.39% (group B), 60.41% (group C) and 61.15% (group D), respectively, the proportion of the phylum *Firmicutes* increased to 42.36%, 31.97% and 22.25% in the same groups.

Genus level diversity of gut bacteria in the four experimental groups is shown in Fig.4.3. The dominant bacterial genus in all samples was *Barnesiella*. Compared to the control group, the genera *Barnesiella*, *Tannerella*, *Parabacteroides*, *Alistipes*, *Bacteroides*, *Klebsiella* and *Exiguobacterium* declined in the lytic phage treatment group. Both phage treatments increased the abundance of members of the genera *Lactobacillus*, *Allobaculum*, *Macellibacteroides*, *Odoribacter*, *Turicibacter*, *Akkermansia* and *Bifidobacterium*, while all these genera decreased and even disappeared after streptomycin treatment except the genus *Allobaculum*. The abundance of members of the genus *Barnesiella* (33.08%), *Tannerella* (12.66%), *Parabacteroides* (1.44%), *Alistipes* (2.89%), *Escherichia*/*Shigella* (4.56%), *Clostridium* IV (0.80%) and *Clostridium* XlVa (1.91%) in the temperate phage treated group were higher than in the lytic phage treated group. Treatment with the lytic and the temperate gut phage for 31 days increased the two beneficial genera *Lactobacillus* (19.50% and 13.93%) and *Bifidobacterium* (1.51% and 0.12%), while the proportion of the genus *Lactobacillus* in the control group was only 7.09% and 3.20% in the streptomycin treatment group. Moreover, no *Bifidobacterium* was detected in groups A and D. The lytic phage treatment did not cause an apparent increase in potentially harmful pathogens. Nonetheless, streptomycin caused a clear increase of the genus *Parabacteroides* (5.70%), *Bacteroides* (4.95%), *Klebsiella* (9.66%) and *Escherichia*/*Shigella* (6.29%).

Table 4.1 The gut microbiota composition at the phylum level (unit, %)

Group	A	B	C	D
Bacteroidetes	80.47±4.57	49.39±7.06	60.41±6.42	61.15±9.50
Firmicutes	15.43±4.52	42.36±4.79	31.97±8.45	22.25±3.67
Proteobacteria	2.92±1.44	4.48±3.50	6.20±2.71	16.27±6.24
Actinobacteria	0.95±0.61	2.27±0.81	0.99±0.16	0.31±0.05
Verrucomicrobia	ND	1.40±0.82	0.35±0.20	ND
Candidatus Saccharibacteria	0.10±0.06	0.08±0.15	0.04±0.07	ND
Tenericutes	0.12±0.15	ND	0.02±0.02	ND
Thermotogae	0.02±0.01	0.02	0.02±0.01	0.01±0.01
Fusobacteria	ND	0.01±0.01	ND	ND
Atribacteria	ND	ND	0.01±0.01	ND

Information of each group: A, the control group; B, the lytic group; C, the temperate group; D, the streptomycin group.

ND= Not detected.

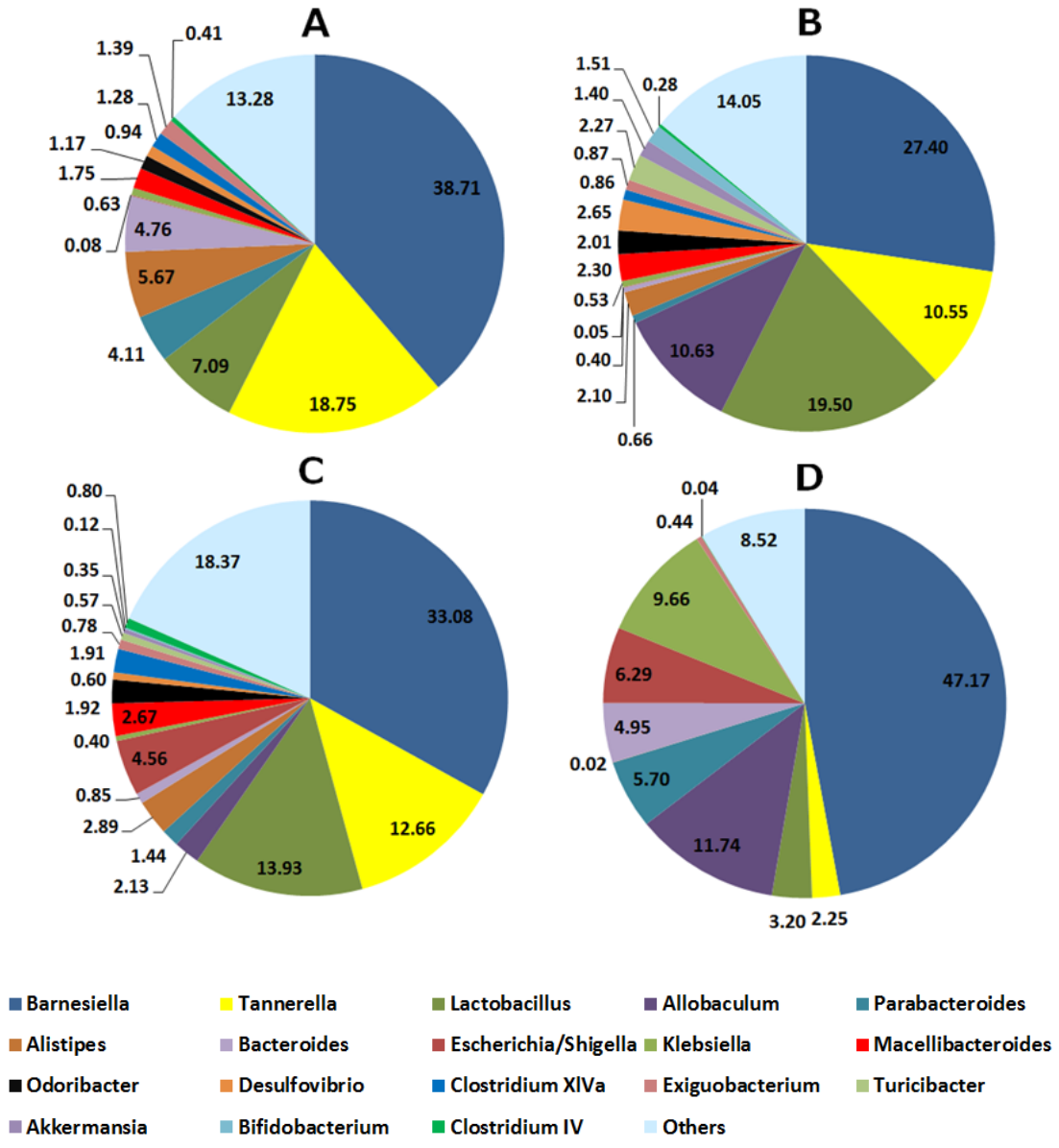

Fig. 4.3 Genus level diversity of gut microbiota in the four experimental groups. Shown are dominant bacterial genera based on OTUs from identified in samples each mouse, the average OTU coverage of each group is displayed as a pie plot. A, the control group; B, the lytic group; C, the temperate group; D, the streptomycin group.

4.3.4 Comparative metagenomic among the bacterial communities

β-diversity analysis using the hierarchical clustering algorithm UPGMA and dimensionality reduction using PCoA for all 15 fecal samples obtained in the study was done (Fig. 4.4 a and 4.4 b). Streptomycin treatment significant reduces the diversity and pattern of the gut microbiota. All groups of treated mice clearly

clustered in their own groups. However, while streptomycin treated mice were clearly separated from the control mice, the lytic and temperate phage treated groups did not show significant differences to the control group.

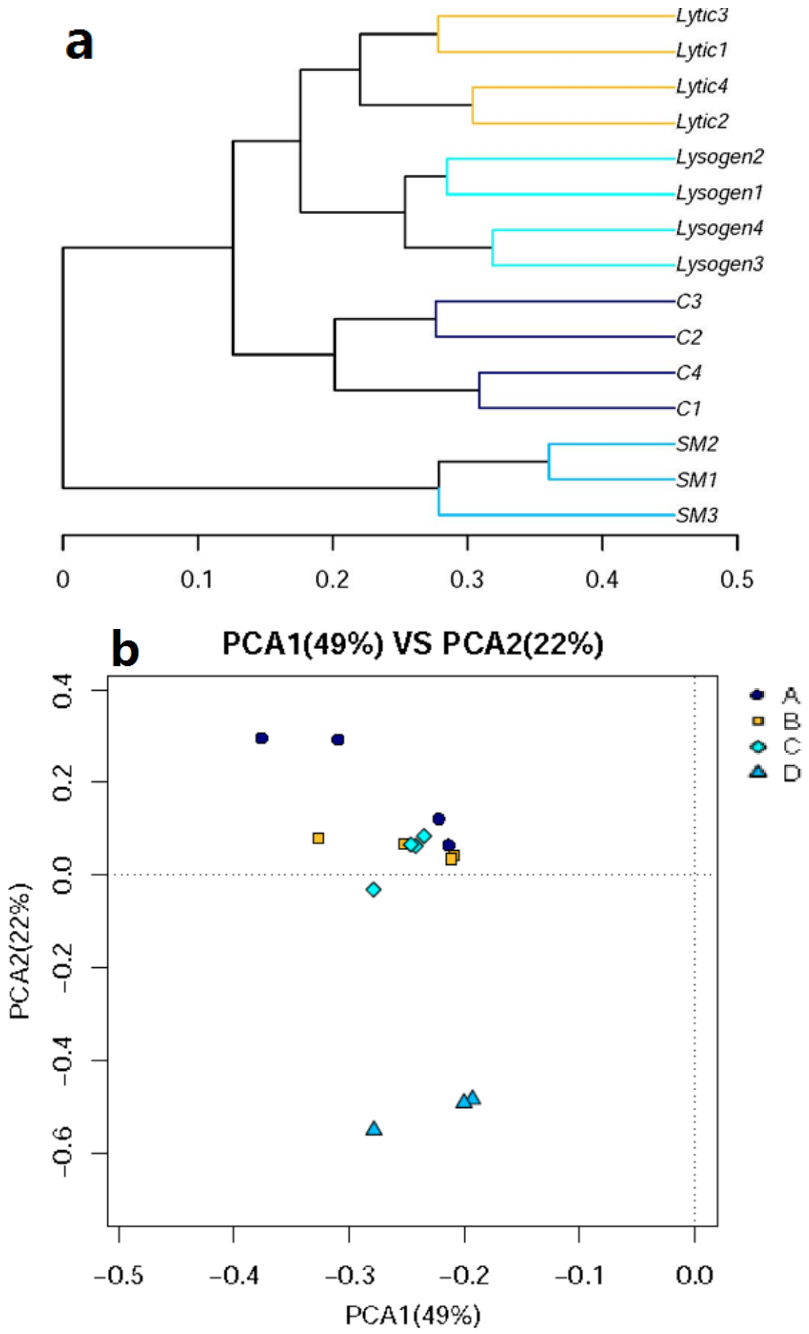

Fig. 4.4 (a) Hierarchical clustering analysis for OTUs of all analyzed samples by UPGMA. (b) Principal coordinate analysis for the gut microbiota patterns depending on the abundances of OTU.

In addition, the community structures within the microbiota were compared using UniFrac for paired comparisons on the distances between samples in regard to the

fraction of evolutionary history that separates the organisms (Fig. 4.5). While samples C1-C4 and Lysogen2, Lysogen3 and Lysogen4 formed clusters, Lysogen1 clustered with samples Lytic1-Lytic4. These three clusters formed a higher-level cluster clearly distant from the cluster of samples SM1- SM3.

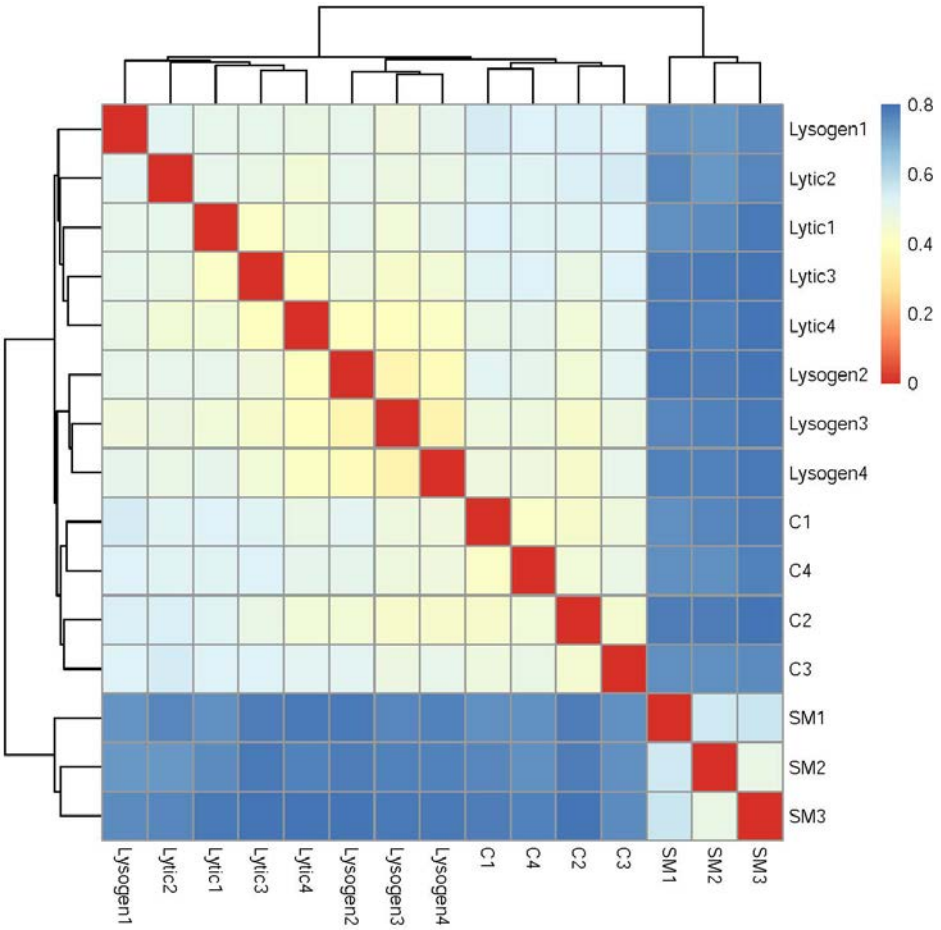

Fig.4.5 The heat map showing the similarity matrix based on hierarchical clustering analysis across the four groups using the weighted UniFrac distance.

4.3.5 The VLPs of fecal samples

Virus-like particles (VLPs) were detected and quantified in feces using confocal microscopy (Fig. 4.6 and 4.7). The average titer was 9.08 log±0.37 VLPs/g for DNA viruses and 8.8 log±0.43 VLPs/g for RNA viruses in the feces of normal C57BJ/6 mice (Fig.4.8). After lytic gut phage or temperate phage treatment, fecal VLPs did not change significantly. However, fecal VLPs increased to 9.89 log ± 0.33 VLPs/g ($p<0.05$) for DNA viruses and 8.99 log ± 0.23 VLPs/g for RNA viruses compared to the control group, after streptomycin treatment.

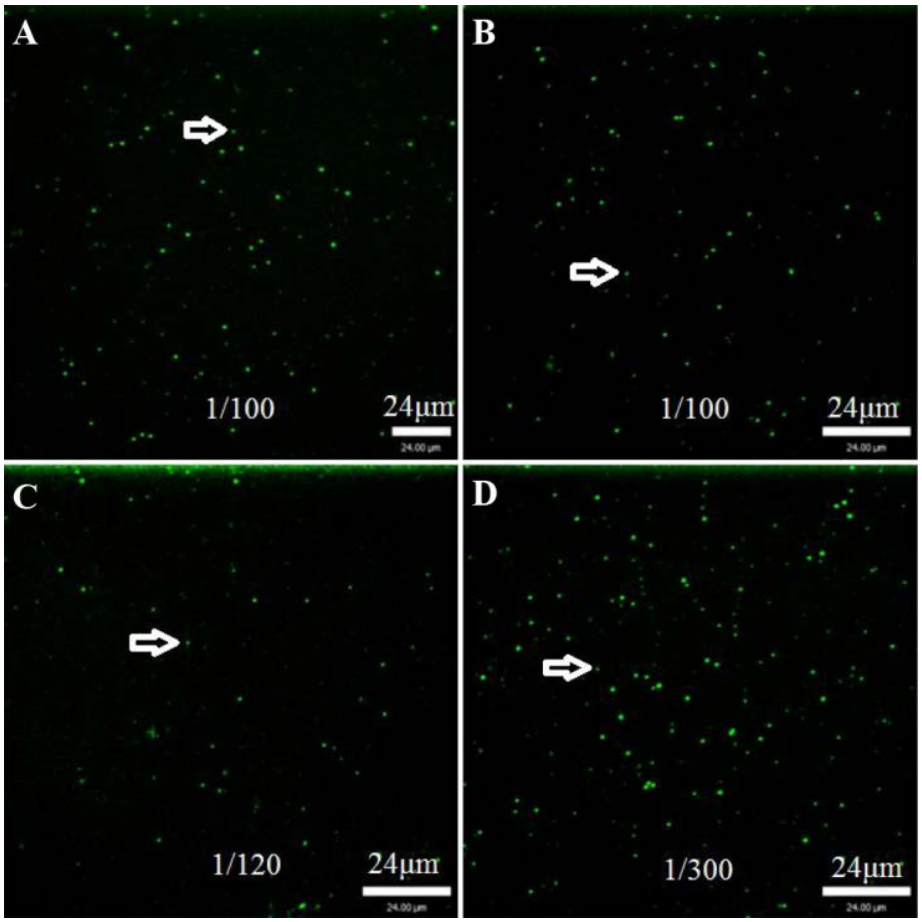

Fig. 4.6 Confocal microscopy images demonstrating the detection of VLPs stained with SYBR Gold. VLPs were dim pinpoints. The yellow arrows refer to VLPs. A, B, C and D represent the control group, the lytic gut phage treated group, the temperate phage treated group and the streptomycin treated group. The representative dilution of each sample is shown at the bottom center of each photo.

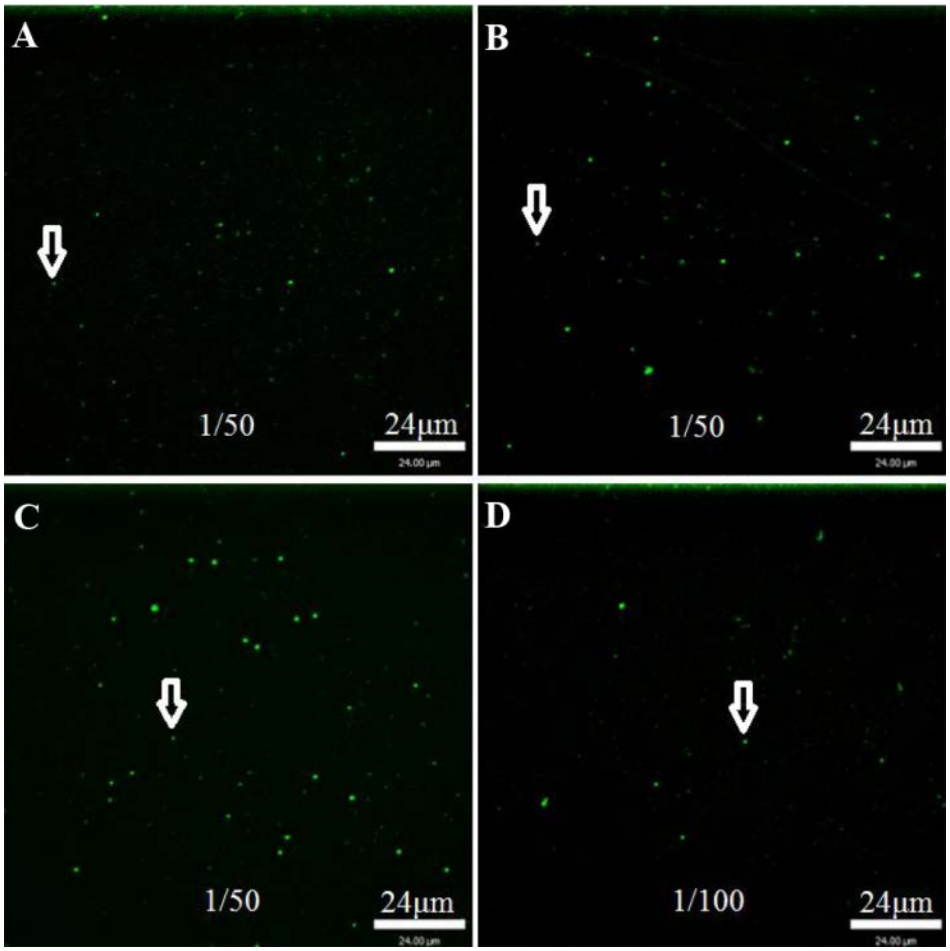

Fig. 4.7 Confocal microscopy images demonstrating the detection of VLPs stained with SYBR GreenⅡ. Filtrates were diluted serially until numerable VLPs were counted in a view. VLPs were dim pinpoints. The yellow arrows refer to VLPs. A, B, C and D represent the control group, the lytic gut phage treated group, the temperate gut phage treated group and the streptomycin treated group.

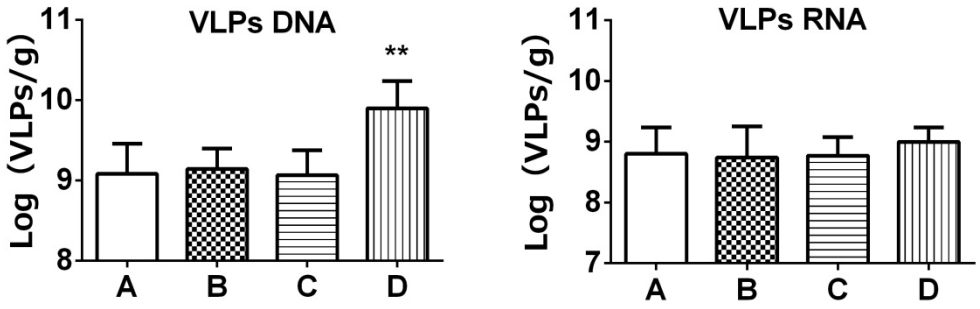

Fig. 4.8 Numbers of VLPs per g of feces of the four tested groups (One-way ANOVA, Duncan's test, **p < 0.01).

4.4 Discussion

Previous metagenomic analyses of feces demonstrated that phages were the most abundant replicating entities (Breitbart et al., 2003), outnumbering bacterial cells by 10 times (Chibani-Chennoufi et al., 2004), with as many as 10^{15} individual phage particles present in the mammalian gut (Lepage, 2013; Mills et al., 2013). Lytic phages appear to perform the predation on sensitive bacteria using the “kill the winner” model (Letarov and Kulikov, 2009). However, research on the role of lytic or temperate gut phages impacting gut microbial populations, especially in healthy animals, is in its early days.

This study indicated that regular, long-term (31 days) administration of lytic or temperate gut phages changed the composition and diversity of gut microbiota. However, previous phage therapy studies indicated that even high titers of lytic phages just reduced the concentration of pathogens but revealed no significant differences of the diversity of gut microbiota (Golomidova et al., 2007; Mai et al., 2010; Tanji et al., 2005). The fundamentally different results in the present study might be due to the long-term treatment of mice instead of the usual short time treatment.

The gut microbial diversity might enhance the host immune response enhancing susceptibility to allergy (Shreiner et al., 2008) and has been related to increase the stability of the ecological balance (Eisenhauer et al., 2012). High diversity of the gut microbiota was more propitious to the holistic health and productivity of animals such as weaned piglets (Zhang et al., 2016) and disease resistance in laboratory mice (Rosshart et al., 2017). Studies targeting the role of probiotic bacteria showed that they can enhance species richness and diversity in the fecal microbiota (Cox et al., 2010; Grazul et al., 2016). The treatment results in this study showed that α-diversity of gut bacteria were higher in both phage treated groups of mice when compared to the control group using both Chao 1 and Shannon diversity indexes. However, in the present study streptomycin treatment significantly reduced the richness and diversity of the intestinal microbial community of mice, matching another recent study (Grazul et al., 2016) indicating that the broad-spectrum antibiotics reduced the gut microbiota of mice to two genera, *Stenotrophomonas* and *Xanthomonas*. These results indicated that the lytic and temperate gut phages used for treatment are beneficial as they

promote the diversity of gut microbiota such as probiotics treatments. The low diversity of microbiota by antibiotic treatment can increase susceptibility to pathogens such as *Salmonella* Typhimurium and *Clostridium difficile* (Barthel et al., 2003; Zhang et al., 2013).

The dominant phyla detected in the distal gut of mice in the earlier study were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Clarke et al., 2012). They were also the dominant phyla in the GIT of mice after 31 days of phage treatments in our study. Nevertheless, the composition of gut microbiota might be influenced additionally by species, diet, water, husbandry, and age as was reported for pigs (Zhao et al., 2015).

The most abundant genus *Barnesiella* (family *Porphyromonadaceae*, phylum *Bacteroidetes*) declined after the lytic phage or temperate phage treatment, but increased after the streptomycin treatment. Although other studies have detected *Barnesiella* as an abundant member of the gut community in mice (Cho et al., 2016), the role of *Barnesiella* and the meaning of their intestinal reduction is currently not clear.

At the genus level, the most obvious difference between groups in this study was the abundance of members of the beneficial genus *Lactobacillus*. The level of these "probiotics" was greater in the lytic and temperate gut phage treated mice than in the other two groups. In addition, the genus *Bifidobacterium* was also enhanced after both phage administrations. This distinctive role of the lytic gut phage on the composition of gut microbiota was similar to the reported treatment with a probiotics cocktail, containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Pediococcus* strains, which significantly increased the numbers of *Bifidobacterium* spp. and *Lactobacillus* spp. in cecal microflora of broilers (Mountzouris et al., 2007). *Lactobacillus* and *Bifidobacterium* species are two commonly used probiotic bacteria, enhancing resistance to pathogens or producing short-chain fatty acid (Kechagia et al., 2013; Rist et al., 2013). In addition, they play an important modulation role in the digestive and in the immune system (Ashraf and Shah, 2014). Mice given either one of the four riboflavin-producing probiotic strains showed that there were lower histologic damage, lower translocation of pathogens, significant $iNOs^{+}$ cells and pro-inflammatory cytokines reduction in the large intestines (Levit et al., 2017).

Lactobacillus murinus treatment could prevent experimental autoimmune encephalomyelitis and salt-sensitive hypertension by modulating T_H17 cells (Wilck et al., 2017), mice gavage-fed *Lactobacillus rhamnosus* GG increased expression of transmembrane Claudin protein (Cldn) 3 and 7 and accelerated maturation of gut barrier function (Ravi et al., 2011). Similarly, *Bifidobacterium* species provided benefits including the protection of animals from enteric pathogens (Fanning et al., 2012; Fukuda et al., 2011) and prevention of inflammatory disorders of the GIT (Gareau et al., 2010). Moreover, in this study the lytic and temperate gut phages significantly decreased the abundance of the genus *Klebsiella*, containing potential pathogens, such as *Klebsiella pneumoniae*, when compared to the streptomycin treatment. Hence, the results demonstrate that lytic and temperate gut phages can regulate the composition and diversity of gut microbiota in mice, resulting in a significant probiotic effect. In contrast, the human norovirus directly induce acute gut inflammation (Basic et al., 2014) and increase the incidence of Crohn's disease (Cadwell et al., 2010).

However, after the temperate phage administration, some bacterial genera such as *Esherichia/Shigella*, including potential pathogens were increased; demonstrating that exposure to temperate phages may establish conditions promoting such organisms. These findings highlight the potential role of temperate phages increasing intestinal permeability (Dalmasso et al., 2104).

This study further analyzed the effect of lytic or temperate phage and streptomycin treatment on the abundance of enteric viruses. VLPs are usually quite high in gut, ranging from 5×10^7 to 1×10^{10} particles/g (Letarov and Kulikov, 2009), thereby matching the data for mice feces, yet these values are probably underestimated. Viral communities and their encoding genes are highly diverse, with only 0.0002% of the global viral gene pool being sequenced (Rohwer, 2003). Deep sequencing of virus DNA/RNA purified from the environment typically yielded a majority of unidentified sequences (Breitbart et al., 2008; Minot et al., 2012; Minot et al., 2011; Reyes et al., 2010). Thus, Mills *et al*. (2013) reported that 81% of VLP-derived metagenomic data is unknown. The enumeration results for VLPs showed that both phages did not cause a significantly alteration of the VLPs number in feces. However, streptomycin treatment resulted in significantly higher DNA VLP titers than seen in control mice possibly because of release of endogenous prophages (Allen et al., 2011). This is

consistent with fluoroquinolone treatment increasing the production of *Clostridium difficile* phages in mammalian feces (Davies et al., 2016). However, this result does not match a study reporting that an antiviral cocktail decreased enteric DNA and RNA viruses significantly in mice (Yang et al., 2016).

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CHAPTER FIVE

THE IMPACT OF PREADMINISTRATION OF LYTIC AND TEMPERATE *SALMONELLA* GUT PHAGE ON GUT MICROBIOTA COMPOSITION AND INTESTINAL INFLAMMATION IN A MOUSE MODEL

5.1 Introduction

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) is an important enteropathogen causing zoonotic diseases, and a serious factor affecting public health worldwide (Ebel et al., 2016). The primary habitat of *Salmonella* spp. is the intestinal tract of diverse animals such as chicken, birds, pigs, humans, and occasionally insects (Kubota et al., 2011). They can colonize the intestinal tract, causing serious infections in domestic animals (Park et al., 2015). The mouse model has been widely used to study the mechanisms of *Salmonella* spp. pathogenesis, immunity, and systemic salmonellosis (Mastroeni and Sheppard, 2004). Non-typhoidal *Salmonella* Typhimurium infections of streptomycin-pretreated mice resemble many aspects of the mammalian enteric salmonellosis (Barthel et al., 2003).

The animal GIT is a very complex ecosystem because of the diverse interactions of invading pathogens, the host immune system, and symbiotic microorganisms colonizing the host (the microbiota) (McKenney and Pamer, 2015; Yurist-Doutsch et al., 2014). There are very limited options for reducing already established *Salmonella* spp. in the GIT (Atterbury et al., 2007). Antibiotics, which are used to treat bacterial infections, are considered to be not efficient for the treatment of gut pathogens (Stecher and Hardt, 2011). As a result of disrupting the balanced species composition in the intestinal system, antibiotic treatments can support the growth of pathogenic bacteria such as *Clostridium difficile* (Issa and Moucari, 2014). Additionally, the emergence of multi-drug resistant strains of *Salmonella* spp., pathogenic strains of *E. coli*, vancomycin-resistant *Enterococcus* spp., and fluoroquinolone-resistant *Campylobacter* spp. were all related to the use of antibiotic (Woodford et al., 2011). Probiotics, which can alleviate enteropathogen carriage, have received a substantial amount of public enthusiasm (Patterson and Burkholder, 2003). Many studies have described the efficacy of probiotics controlling pathogens. For example, when pigs were orally inoculated with the cocktail of two *Lactobacillus* strains, the counts of *Enterobacteriaceae* were decreased (Gardiner et al., 2004), and *Salmonella enterica*

serovar Typhimurium in the GIT were also reduced when pigs were treated with a five-strain probiotic combination (Casey et al., 2007). Although more attention has been paid to the use of probiotics as prophylactic and therapeutic preparation in animal husbandry (Neal-McKinney et al., 2012; Potocnjak et al., 2017; Wang, et al., 2013), relatively few probiotic strains have demonstrated efficacy *in vivo* (Gardiner et al., 2004; Steinberg et al., 2014). As the exact mode of action of probiotics is still unknown (Mengheri, 2008), searching for new alternatives to treat gastrointestinal pathogen infections is an urgent matter.

Phages are extremely abundant viruses, and are at least ten times of bacteria on the earth (Casjens, 2005). Interestingly, phage therapy is completely different from antibiotic treatment to prevent pathogenic infections. Prophylactic use of phages resembles that used for probiotics (Cisek et al., 2017). Smith et al. (1987) demonstrated that in orally treated calves, phage titers increased in feces over time with a concomitant decrease in pathogenic *E. coli* counts. Moreover, calves could no longer be infected once phages occupied the gastrointestinal tract, manifesting so-called “infectious protection”. A study by Bardina et al. (2012) indicated that the prophylactic administrations of phage to chickens for a long-term are able to reduce the concentration of *Salmonella* spp. significantly. Furthermore, phages were administered orally to eliminate diarrheic pathogens like *Salmonella* spp. (Toro et al., 2005), *Clostridium difficile* (Ramesh, 1999) and *E. coli* O157: H7 (Raya et al., 2006). All of the successful phage therapy studies depended on using specific phages. However, phages inevitably encounter diverse enteric pathogens in the highly complex gut ecosystem (Olivo et al., 2016). The effects of non-specific lytic gut phages or temperate phages on infections caused by bacterial pathogens are still not well established.

Results reported in chapter four confirmed that lytic gut phages potentially promote gut health through alteration of the composition of gut microbiota while temperate phages enhanced the diversity and changed the composition of gut microbiota. The question is whether phages can alleviate intestinal inflammation and improve mammalian health. In this study, the hypothesis that phage pretreatment causes changes in gut microbiota and that non-specific phages can prevent intestinal infections, was therefore examined.

5.2 Materials and Methods

5.2.1 Phages and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain

The lytic gut phage PA13076 and the temperate gut phage BP96115 were amplified and purified according to the methods described in chapter two, using their specific host. They were purified through CsCl gradient ultracentrifugation, dialysis and ultrafiltration. Solutions of lytic phage PA13076 or temperate phage Bp96115, at 1×10^8 PFU/mL in drinking water, were prepared using the method described in chapter three. To confirm that the *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain used for the challenge was not infected by the lytic or the temperate gut phage, spot tests were employed. *Salmonella* Typhimurium 002 was chosen and grown overnight from a colony at 37℃ in TSB (Beijing Land Bridge Technology Co., LTD, Beijing). Cells were diluted by 1:20 in fresh sterile medium, and subcultured for 4 h at the same temperature, washed twice in ice-cold PBS (pH 7.2, Hyclone, Logan, Utah) and suspended in cold PBS to 2×10^8 CFU/200 μL.

5.2.2 Animals

C57BL/6 female mice (6 to 8 weeks old) were purchased from the Comparative Medical Center of Yangzhou University (Yangzhou, China). For the experiments, mice were reared in groups of up to five animals and maintained under specific pathogen-free conditions in the experimental animal center in the Jiangsu Academy of Agricultural Sciences (JAAS). Experiments were done following the ARRIVE guidelines (Kilkenny et al., 2010). Mice were fed ad libitum with a commercial grain formulation (SHOOBREE Rat and Mouse Maintenance Die, Jinagsu xietong organism Co., Ltd., Nanjing) and sterilized mineral water (Evian; pH 7.2; HCO_3^-, 489 mg/L), and allowed to acclimatize for two weeks before commencement of experiments. They were weighed before and after the experiments. Fresh fecal samples were collected after infection and treatment.

5.2.3 Experimental design

Two trials were done as follows. Trial 1 evaluated the regulating efficacy of pretreatment with lytic or temperate phage on gut microbiota diversity and composition after being challenged with *Salmonella* Typhimurium 002. Fifty mice

were randomly allocated into five groups (n=10): Group A (10 mice, the control group) was without any treatment; Group F (10 mice, the ST group) drank sterilized mineral water daily for 31 days before and after being challenged with *Salmonella* Typhimurium 002 (2×10^8 CFU) by oral gavage; Group G (10 mice, the LyticST group) was treated with 1×10^8 PFU/mL of lytic phage PA13076 (thus making approximately 4×10^8 PFU/ mouse daily) for 31 days before and after being challenged with 2×10^8 CFU of *Salmonella* Typhimurium 002 by oral gavage; Group H (10 mice, the LysogenST group) was treated with 1×10^8 PFU/mL of temperate phage BP96115 daily (approximately 4×10^8 PFU/mouse), the same titer as for the lytic phage, for 31 days before and after being challenged with 2×10^8 CFU of *Salmonella* Typhimurium 002 by oral gavage and Group I (10 mice, the SMST group) received 10 mg/mL of streptomycin solution (approximately 40mg/mouse) daily for 31 days before and after being challenged with 2×10^8 CFU of *Salmonella* Typhimurium 002 by oral gavage.

Trial 2 was conducted to evaluate the pretreatment of lytic or temperate phage to prevent intestinal inflammations. The preadministration of phages or the streptomycin solution were the same as for trial 1. However, after 31 days of preadministration, mice were gavaged with a single dose of 20 mg of streptomycin 24 hours before being challenged with 2×10^8 CFU of *Salmonella* Typhimurium 002. In this trial, each group matched a corresponding group from trial 1, thus, Group a (10 mice, the control group), Group f (10 mice, the ST group), Group g (10 mice, the LyticST group), Group h (10 mice, the LysogenST group), and Group i (10 mice, the SM group).

5.2.4 Trial 1

5.2.4.1 16S rRNA gene sequencing and analysis

At 48h post bacteria challenge, a total of eighteen fecal samples from five groups (four samples of each group, except two samples of the SMST group) were chosen from trial 1. 16S rRNA gene sequencing was used to determine the composition of gut microbiota as described in chapter four. In brief, the DNA of each sample was extracted using QIAamp® DNA stool Mini kit (MP Biomedicals, CA). The DNA extractions were verified using a Qubit® 3.0 fluorometer (Life, Madison, WI), DNA integrity were assessed by gel electrophoresis, and amplified using universal primers targeting the V3-V4 regions of 16S rRNA of Bacteria (Klindworth et al., 2013). The amplicons were sequenced on the Illumina Miseq platform (2×300 bp) (Illumina, CA)

at Sangong biotechnology Co. Ltd. (Shanghai, China). The sequencing data from this study were processed and analyzed using the same method of chapter four, and uploaded to the NCBI Sequence Read Archive under accession no. SRP144014.

5.2.4.2 Enumeration of members of the *Enterobacteriaceae* and *Lactobacillus* spp. in cecum samples

After finishing feces collection, mice were sacrificed by CO_2 asphyxiation, and cecal contents were tested for the presence of members of the *Enterobacteriaceae* and *Lactobacillus* spp. Each content was diluted (10-fold increments) in ice-cold PBS (pH 7.2, Hyclone), and then a volume of 100 μL of appropriate decimal dilutions was plated on selective agars. Viable counts (CFU/g) were determined in duplicate using SS agar (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, China) for members of the *Enterobacteriaceae* and MRS agar (Difco, BD science, USA) for *Lactobacillus* spp. The detection limit was 100 CFU/g. Each phage pretreated or streptomycin pretreated sample was compared to the control by one-way ANOVA. Significant differences were determined using SPSS 16.0 (SPSS Inc. CA) with Duncan's test set at $p<0.01$.

5.2.5 Trial 2

At 24h post infection, mice were sacrificed by CO_2 asphyxiation. Serum for LPS and DAO analysis was separated from the blood and tissue samples from the intestinal tracts and used for analysis.

5.2.5.1 Histopathological analysis

Representative portions of ileum and cecum were put in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin-eosin (H&E) according to standard procedures (Cardiff et al., 2014). Based on an earlier study (Barthel et al., 2003), the pathological scores of each tissue were determined. The scores ranged between 0 and 13, the numbers represented: 0, intestine intact without any signs of inflammation; 1~2, minimal signs of inflammation; 3~4, mild inflammation; 5~8, moderate inflammation; 9~13, serious inflammation.

5.2.5.2 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from jejunum tissues using TRIzol (Invitrogen, CA), and cDNA was synthesized using a PrimeScript^{TM} RT reagent kit with gDNA Eraser (Takara, Japan). The products were used as a template for real-time PCR. The targeting primers were designed using Primer 5.0 software, based on the published GenBank sequences (Table 5.1). q-PCR was performed using SYBR® Premix EX Taq^{TM} kit (Takara, Japan) and LightCycler 480 (Roch, Basel, Switzerland). The program was followed by 40 cycles of 94°C for 10 s, 60°C for 20 s, and 68°C for 20 s. Melting curves were subsequently created, which consisted of 95°C for 15 s and 60°C for 20 min increasing by 0.5°C per minute to a final temperature of 95°C. Each experiment was performed in triplicate. The GADPH gene was used as an internal standard, and the values were calculated based on the $2^{-\Delta\Delta Ct}$ method .

Table 5.1 Primers used in this study

Primer name		Sequence (5′-3′)	Amlicon size(bp)	Gene ID
GAPDH	Forward	5'-TGAAGGGTGGAGCCAAAAG-3'	227	14433
	reverse	5'-AGTCTTCTGGGTGGCAGTGAT-3'		
IL-10	Forward	5'- GGTTGCCAAGCCTTATCGG-3'	191	16153
	reverse	5'- ACCTGCTCCACTGCCTTGC-3'		
IFN-γ	Forward	5'-GATGCATTCATGAGTATTGCCAAGT-3'	118	15978
	reverse	5'-GTGGACCACTCGGATGAGCTC-3'		
IL-4	Forward	5'-AGATGGATGTGCCAAACGTCCTCA-3'	104	16189
	reverse	AATATGCGAAGCACCTTGGAAGCC-3'		
IL-1β	Forward	5'- GGGCCTCAAAGGAAAGAATCT-3'	195	16176
	reverse	5'- GAGGTGCTGATGTACCAGTTGG-3'		
TNF-α	Forward	5'- CCCCAAAGGGATGAGAAGTT-3'	212	21926
	reverse	5'- TGGGCTACAGGCTTGTCACT-3'		
IL-6	Forward	5'- GAGGATACCACTCCCAACAGACC -3'	195	16193
	reverse	5`- AAGTGCATCATCGTTGTTCATACA -3`		

5.2.5.3 Intestinal permeability analysis

LPS were measured using LAL kit (Thermo Fisher, Sweden) and DAO Assay Kit (Jiancheng Bio-Technology Co., Ltd, Nanjing, China) according to the manufacturers' instructions. Each sample was analyzed in triplicate.

5.3 Results

5.3.1 Gut microbiota analysis and comparison

High-throughput sequencing of 16S rRNA gene amplicons was conducted to exploit the role of lytic and temperate gut phage on dysbiosis of gut microbiota caused by *Salmonella* Typhimurium 002 challenge. A total of 35,441 to 47,729 merged reads were obtained for each sample. As shown in Table 5.2, *Bacteriodetes*, *Firmicutes* and *Proteobacteria* was the top abundant phylum in the control group, accounting for 80.47%, 15.43% and 2.92%, respectively. However, when administrating the lytic or the temperate phage for 31 days prior to the *Salmonella* Typhimurium 002 challenge, the *Firmicutes* became the most abundant phylum, accounting for 60.62% and 43.01% of reads, respectively. The abundance of *Actinobacteria* increased and *Tenericutes* reduced in group G and H compared to group A and F. Furthermore, the phylum *Proteobacteria* increased from 5.82% in group F (challenged with *Salmonella* Typhimurium 002) to 24.49% in group I (pretreated with streptomycin for 31 days before and after *Salmonella* Typhimurium 002 challenge). The number of phyla decreased in group I. Meanwhile, Bray-Curtis similarity cluster analysis indicated that group I was clearly separated from the other groups, and group G and group H were strongly correlated (Fig 5.1). Fig. 5.2 (1) Indicated that the diversity of bacterial communities in groups F, G and H was higher compared to the control group (A), and the diversity declined clearly in I group ($p=0.083$). The same trend was observed when comparing the Shannon index (Fig. 5.2 (2) with that using the Chao 1 diversity index. Principal coordinate analysis revealed differences based on relative OTU abundance at family level between groups. It showed that groups receiving pretreatment with streptomycin mostly differed from groups pretreated with phages and control samples (Fig. 5.3). The changes in bacterial composition at the genus level for selected members of the gut community are shown in Fig. 5.4. Pretreatment with the lytic and the temperate phage before *Salmonella* Typhimurium 002 challenge

increased two beneficial genera, *Lactobacillus* (14.73% and 21.02%) and *Bifidobacterium* (2.43% and 2.05%). While the genus *Lactobacillus* in group F was abundant at 14.04%, the genus *Bifidobacterium* was not detected. However, in group H an increased abundance of the genus *Escherichia*/*Shigella* (10.69%) is evident. The genus *Allobaculum*, belonging to the phylum *Firmicutes*, was also present in higher relative abundances after pretreatment with gut phage. On the other hand, the genus *Klebsiella* declined in groups G and H compared to groups F and I where it was high as 19.75%.

Table 5.2 Composition of the gut microbiota at phylum level (%) various experimental groups with or without challenged with *Salmonella* Typhimurium 002

Group	A	F	G	H	I
Bacteroidetes	80.47±4.94	58.99±11.07	30.29±5.29	30.78±15.17	55.08±0.40
Firmicutes	15.43±5.43	33.19±10.19	60.62±3.88	43.01±23.75	20.14±3.93
Proteobacteria	2.92±1.56	5.82±4.75	3.92±1.79	17.51±9.58	24.49±4.49
Actinobacteria	0.95±0.61	0.49±0.21	4.17±1.20	2.98±2.22	0.27±0.14
Verrucomicrobia	ND	1.24±1.39	0.87±0.82	5.69±9.58	ND
Candidatus Saccharibacteria	0.09±0.06	0.11±0.09	0.09±0.14	0.01±0.005	ND
Tenericutes	0.12±0.15	0.11±0.16	0.01±0.01	0.003±0.005	ND
Thermotogae	0.02±0.01	0.03±0.01	0.01±0.01	0.025±0.01	0.02±0.01
Fusobacteria	ND	ND	0.01±0.02	ND	ND
Cyanobacteria	ND	ND	ND	ND	0.02±0.02
Synergistetes	ND	0.003±0.005	ND	ND	ND
Euryarchaeota	ND	0.003±0.005	ND	ND	ND
Lentisphaerae	ND	0.003±0.005	ND	ND	ND
Planctomycetes	ND	0.003±0.005	ND	ND	ND

Note: Information of each group of trial 1: A, the control group, without any treatment; F, the ST group, drank sterilized mineral water daily for 31 days before and after being challenged with *Salmonella* Typhimurium 002; G, the LyticST group, treated with the lytic gut phage PA13076 for 31 days before and after being challenged with *Salmonella* Typhimurium 002; H, the LysogenST group, treated with the temperate phage BP96115 for 31 days before and after being challenged with *Salmonella* Typhimurium 002 and I, the SMST group, received streptomycin solution for 31 days before and after being challenged with *Salmonella* Typhimurium 002.

ND=Not detected.

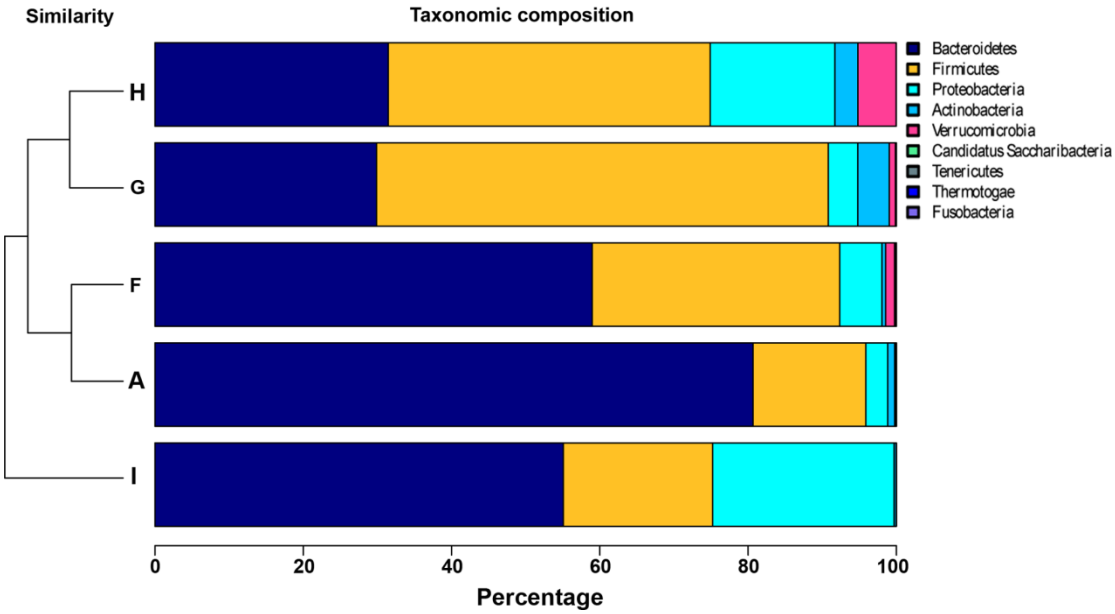

Fig. 5.1 Bray-Curtis similarity cluster analysis combined with the composition of gut microbiota at phylum level. A, the control group; F, the ST group; G, the LyticST group; H, the LysogenST group and I, the SMST group.

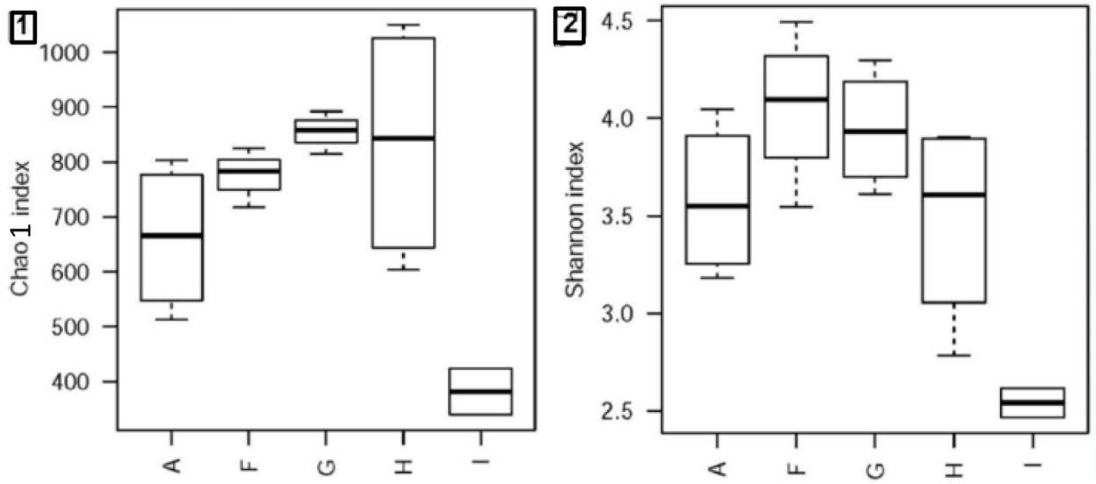

Fig. 5.2 The alpha diversity of gut microbiota in the different groups based on the Chao 1 (1) and the Shannon index (2) of diversity. A, the control group; F, the ST group; G, the LyticST group; H, the LysogenST group and I, the SMST group.

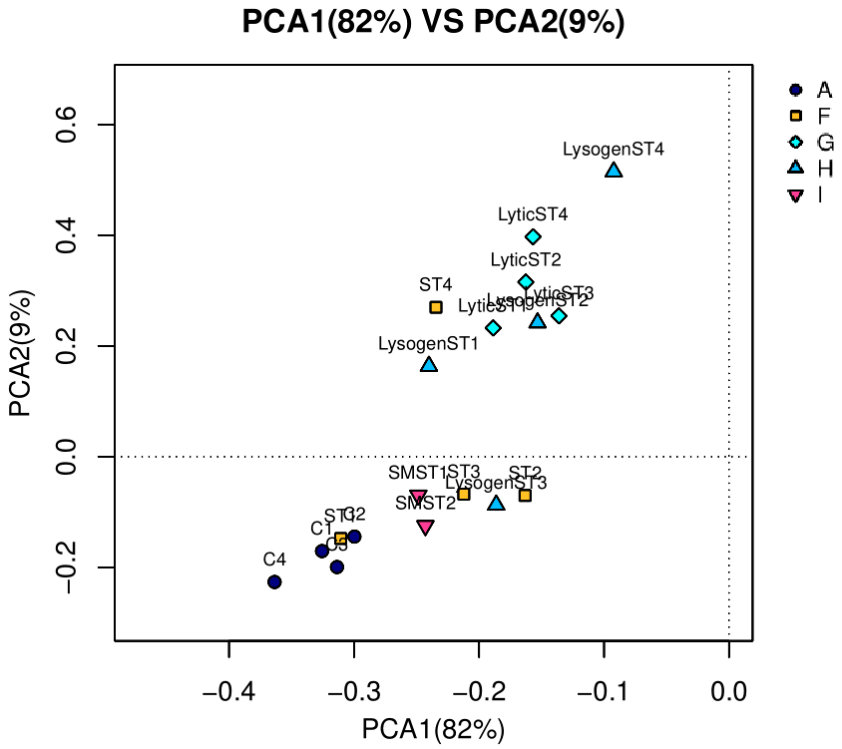

Fig. 5.3 Principal coordinate analysis (PCoA) of 18 metagenomic samples at family level for the different groups. Points represent samples, and the color indicates group information.

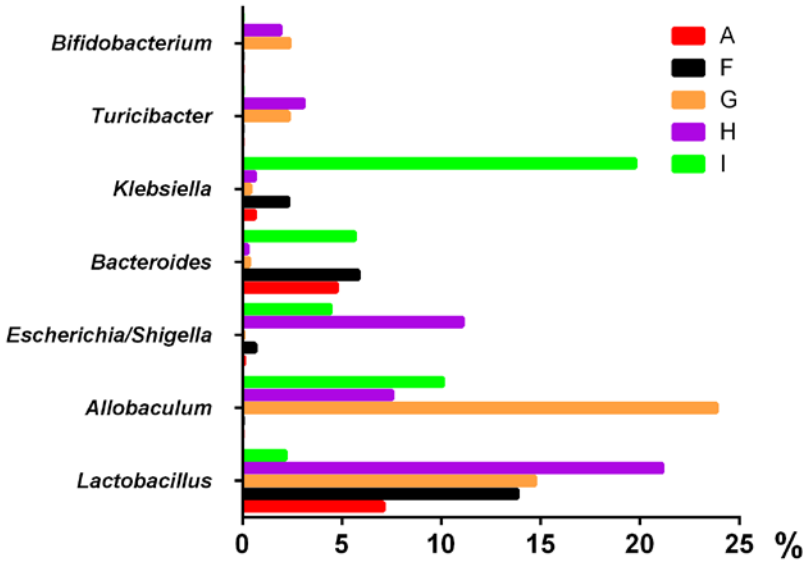

Fig. 5.4 Relative abundance of selected members of the gut bacterial community at genus level. A, the control group; F, the ST group; G, the LyticST group; H, the LysogenST group and I, the SMST group.

5.3.2 Enumeration of members of the *Enterobacteriaceae* and *Lactobacillus* spp. in cecal contents

Phage pretreatment did not significantly change counts of the *Enterobacteriaceae* in cecal contents. However, streptomycin pretreatment reduced the viable counts for the *Enterobacteriaceae* to 4.32±0.12 log (cfu/g) in cecal samples (Fig. 5.5-1). In addition, pretreatment with the lytic and temperate gut phage for 31 days increased the *Lactobacillus* spp. counts (>8.0 cfu/g), even though the mice were challenged with *Salmonella* Typhimurium 002, while pretreatment with streptomycin decreased the *Lactobacillus* spp. counts to 6.16±0.41(cfu/g).

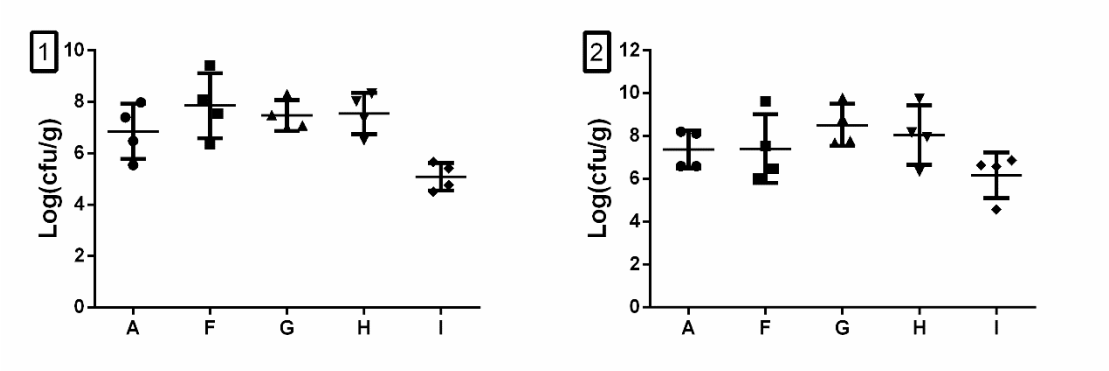

Fig. 5.5 Viable counts of *Enterobacteriaceae* (1) and *Lactobacillus* spp. (2) in cecal content of mice after being challenged with *Salmonella* Typhimurium 002. Results are expressed as base-10 logarithm of colony-forming units per gram. Error bars represent standard deviations. A, the control group; F, the ST group; G, the LyticST group; H, the LysogenST group and I, the SMST group.

5.3.3 Histopathological changes

In order to characterize the regulatory role of phages in the process of intestinal inflammation, the mice were treated orally with the lytic or the temperate phage for 31 days. Intestinal inflammation was induced via oral pretreatment with streptomycin before the *Salmonella* Typhimurium 002 challenge. Histologic changes of ileum and cecum were analyzed by bright field microscopy and representative pictures are shown (Fig. 5.6, 1-2). Compared with group f (the ST group) and group i (the SMST group), the phenomena of villus break and loss of crypts were absent in ileum samples of groups g (the LyticST group) and h (the LysogenST group). However, compared

with group a (the control group), pathophysiological changes (epithelial edema, villus break and thinner villi) were still present. In the cecum samples of group f, the destruction of the crypt structure, the decrease of goblet cells, epithelial erosion and PMN infiltration of the submucosa were observed. However, no signs of serious inflammation were observed in the cecum of groups g and h of mice. The histological severity of effects observed in treated mice was quantified by pathologic scoring (Fig.5.6-3). According to the analysis of pathological changes, preadministration of the lytic (group g) and the temperate (group h) gut phage reduced ileum and cecum inflammation.

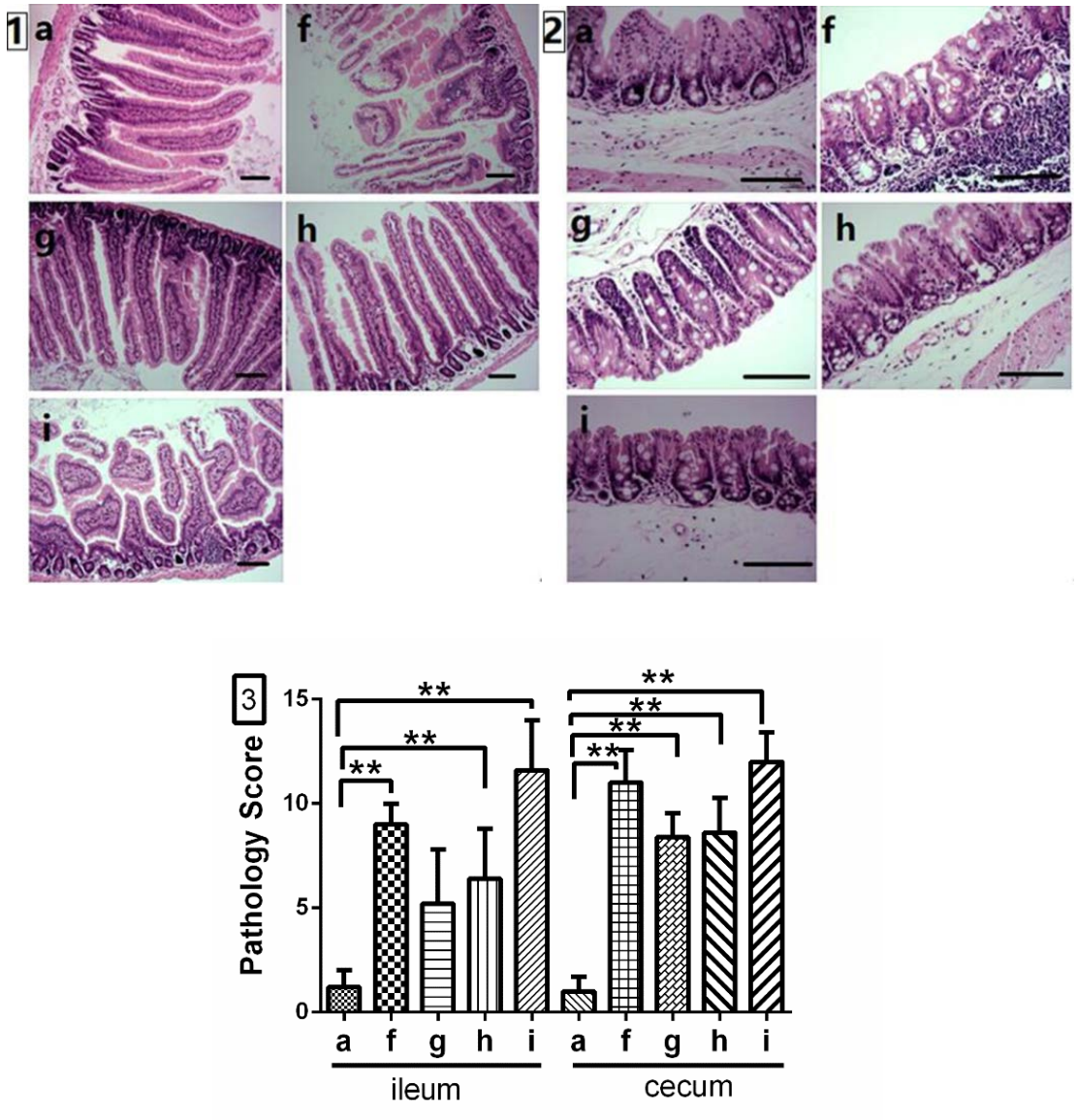

Fig. 5.6 Representative images of hematoxylin-eosin staining of ileum (1) and cecum (2) samples, n = 5 mice per group. Scale bar, 100 μm. (3) The cumulative

pathology scores from 5 mice per group (range, 0-13). ** differed by p<0.01. Information of each group of trial 2: Group a, the control group; group f, the ST group; group g, the LyticST group; group h, the LysogenST group and group i, the SMST group.

5.3.4 Gene expression of inflammatory cytokines in Jejunum tissue

To further assess pathologic changes, mRNA levels were analyzed for cytokines IL-1β, IFN-γ, TNF-α, IL-6, IL-4 and IL-10. The expression of each of these tested cytokine genes was elevated 3.85, 3.78, 3.92, 3.95,4.32, and 4.28 fold in average in the jejunum at 24h post challenge in the f group (ST group). Compared to the group f, the expression of all detected cytokine genes was not changed significantly in treated groups (Fig. 5.7). However, oral pretreatment with the lytic or the temperate gut phage resulted in lower gene expression of IL-1β, IFN-γ, TNF-α, IL-6 and IL-4 than in group f.

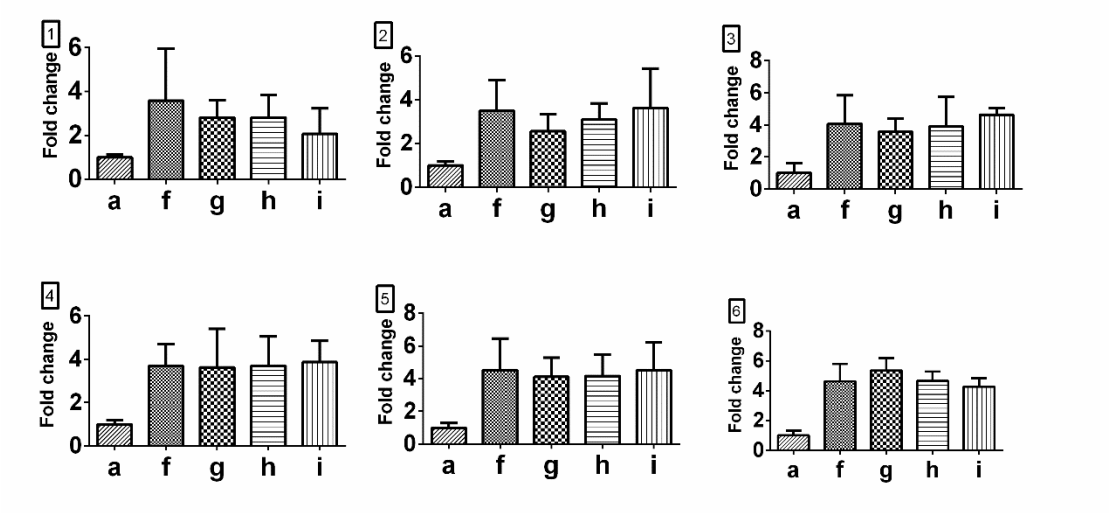

Fig. 5.7 Effects of the pretreatment with the lytic or the temperate gut phage on expression levels of inflammatory cytokines in jejunum tissue. (1) IL-1β, (2) TNF-α, (3) IFN-γ, (4) IL-6, (5) IL-4, (6) IL-10. Group a, the control group; group f, the ST group; group g, the LyticST group; group h, the LysogenST group and group i, the SMST group.

5.3.5 DAO and ET levels after phage treatment

Serum Lipopolysaccharide (LPS) and diamine oxidase (DAO) levels can be used indicators for assessing intestinal injury and monitoring intestinal permeability

(Hartmann et al., 2012). Fig. 5.8 showed that pretreatment with the lytic or the temperate gut phage before intestinal inflammation resulted in slightly reduced DAO levels in serum compared to group f. In addition, the ET level was also reduced but the difference was statistically not significant. However, even pretreatment with streptomycin increased the levels of DAO and ET.

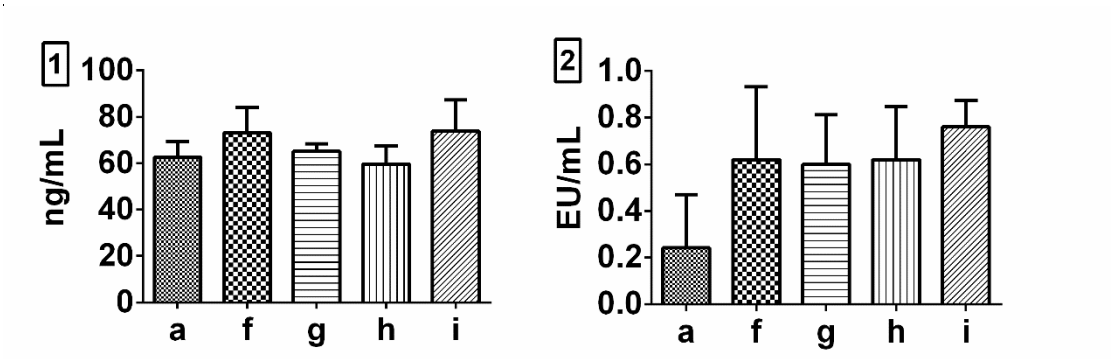

Fig. 5.8 The concentration of DAO (1) and ET (2) in serum. Group a, the control group; group f, the ST group; group g, the LyticST group; group h, the LysogenST group and group i, the SMST group. Error bars represent standard deviations.

5.4 Discussion

Phages are the most abundant bacterial viruses on the earth and it was estimated that as many as 10^{15} individual phage particles are present in the mammalian gut (Dabrowska et al., 2005; Dalmasso et al., 2014). Several phage therapy studies indicated that phages minimally impact health-protecting bacteria of the normal gut flora (Galtier et al., 2016; Mai et al., 2010). The reasons were that they infected only a few strains of a particular bacterial species, and the short administration time and low repetitions safeguarded that no detrimental effects took place. The ability of phages to regulate dysbiosis of gastrointestinal microbiota caused by *Salmonella* Typhimurium challenge in a mouse model was explored in this study. As the challenged bacteria were not the specific host of the used phages and the phages were repeatedly preadministrated for an extended period, this study was different from previous studies evaluating phage therapy against bacterial pathogen infections in laboratory animals (Ahmadi et al., 2016; Fukuda et al., 2012; Tanji et al., 2005). The present study was based on a concept of probiotic intervention (Abedon et al., 2011), which would employ phage therapy prophylactically rather than therapeutically. Although phages are not considered to be probiotics, they actually conform to the definition of

probiotics by the Food and Agriculture Organization (FAO) of the United Nations (UN) and the World Health Organization (WHO) (Abedon et al., 2011): “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (WHO, 2001).

Several studies reported that phages may have a potential role in shaping the composition of the gut bacterial community (Gorski et al., 2003; Gorski and Weber-Dabrowska, 2005; Mills et al., 2013) and that phages are a good maker for health and disease (Dalmasso et al., 2014; Lepage et al., 2008). An earlier study in Chapter 4 confirmed that not only the lytic but also the temperate gut phage enhanced the diversity and altered the composition of gut microbiota when the mice were orally administrated with these phages over 31 days. In this study, the result of higher alpha diversity in phage pretreated mice was consistent with similar studies by Valeriano et al. (2017) from probiotic studies. However, this experiment results of higher alpha diversity are contrary to the experimental results of mice study of HPLCD diet (30% casein and 57% carbohydrate) (Kim et al., 2016), and the rat models of colorectal cancer and ulcerative colitis (Zhu et al., 2014). The diversity of intestinal microflora is related to some health deterioration and will be an indication of illness (Kim et al., 2016). This suggests that the pretreated mice with phage may have good benefits on intestinal health by increasing the diversity of intestinal microflora.

Furthermore, the lytic gut phage provided a healthy gut ecosystem, which matches probiotics (Valeriano et al., 2017). Probiotics are considered to modulate gut microbiota imbalances and modify population of the gut microbiota to improve the health of animal body (Rahmat Ullah et al., 2017; Stough et al., 2017). Similarly, results obtained in this study indicate that preadministration of phages for 31 days before the bacterial challenge can enhance the diversity of gut bacterial populations, increase the *Firmicutes*/*Bacteroidetes* ratio and specifically increase the beneficial genera *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* and *Bifidobacterium* can lower the pH value of intestinal digesta and prevent pathogens colonization, so that, they have the potential ability to facilitate the development of intestinal functions (Dunkley et al., 2009; Haghighi et al., 2008) and enhancing animal immune functions (Haghighi et al., 2005). One probiotic study indicated that the application of *Lactobacillus plantarum* G83 not only increased genera *Lactobacillus* and *Bifidobacterium*, but also reduced the number of *Bacteroidetes* and

Enterobacteriaceae in the mice colon (Oliveira et al., 2016). Members of the genus *Bifidobacterium*, along with *Lactobacillus*, represent well-known probiotics and are the major bacterial genera making up the bacterial flora in healthy mammals (Unkauf et al., 2016). Probiotic *Bifidobacterium* spp. enhanced the abundance of *Bifidobacterium*, accompanied by reduced IL-10 and TNF-α levels in the elderly, demonstrating that *Bifidobacterium* could decrease the age-dependent inflammatory status (Ouwehand et al., 2008). Furthermore, using *Bifidobacterium* spp. as a probiotic in the conventional treatment of ulcerative colitis was associated with improved rates and maintenance of remission (Fernandez et al., 2017).

Phage-based "probiotic products" do not carry pathogenic bacteria and can improve overall balance of the gastrointestinal tract. Moreover, a previous study verified that a phage cocktail as the additive in the dietary improved the growth performance of weaning pigs (Wang, et al., 2013). However, the broad-spectrum of antibiotics leads to microflora dysbiosis and overgrowth of pathogens, and potentially life-threatening secondary infections (Schokker et al., 2015). Feeding antibiotics particularly reduced the relative abundance of the beneficial genus *Lactobacillus* (Collier et al., 2003; Looft et al., 2012).

Pretreatment of mice with streptomycin disturbs the microbiota community structure, and the *Salmonella* Typhimurium invasion into the gut tissue further triggers gut inflammation. Streptomycin-pretreated mice provide a mouse model for *Salmonella* Typhimurium colitis (Barthel et al., 2003; Miki et al., 2017; Pedicord et al., 2016). Intestinal microbial composition appears to benefit for systemic balance and even modulate inflammatory responses caused by diseases for mice (Kinross et al., 2011; Pedicord et al., 2016). Moreover, it has been shown that gut microbiota of the wild mice promoted host adaptability and alleviated inflammation (Rosshart et al., 2017). Microbiome-based therapeutics via the preadministration of phage may present a feasible approach to affect the microbial structure or activity of the gut microbiota (Pedicord et al., 2016).

Inflammatory cytokines are usually expressed at elevated levels following infections with pathogens (Pinsky et al., 1993). However, it was shown that phages did not stimulate the production of inflammatory mediators *in vitro* and in mice (Weber-Dabrowska et al., 2000), but also played a strong anti-inflammatory roles, and

the mechanisms appear to be inconsistent with the antibacterial effects (Gorski et al., 2017). It was also shown that alteration of the gut microbiota composition may play a role in ensuring immune response (Cho et al., 2016). In this study, oral pretreatment with the lytic or the temperate gut phage resulted in decreased expression of IL-1β, IFN-γ, TNF-α, IL-6 and IL-4 genes. These results match a study showing that phage can reduce inflammation and depress the cytokine levels of TNF-alpha and neutrophil in bronchoalveolar lavage fluid of mice (Carmody et al., 2010).

The effects of phages on the immunologic system have been studied. Firstly, phages reduce bacterial numbers to relative lower levels, and then the immune systems help to scavenge bacteria together (Gorski and Weber-Dabrowska, 2005). The immunogenicity could be produced after phage causing repeated lytic infections of the normal bacterial flora (Biswas et al., 2002; Gorski and Weber-Dabrowska, 2005).

In this study, histological analysis confirmed that the degree of ileum and cecum damage in groups of mice pretreated with phages was lower than that in groups of mice pretreated with streptomycin, which was confirmed by lower inflammatory scores. These observations are consistent with phage therapies ameliorating the histological damage in the colon (Jun et al., 2014) and in the vital organs: liver, kidney, lung, and spleen (Shivshetty et al., 2014). Intestinal pathogens usually alter the permeability of the mucosal barrier by destroying the tight junction and function and secreting proteases, so that they can translocate from gut to the other parts of the body (Berkes et al., 2003). The concentration of serum LPS was associated with inflammation and bacterial tissue invasion (Rosenfeld and Shai, 2006) and DAO was related to the capability of the mucosal barrier (Sun et al., 2013). Overgrowth of the gut community by Gram-negative bacteria and gut inflammation increase circulation of LPS, conversely, the increased LPS activates host immune cells to produce inflammatory factors and perpetuate the inflammatory cycle. Previous studies indicated that the activity of serum DAO was increased significantly in infected rats and the secretion of TNF-α, IFN-γ and IL-8 in the intestinal tissues were also higher compared to the healthy rats (Xin et al., 2016).

In a word, phages can act as modulating agents regulating gut microbiota, showing that phage based prophylaxis can limit infection by bacterial pathogens.

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CHAPTER SIX

GENERAL OVERVIEW AND CONCLUSIONS

6.1 Summary of findings

The worldwide misuse of antibiotics in animal feed and particularly the rapid emergence of multi-drug-resistant *Salmonella* spp. due to antibiotic overuse have received global attention and raised concern (Martin et al., 2015; Park et al., 2017). Since 2005, many countries began to restrict the use of antibiotics as growth promoters for farm animals (EC, 2005). Currently, the most promising alternatives to antibiotics for controlling bacteria are bacteriophages (Cisek et al., 2017; Golkar et al., 2014).

Phages are probably the most diverse and abundant biological entities on earth. Furthermore, they are usually present in a very high concentration (approximately 10^{15} phages) in the mammalian gut (Dalmasso et al., 2014). It is noteworthy that the majority of phages are prophages, which can be spontaneously induced as active phages (Kim and Bae, 2018). Furthermore, when lytic phages are orally used to treat pathogen infections *in vivo*, they encounter both pathogens and commensal microorganisms in the complex gut of the animal (Olivo et al., 2016). Except of phages being used as a lytic bio-bactericide, other beneficial impacts of phages on intestinal epithelial cells or even the animal body also can be exploited to help in the treatment of various diseases.

Phages are diverse and largely unexplored in the animal gut, and most of the genome sequences of gut bacteriophages are still unknown (Rohwer, 2003; Ventura et al., 2011). Lepage et al. (2008) suggested that gut phages likely have a strong influence on the diversity and population structure of bacterial communities in the intestinal tract; however, little is known of the role of abundant phages in the gastrointestinal tract (GIT) in shaping the mammalian gut microbiota and their relationships with health and disease.

This thesis established that:

- A lytic gut phage vB_SenM-PA13076 was isolated from fecal samples of chicken and characterized physiologically and genetically. This phage demonstrated a broad host range and contained no undesirable genes such as those for lysogeny, virulence

factors, and antimicrobial resistance genes. PA13076 is a virulent phage that has potential for treating animals to prevent infections by several *Salmonella*. In addition, a temperate gut phage designated as vB_SpuP-BP96115 was induced from *Salmonella* Pullorum strains using mitomycin C and characterized. Several lysogeny modules for phage lysogenization, such as cI, cro, integrase, and Q, were identified from the genome of this phage. Furthermore, according to the comparative analysis of the putative proteins, many proteins showed high similarity to proteins from other temperate phages indicating that this phage is a real temperate phage (Zinno et al., 2014).

- The lytic phage PA13076, and the temperate phage BP96115, were purified using ultracentrifugation and ultrafiltration before phage administrations to mice. The endotoxin levels of lytic gut phage PA13076 and temperate phage BP96115 solutions were reduced to <1 EU/mL. Substantial phage titers were observed in the GIT after oral administration to mice, and both phages were mainly located in the cecum and feces, with titers reaching up to 10^4 and 10^6 PFU/g in cecum for PA13076 and BP96115, respectively. Simultaneously, the phages were detected in blood and spleen samples of mice. Overall, the current study demonstrated that a lytic and a temperate phage were distributed in the intestinal tract of mice and colonized it after oral intake, preferentially colonizing the posterior of the gut.

- The metagenomic analyses of fecal samples showed that treatment of mice with the lytic and the temperate gut phage increased the gut bacterial diversity. Furthermore, the lytic gut phage significantly increased the alpha diversity and both phages changed the composition of the mice gut microbiota. Two beneficial genera, *Lactobacillus* and *Bifidobacterium*, were enhanced by treatment with the lytic and the temperate gut phage. While the lytic gut phage treatment did not cause an increase in potentially harmful bacterial pathogens, the temperate phage treatment of mice gave rise to the genus *Shigella*. Both phages did not change fecal VLPs significantly. Therefore, this study demonstrated that a long-term phage administration undoubtedly influences the dominant bacterial groups in the mammalian intestine and therefore health. The lytic gut phage promoted a beneficial gut ecosystem, while the temperate phage may promote conditions enabling diseases to occur.

- Mice pretreated with the lytic and the temperate gut phage before *Salmonella*

Typhimurium challenge had an increased diversity of the gut microbiota not only at phylum but also at genus level. These results suggest that pretreatment of mice with lytic gut phage maintained a stable and healthy gut ecosystem, altered the composition of the bacterial community and enhanced the capability of mice to resist the pathogen challenge. The altered intestinal permeability and expression of inflammatory cytokines confirmed that the pretreatment of mice with the lytic or temperate gut phage reduced the pathogen induced intestinal inflammation. Hence, the use of a phage-based "probiotic product" affected the abundance and composition of gut microbiota as a regulatory therapy by increasing beneficial bacteria and thereby restricting intestinal inflammation. This study provides a first insight regarding the potential of phages as modulating agents for gastrointestinal microbial communities, potentially allowing for the development of phage-based strategies to prevent pathogen infection.

6.2 Conclusions

Due to the rapid emergence of multi-drug resistant *Salmonella* spp. causing infections of animal intestines and the overuse of antibiotics in animal feed, there is an urgent need to use phage-based bio-bactericides to promote animal health instead of antibiotics (Watts, 2017; Zelasko et al., 2017). Therefore, lytic phages seem to be a potential agent to improve the treatment of infections caused by bacterial pathogens (Cisek et al., 2017). However, unlike lytic phages, temperate phages are not suitable to kill bacterial pathogens (Cadwell, 2015). Nevertheless, temperate phages dominate in the animal gut, and perform a remarkable level of horizontal gene transfer and enter a preferred prophage life cycle upon infection (De Paepe et al., 2014). From an evolutionary perspective, phages will undoubtedly exert selective pressure on the commensal bacterial populations in the animal gut. However, so far there are limited numbers of studies, which have focused on the regulatory role of phages in the animal gut.

To gain a better understanding of the governing capability of phages in the animal gut, One lytic and one temperate phage, which were originally isolated from animal gut were characterized and sequenced. The two genomes were analyzed using a comparative genomics approach. This is important as our knowledge of the genomes and the diversity of gut phages is very limited (Moreno Switt et al., 2013). Phage

PA13076 was confirmed as a virulent phage based on the absence of genes coding for integrase, repressor or transposase proteins and its high similarities to the genomes of lytic phage BP63 and UPF_BP2. Similarly, phage BP96115 shared a high degree of similarity with other temperate phages such as *Salmonella* phage P22 (Vander Byl and Kropinski, 2000), and enterobacteria phage ST104 (Tanaka et al., 2004). Additionally, the genomic elements, such as the functional serotype conversion cassette, integrase, excisionase, Abc1, and superinfection exclusion were found in BP96115, strongly suggesting that this phage is a temperate phage (Yoon and Chang, 2015). A better understanding of phage genomes is essential as gut phages may have an important role in protecting the host from pathogen infections and in regulating the bacterial population of the gut (Hurley et al., 2008; Lusiak-Szelachowska et al., 2017).

Efficient removal of LPS from phage preparations is a key process to produce high quality phage formulations for clinical application. A combination of ultracentrifugation and ultrafiltration was efficient in removing bacterial LPS from prepared phage solutions, thereby matching previous studies (Dufour et al., 2016; Hashemi et al., 2013; Henry et al., 2013). Oral administration of phages is an important way to treat gastrointestinal infections and containing systemic infections (Ryan et al., 2011). However, phage survival as well as the preferred colonization and distribution in the GIT of treated animals are poorly understood. This study demonstrated that the lytic phage PA13076 and the temperate phage BP96115 can translocate across the gut mucosal barrier and migrate to peripheral blood and local tissues, which is in good agreement with previous studies (Gorski et al., 2006). In the present study, highest phage titers were detected in the cecum and in feces of mice. This result matches the report by Hoffmann (1965), showing that phage T3 preferentially located in the higher or deeper sections of the intestine. Moreover, it was verified in this thesis that the temperate phage was more numerous than the lytic phage in mammalian feces, which matches the result of Dhillon et al. (1976).

This study indicated that long-term (31 days) administration of the lytic or temperate gut phages in healthy mice changed the composition and diversity of gut microbiota. This result matched previous studies highlighting that phages have the potential to exert selective pressure upon and regulate selected members of the mammalian intestinal microbiota (De Paepe, et al., 2014; Lusiak-Szelachowska et al., 2017; Mills et al., 2013). The alpha diversity of gut microbiota was higher in both phage treated

groups of mice when compared to the control group. It has been shown that a high diversity of the gut microbiota is good for the overall health and productivity of animals such as weaned piglets and disease resistance in laboratory mice (Rosshart et al., 2017; Zhang et al., 2016). Furthermore, the abundance of members of two beneficial bacterial genera, *Lactobacillus* and *Bifidobacterium*, was higher in the lytic and temperate gut phage treated mice than in mice of control group. These enhanced beneficial gut probiotics can provide protection from enteric pathogen infection in animals (Fanning et al., 2012; Ravi, 2011). However, the abundance of the genus *Escherichia*/*Shigella* was higher in mice after temperate phage administration than in the control group. These results demonstrate that a long-term lytic gut phage administration promoted a beneficial gut ecosystem, while using the temperate phage may promote conditions enabling diseases to occur.

The ability of phages to regulate dysbiosis of gut microbiota caused by *Salmonella* Typhimurium challenge in a mouse model was further explored. It was shown that the prophylactic use of phages for 31 days can prevent gut microbiota imbalances and improve gut health via modifying the gut microbial population based on a phage-based “probiotic product” (Abedon et al., 2011). Phage-based prophylaxis may therefore present a feasible approach to promote host fitness and limit pathogenic infection (Pedicord et al., 2016; Rosshart et al., 2017). Phages did not stimulate inflammatory mediator production *in vitro*, but can exert strong anti-inflammatory effects (Gorski et al., 2017). In addition, phage-based prophylaxis can reduce intestinal inflammation levels by ameliorating the histological damage in the ileum and cecum (Jun et al., 2014). In the present study, the mucosal barrier function was somewhat enhanced based on detecting LPS and DAO activity. This is closely related to the lower inflammatory level and the degree of damage of the intestinal mucosa (Xin et al., 2016). Therefore, phages can act as modulating agents regulating gut microbiota, showing that phage based prophylaxis can limit infection by bacterial pathogens.

6.3 Further studies

Gut health is very important for safeguarding animal health, performance, and welfare (Ohland and Jobin, 2015; Tuddenham and Sears, 2015). The results of this thesis indicated that long-term use of phages increased the diversity of gut microbiota and

enhanced the abundance of beneficial bacteria, thereby reducing the intestinal inflammation caused by bacterial infection. Hence, there is a need to expand our understanding of the diversity and abundance of phages as a large proportion of phage genes detected in the gut are unknown and cannot be assigned to known phages (Minot et al., 2011; Rohwer, 2003). This highlights the need for the application of in depth metagenomic studies to detect currently nonculturable phages (Breitbart et al., 2003; Reyes et al., 2010). An improved understanding of the mechanisms by which gut phages shape the diversity and composition of commensal bacteria is also desirable, as this will provide a basis to develop new, more effective ways to control bacterial infections or to find new effective applications to promote the gut health of animals.

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SUPPLEMENTARY MATERIAL

Table S2.1 Host ranges of lytic phage PA13076

No.	*Salmonella* strains	Serotype	Lytic phage PA13076 susceptibility[a]	Sources
1	50336	Enteritidis	+	Ⅰ
2	994	Enteritidis	+	Ⅰ
3	T48	Enteritidis	++	Ⅰ
4	T49	Enteritidis	++	Ⅰ
5	T64	Enteritidis	+	Ⅰ
6	CVCC1	Pullorun	-	Ⅰ
7	S11-2	Pullorun	-	Ⅰ
8	S11-3	Pullorun	-	Ⅰ
9	S11-4-1	Pullorun	+	Ⅰ
10	S11-4-2	Pullorun	+	Ⅰ
11	S11-5	Pullorun	+	Ⅰ
12	S12-2-1	Pullorun	+	Ⅰ
13	S11-2-2	Pullorun	+	Ⅰ
14	C1	Enteritidis	+	Ⅱ
15	C2	Enteritidis	+	Ⅱ
16	C5	Enteritidis	+	Ⅱ
17	C7	Enteritidis	+	Ⅱ
18	C8	Enteritidis	-	Ⅱ
19	C11	Enteritidis	+	Ⅱ
20	C12	Enteritidis	+	Ⅱ
21	C14	Enteritidis	+	Ⅱ
22	C15	Enteritidis	+	Ⅱ
23	C16	Enteritidis	+	Ⅱ
24	C17	Enteritidis	+	Ⅱ
25	C18	Enteritidis	+	Ⅱ
26	C19	Enteritidis	±	Ⅱ
27	C20	Enteritidis	±	Ⅱ
28	C21	Enteritidis	±	Ⅱ
29	C22	Enteritidis	±	Ⅱ
30	C23	Enteritidis	±	Ⅱ
31	C24	Enteritidis	±	Ⅱ
32	C25	Enteritidis	±	Ⅱ

33	C26	Enteritidis	-	II
34	C27	Enteritidis	+	II
35	C29	Enteritidis	+	II
36	C30	Enteritidis	+	II
37	C31	Enteritidis	+	II
38	C32	Enteritidis	+	II
39	C36	Enteritidis	+	II
40	C39	Enteritidis	+	II
41	C40	Enteritidis	+	II
42	C41	Enteritidis	+	II
43	C43	Enteritidis	+	II
44	C44	Enteritidis	+	II
45	C46	Enteritidis	+	II
46	C53	Enteritidis	+	II
47	C56	Enteritidis	+	II
48	C57	Enteritidis	+	II
49	C58	Enteritidis	-	II
50	C59	Enteritidis	-	II
51	C60	Enteritidis	-	II
52	C61	Enteritidis	-	II
53	C63	Enteritidis	+	II
54	C65	Enteritidis	+	II
55	C71	Enteritidis	+	II
56	C72	Enteritidis	+	II
57	C76	Enteritidis	+	II
58	C79	Enteritidis	+	II
59	C80	Enteritidis	+	II
60	C83	Enteritidis	+	II
61	C88	Enteritidis	+	II
62	C89	Enteritidis	+	II
63	C94	Enteritidis	+	II
64	C96	Enteritidis	+	II
65	C99	Enteritidis	+	II
66	C102	Enteritidis	+	II
67	C105	Enteritidis	+	II
68	C106	Enteritidis	+	II
69	C110	Enteritidis	+	II

70	C118	Enteritidis	+	II
71	C124	Enteritidis	+	II
72	C125	Enteritidis	+	II
73	C128	Enteritidis	+	II
74	C130	Enteritidis	+	II
75	C133	Enteritidis	-	II
76	C135	Enteritidis	+	II
77	C137	Enteritidis	-	II
78	C138	Enteritidis	+	II
79	C139	Enteritidis	+	II
80	C144	Enteritidis	+	II
81	C145	Enteritidis	+	II
82	C146	Enteritidis	+	II
83	C149	Enteritidis	+	II
84	C150	Enteritidis	+	II
85	C152	Enteritidis	+	II
86	C154	Enteritidis	+	II
87	C155	Enteritidis	+	II
88	C156	Enteritidis	+	II
89	C158	Enteritidis	+	II
90	C161	Enteritidis	+	II
91	C164	Enteritidis	+	II
92	C165	Enteritidis	+	II
93	C167	Enteritidis	+	II
94	2	Enteritidis	-	II
95	4	Enteritidis	-	II
96	8	Enteritidis	+	II
97	21	Enteritidis	+	II
98	31	Enteritidis	+	II
99	32	Enteritidis	+	II
100	34	Enteritidis	+	II
101	36	Enteritidis	+	II
102	37	Enteritidis	+	II
103	42	Enteritidis	+	II
104	43	Enteritidis	-	II
105	59	Enteritidis	+	II
106	71	Enteritidis	+	II

107	SM-1-KDE	Enteritidis	+	Ⅱ
108	SM-3-KDE	Indiana	+	Ⅱ
109	SM-4-KDE	Typhimurium	-	Ⅱ
110	SM-5-KDE	Typhimurium	-	Ⅱ
111	SM-6-KDE	Typhimurium	-	Ⅱ
112	SM-7-KDE	Typhimurium	-	Ⅱ
113	SM-8-KDE	Typhimurium	+	Ⅱ
114	SM-10-KDE	Enteritidis	+	Ⅱ
115	SM-12-KDE	Typhimurium	-	Ⅱ
116	SM-14-KDE	Enteritidis	+	Ⅱ
117	SM-16-KDE	Enteritidis	+	Ⅱ
118	SM-17-KDE	Enteritidis	+	Ⅱ
119	SM-22-KDE	Newport	+	Ⅱ
120	SM-23-KDE	Enteritidis	+	Ⅱ
121	SM-27-KDE	Sanftenberg	+	Ⅱ
122	SM-28-KDE	Indiana	+	Ⅱ
123	SM-29-KDE	Kottbus	+	Ⅱ
124	SM-30-KDE	Indiana	+	Ⅱ
125	SM-31-KDE	Choleraesuis	+	Ⅱ
126	SM-32-KDE	Enteritidis	+	Ⅱ
127	SM-34-KDE	Enteritidis	+	Ⅱ
128	SM-38-KDE	Enteritidis	+	Ⅱ
129	SM-39-KDE	Enteritidis	++	Ⅱ
130	SM-41-KDE	Enteritidis	-	Ⅱ
131	SM-46-KDE	Enteritidis	+	Ⅱ
132	SM-49-KDE	Enteritidis	+	Ⅱ
133	SM-52-KDE	Enteritidis	+	Ⅱ
134	SM-53-KDE	Enteritidis	+	Ⅱ
135	SM-54-KDE	Enteritidis	+	Ⅱ
136	SM-55-KDE	Enteritidis	+	Ⅱ
137	SM-56-KDE	Indiana	+	Ⅱ
138	SM-57-KDE	Indiana	+	Ⅱ
139	SM-58-KDE	Typhimurium	-	Ⅱ
140	SM-61-KDE	Newport	++	Ⅱ
141	SM-SC-XT-1	Enteritidis	-	Ⅱ
142	SM-SC-XT-2	Enteritidis	-	Ⅱ
143	SM-SC-XT-3	Enteritidis	+	Ⅱ

144	SM-SC-XT-4	Enteritidis	+	Ⅱ
145	SM-SC-XT-5	Enteritidis	-	Ⅱ
146	SM-SC-XT-6	Enteritidis	-	Ⅱ
147	SM-SC-XT-7	Enteritidis	+	Ⅱ
148	SM-SC-XT-8	Enteritidis	+	Ⅱ
149	SM-SC-XT-9	Enteritidis	+	Ⅱ
150	SM-SC-XT-10	Enteritidis	+	Ⅱ
151	SM-SC-XT-11	Enteritidis	+	Ⅱ
152	SM-SC-XT-12	Enteritidis	-	Ⅱ
153	SM-SC-XT-13	Enteritidis	+	Ⅱ
154	SM-SC-XT-14	Enteritidis	-	Ⅱ
155	SM-SC-XT-15	Enteritidis	-	Ⅱ
156	SM-SC-XT-16	Enteritidis	-	Ⅱ
157	SM-SC-XT-17	Enteritidis	-	Ⅱ
158	SM-SC-XT-18	Enteritidis	-	Ⅱ
159	SM-SC-XT-19	Enteritidis	+	Ⅱ
160	SM-SC-XT-20	Enteritidis	-	Ⅱ
161	SM-SC-XT-21	Enteritidis	-	Ⅱ
162	SM-SC-XT-22	Enteritidis	-	Ⅱ
163	SM-SC-XT-23	Enteritidis	-	Ⅱ
164	SM-SC-XT-24	Enteritidis	-	Ⅱ
165	SM-SC-XT-25	Enteritidis	-	Ⅱ
166	SM-SC-XT-26	Enteritidis	-	Ⅱ
167	SM-SC-XT-27	Enteritidis	+	Ⅱ
168	SM-SC-XT-28	Enteritidis	+	Ⅱ
169	SM-SC-XT-29	Enteritidis	+	Ⅱ
170	SM-SC-XT-30	Enteritidis	-	Ⅱ
171	SM-SC-XT-31	Enteritidis	-	Ⅱ
172	SM-SC-XT-32	Enteritidis	+	Ⅱ
173	SM-SC-XT-33	Enteritidis	+	Ⅱ
174	SM-SC-XT-34	Enteritidis	+	Ⅱ
175	SM-SC-XT-35	Enteritidis	-	Ⅱ
176	SM-SC-XT-36	Enteritidis	+	Ⅱ
177	SM-SC-XT-37	Enteritidis	+	Ⅱ
178	SM-SC-XT-38	Enteritidis	+	Ⅱ
179	SM-90-1	Enteritidis	-	Ⅱ
180	SM-90-2	Enteritidis	-	Ⅱ

181	SC-81	Enteritidis	-	Ⅱ
182	SC-91	Typhimurium	++	Ⅱ
183	SC-131	Enteritidis	-	Ⅱ
184	L3	Enteritidis	-	Ⅱ
185	L5	Enteritidis	+	Ⅱ
186	L6	Enteritidis	+	Ⅱ
187	L7	Enteritidis	-	Ⅱ
188	L10	Enteritidis	-	Ⅱ
189	L11	Enteritidis	-	Ⅱ
190	L13	Enteritidis	+	Ⅱ
191	L20	Enteritidis	-	Ⅱ
192	L24	Enteritidis	-	Ⅱ
193	L35	Enteritidis	+	Ⅱ
194	L40	Enteritidis	+	Ⅱ
195	L43	Enteritidis	-	Ⅱ
196	L44	Enteritidis	+	Ⅱ
197	L56	Derby	-	Ⅱ
198	L80	Indiana	+	Ⅱ
199	L98	Indiana	+	Ⅱ
200	L333	Enteritidis	+++	Ⅱ
201	205	Enteritidis	+	Ⅱ
202	206	Enteritidis	+	Ⅱ
203	213	Enteritidis	-	Ⅱ
204	225	Indiana	+	Ⅱ
205	228	Enteritidis	-	Ⅱ
206	230	Indiana	+	Ⅱ
207	234	Enteritidis	-	Ⅱ
208	243	Indiana	-	Ⅱ
209	269	Enteritidis	+	Ⅱ
210	275	not belonging to A-f group	++	Ⅱ
211	283	Indiana	+	Ⅱ
212	300	Indiana	+	Ⅱ
213	307	Indiana	+	Ⅱ
214	312	Enteritidis	-	Ⅱ
215	315	Indiana	+	Ⅱ
216	324	Enteritidis	-	Ⅱ

217	325	Enteritidis	-	II
218	334	Enteritidis	+	II
219	342	Indiana	+	II
220	343	Indiana	+	II
221	354	Enteritidis	+	II
222	359	Indiana	+	II
223	360	Enteritidis	+	II
224	365	Enteritidis	-	II
225	404	Indiana	+	II
226	407	Typhimurium	-	II
227	410	Enteritidis	+	II
228	414	Indiana	+	II
229	415	Indiana	+	II
230	418	Enteritidis	+	II
231	420	Indiana	+	II
232	421	Enteritidis	+	II
233	431	Enteritidis	-	II
234	433	Enteritidis	+	II
235	450	Enteritidis	+	II
236	457	Enteritidis	-	II
237	460	Indiana	+	II
238	462	Indiana	+	II
239	470	Enteritidis	+	II
240	472	Enteritidis	+	II
241	505	Enteritidis	+	II
242	507	Enteritidis	+	II
243	508	Enteritidis	-	II
244	513	Enteritidis	+	II
245	515	Enteritidis	+	II
246	517	Enteritidis	++	II
247	521	Typhimurium	+	II
248	SPu-109	Pullorum	-	III
249	SPu-13	Pullorum	-	III
250	SPu-102	Pullorum	-	III
251	SPu-45	Pullorum	-	III
252	SPu-115	Pullorum	-	III
253	SPu-116	Pullorum	-	III

254	SPu-905	Pullorum	-	Ⅲ
255	SPu-27	Pullorum	-	Ⅲ
256	SPu-01	Pullorum	-	Ⅲ
257	SPu-49	Pullorum	-	Ⅲ
258	SPu-103	Pullorum	-	Ⅲ
259	SPu-85	Pullorum	-	Ⅲ
260	E2	Typhimurium	++	Ⅲ
261	E6	Typhimurium	+	Ⅲ
262	E10	Typhimurium	+	Ⅲ
263	E12	Typhimurium	+	Ⅲ
264	E16	Typhimurium	+	Ⅲ
265	E17	Anatum	+	Ⅲ
266	D9	London	+	Ⅲ
267	S3	Typhimurium	+	Ⅲ
268	S8	Typhimurium	+	Ⅲ
269	S9	Typhimurium	+	Ⅲ
270	S20	Enteritidis	+	Ⅲ
271	DHLM-1	Typhimurium	+	Ⅲ
272	DHLM-2	Typhimurium	+	Ⅲ
273	JKSen	Enteritidis	+	Ⅲ
274	C50041	Enteritidis	-	Ⅲ
275	CYR1	Enteritidis	+++	Ⅲ
276	CYR2	Enteritidis	+	Ⅲ
277	CYR3	Enteritidis	+	Ⅲ
278	CYR4	Enteritidis	+	Ⅲ
279	CYR5	Enteritidis	+	Ⅲ
280	ATCC13076	Enteritidis	+++	Ⅳ
281	ATCC13311	Typhimurium	-	Ⅳ
282	ATCC50073	Paratyphi A	-	Ⅳ
283	CVCC2184	Enteritidis	+	Ⅲ
284	CMCC533	Pullorum	-	Ⅲ
285	HNER027	Derby	+	Ⅴ
286	HNER121	Enteritidis	+	Ⅴ
287	HNER055	Derby	+	Ⅴ
288	HNER127	Derby	+	Ⅴ
289	HNER004	Derby	+	Ⅴ
290	HNER067-1	Derby	++	Ⅴ

291	HNER067-2	Derby	+	Ⅴ
292	HNER117	Derby	++	Ⅴ
293	HNER102	Derby	+	Ⅴ
294	HNER047	Derby	+	Ⅴ
295	HNER178	Typhimurium	+	Ⅴ
296	HNER086	Derby	++	Ⅴ
297	CDER157	O:10, H:1,w	-	Ⅴ
298	CDER160	Derby	+	Ⅴ
299	CDER188	Derby	+	Ⅴ
300	DBER023	Typhimurium	+	Ⅴ
301	DLJR01	Arizonae	-	Ⅴ
302	DBJR151	Enteritidis	+	Ⅴ
303	DBJR193-2	Derby	+	Ⅴ
304	DBJR236	Enteritidis	+	Ⅴ
305	1769	Derby	+	Ⅵ
306	1401	Pullorum	-	Ⅵ
307	1402	Pullorum	-	Ⅵ
308	1403	Pullorum	-	Ⅵ
309	1404	Pullorum	-	Ⅵ
310	1405	Pullorum	-	Ⅵ
311	1406	Pullorum	-	Ⅵ

Sources: I Guo-qiang Zhu (Yangzhou University, Yangzhou, China); II Yu-qing Liu (Shandong Academy of Agricultural Sciences, Jinan, China); III Lab stock; IV Guo-xiang Cao (Chinese Academy of Agricultural Science, Yangzhou, China); VYan-bin Zeng (Jiangxi Academy of Agricultural Sciences, Nanchang, China);VI Jian-sen Gong(Poultry institute, Chinese Academy of Agricultural Sciences, Yangzhou, China, Yangzhou, China)

[a] +++ , complete lysis; ++, lysis; +, turbid lysis; -, no plagues.

Table S2.2 Host range of temperate phage BP96115

No.	*Salmonella* strains	Serotype	Temperate phage BP96115 susceptibility[a]	Sources
1	S11-2	Pullorum	-	I
2	SPu-115	Pullorum	-	III
3	SPu-109	Pullorum	+++	III
4	SPu-01	Pullorum	-	III
5	S11-3	Pullorum	++	I
6	SPu-905	Pullorum	+	III
7	SPu-45	Pullorum	++	III
8	S11-5	Pullorum	-	I
9	SPu-13	Pullorum	-	III
10	SPu-103	Pullorum	-	III
11	SPu-116	Pullorum	+	III
12	SPu-27	Pullorum	++	III
13	SPu-85	Pullorum	-	III
14	CYR1	Enteritidis	-	III
15	CYR2	Enteritidis	-	III
16	CYR3	Enteritidis	-	III
17	CYR4	Enteritidis	-	III
18	CYR5	Enteritidis	-	III
19	D9	London	-	III
20	SPu-102	Pullorum	-	III
21	SPu-49	Pullorum	-	III
22	SPu-95	Pullorum	++	III

23	DHLM-2	Typhimurium	-	III
24	HNER 067-2	Derby	-	V
25	HNER 178	Typhimurium	++	V
26	SM-SC-XT-35	Enteritidis	++	II

The information of the bacteria is the same with the bacteria's in table S2.1.

Table S2.3 ORF features, database matches and functional assignments of coding sequences (cds) of the lytic phage PA13076 genome for which homologies (e-value < 0.01) to known proteins could be found

ORF#	Start	Stop	Strand	Predicted product	Organism, gene or product	Coverage	E value	Identity	Accession no	Predicted Domain
1	<2	106	-	Hypothetical protein BP63_38	*Salmonella* phage BP63	100%	3.00E-15	100%	YP_009302957.1	Uncharacterized protein
2	106	2682	-	Hypothetical protein BP63_36	*Salmonella* phage BP63	64%	0	98%	YP_009302955.1	Uncharacterized protein
3	2845	3273	-	Hypothetical protein BP63_34	*Salmonella* phage BP63	100%	.00E-99	100%	YP_009302953.1	Uncharacterized protein
4	3292	3723	-	Hypothetical protein BP63_33	*Salmonella* phage BP63	100%	5.00E-101	100%	YP_009302952.1	Uncharacterized protein
5	3735	4874	-	Hypothetical protein BP63_32	*Salmonella* phage BP63	100%	0	99%	YP_009302951.1	Uncharacterized protein
6	4961	7090	-	Cell wall/surface repeat-containing protein	*Salmonella* phage BP63	100%	0	98%	YP_009302950.1	Cell wall/surface repeat-containing protein
7	7102	7740	-	Ig domain protein group 2 domain protein	*Salmonella* phage BP63	100%	2.00E-139	99%	YP_009302949.1	Ig domain protein group 2 domain protein
8	7808	10765	-	Tail protein	Salmonella phage BP63	100%	0	99%	YP_009302948.1	Tail-associated protein
9	10765	11388	-	Putative tail fiber protein	*Salmonella* phage BP63	100%	1.00E-144	99%	YP_009302947.1	Putative tail fiber protein
10	11388	11696	-	Hypothetical protein BP63_27	*Salmonella* phage BP63	100%	7.00E-68	99%	YP_009302946.1	Uncharacterized protein
11	11696	11839	-	Unkown						leucine-rich repeat and death domain-containing protein 1-like
12	11839	12216	-	Hypothetical protein BP63_26	*Salmonella* phage BP63	100%	5.00E-90	100%	YP_009302945.1	Uncharacterized protein
13	12216	12704	-	Hypothetical protein BP63_25	*Salmonella* phage BP63	100%	2.00E-115	99%	YP_009302944.1	Uncharacterized protein
14	12704	13204	-	Hypothetical protein BP63_24	*Salmonella* phage BP63	100%	2.00E-116	97%	YP_009302943.1	Virion protein
15	13309	14286	-	Major capsid protein	*Salmonella* phage BP63	100%	0	99%	YP_009302942.1	Uncharacterized protein
16	14290	15003	-	Scaffold protein	*Salmonella* phage BP63	100%	3.00E-171	100%	YP_009302941.1	Scaffold protein
17	15181	16569	-	Portal protein	*Salmonella*	100%	0	100%	YP_009302	Portal protein

					phage BP63				940.1	
18	16745	18193	-	Terminase large subunit	*Salmonella* phage BP63	100%	0	99%	YP_009302939.1	Putative phage terminase large subunit
19	18197	18487	-	Hypothetical protein BP63_19	*Salmonella* phage BP63	100%	8.00E-62	99%	YP_009302938.1	no hits
20	18665	18955	-	Hypothetical protein BP63_18	*Salmonella* phage BP63	100%	1.00E-65	99%	YP_009302937.1	Uncharacterized protein
21	18955	19647	-	Fibrous sheath CABYR-binding protein	*Salmonella* phage BP63	100%	2.00E-158	91%	YP_009302936.1	fibrous sheath CABYR-binding protein
22	19644	20150	-	Thymidylate kinase	*Salmonella* phage BP63	100%	5.00E-120	99%	YP_009302935.1	thymidylate kinase
23	20147	20821	-	Hypothetical protein BP63_15	*Salmonella* phage BP63	100%	1.00E-164	98%	YP_009302934.1	PHIKZ004
24	20814	21299	-	Hypothetical protein BP63_14	*Salmonella* phage BP63	98%	1.00E-113	98%	YP_009302933.1	Uncharacterized protein
25	22121	22423	+	unknown						No hits
26	22519	23088	+	Hypothetical protein BP63_11	*Salmonella* phage BP63	99%	5.00E-117	93%	YP_009302930.1	Uncharacterized protein VR7ORF147c
27	23153	23311	+	Hypothetical protein BP63_10	*Salmonella* phage BP63	100%	2.00E-28	100%	YP_009302929.1	TPR repeat protein
28	23317	23574	+	Hypothetical protein BP63_09	*Salmonella* phage BP63	100%	3.00E-52	96%	YP_009302928.1	Uncharacterized protein (Fragment)
29	23571	23744	+	Hypothetical protein BP63_08	*Salmonella* phage BP63	100%	8.00E-32	100%	YP_009302927.1	Uncharacterized protein
30	23818	24207	+	Hypothetical protein BP63_07	*Salmonella* phage BP63	100%	2.00E-88	96%	YP_009302926.1	All3346 protein
31	24207	24407	+	Hypothetical protein BP63_06	*Salmonella* phage BP63	100%	3.00E-40	94%	YP_009302925.1	Uncharacterized protein
32	24520	24645	+	TPR repeat:Kinesin light chain	*Salmonella* phage BP63	100%	4.00E-19	93%	YP_009302924.1	TPR repeat:Kinesin light chain
33	24657	24824	+	Hypothetical protein BP63_04	*Salmonella* phage BP63	100%	8.00E-31	100%	YP_009302923.	Uncharacterized protein
34	24880	25059	+	Hypothetical protein BP63_03	*Salmonella* phage BP63	100%	2.00E-36	98%	YP_009302922.1	Predicted protein
35	25238	25669	-	Hypothetical protein BP63_01	*Salmonella* phage BP63	100%	3.00E-104	100%	YP_009302920.1	Uncharacterized protein
36	26149	28683	-	Primase	*Salmonella* phage BP63	100%	0	98%	YP_009302995.1	Putative primase/helicase
37	28658	28990	-	Hypothetical protein BP63_75	*Salmonella* phage BP63	100%	3.00E-75	100%	YP_009302994.1	Putative transcriptional regulator

38	29144	29581	+	Hypothetical protein BP63_74	*Salmonella* phage BP63	100%	3.00E-100	96%	YP_009302993.1	Unplaced genomic scaffold CY34scaffold_122, whole genome shotgun sequence (Fragment)
39	29863	30312	+	Putative DNA binding protein	*Salmonella* phage BP63	100%	3.00E-105	100%	YP_009302991.1	Putative DNA binding protein
40	30468	30659	+	Hypothetical protein BP63_71	*Salmonella* phage BP63	93%	3.00E-30	88%	YP_009302990.1	Uncharacterized protein
41	30750	31040	+	Hypothetical protein BP63_70	*Salmonella* phage BP63	100%	8.00E-62	97%	YP_009302989.1	Uncharacterized protein
42	31037	31327	+	Hypothetical protein BP63_69	*Salmonella* phage BP63	100%	3.00E-65	100%	YP_009302988.1	Uncharacterized protein
43	31442	32347	+	PAS domain S-box	*Salmonella* phage BP63	100%	0	99%	YP_009302987.1	PAS domain S-box
44	32396	32842	+	Hypothetical protein BP63_67	*Salmonella* phage BP63	100%	3.00E-105	99%	YP_009302986.1	Uncharacterized protein (Fragment)
45	32903	33133	+	Hypothetical protein BP63_66	*Salmonella* phage BP63	100%	5.00E-49	99%	YP_009302985.1	Regulator of chromosome condensation, RCC1
46	33133	34017	+	Nucleotide-binding protein	*Salmonella* phage BP63	100%	0	98%	YP_009302984.1	Phage nucleotide-binding protein
47	34125	34670	+	Hypothetical protein BP63_64	*Salmonella* phage BP63	100%	1.00E-128	98%	YP_009302983.1	Uncharacterized protein
48	34732	35217	+	Deoxycytidylate deaminase	*Salmonella* phage BP63	100%	5.00E-116	99%	YP_009302982.1	Uncharacterized protein
49	35201	36079	+	Hypothetical protein BP63_62	*Salmonella* phage BP63	100%	0	99%	YP_009302981.1	Exonuclease
50	36138	37808	+	DEAD box helicase	*Salmonella* phage BP63	100%	0	99%	YP_009302980.1	Superfamily II DNA/RNA helicase
51	38123	38590	+	Hypothetical protein BP63_59	*Salmonella* phage BP63	100%	2.00E-101	100%	YP_009302978.1	50S ribosomal protein L19
52	39268	39666	+	Hypothetical protein BP63_57	*Salmonella* phage BP63	100%	2.00E-91	98%	YP_009302976.1	No hits
53	39653	40246	+	Hypothetical protein BP63_56	*Salmonella* phage BP63	100%	2.00E-144	99%	YP_009302975.1	Uncharacterized protein
54	40246	41130	+	Thymidylate synthase	*Salmonella* phage BP63	100%	0	99%	YP_009302974.1	Thymidylate synthase
55	41161	42156	+	DNA polymerase beta subunit	*Salmonella* phage BP63	100%	0	99%	YP_009302973.1	DNA polymerase beta subunit
56	42156	44123	+	DNA polymerase	*Salmonella*	100%	0	99%	YP_009302	DNA polymerase A

					phage BP63				972.1	
57	44139	44414	+	Hypothetical protein BP63_52	*Salmonella* phage BP63	100%	1.00E-56	97%	YP_009302971.1	Uncharacterized protein
58	44414	44638	+	Zinc finger protein 729	*Salmonella* phage BP63	100%	9.00E-46	97%	YP_009302970.1	Zinc finger protein 729 (Fragment)
59	44856	45185	-	Hypothetical protein BP63_49	*Salmonella* phage BP63	100%	1.00E-68	96%	YP_009302968.1	Uncharacterized protein
60	45164	45670	-	Lysozyme	*Salmonella* phage BP63	100%	2.00E-123	99%	YP_009302967.	Lysozyme
61	45684	45944	-	Hypothetical protein BP63_47	*Salmonella* phage BP63	100%	1.00E-54	100%	YP_009302966.1	Uncharacterized protein
62	46047	46550	-	Putative tail fiber assembly protein	*Salmonella* phage BP63	100%	2.00E-118	98%	YP_009302965.1	Putative tail fiber assembly protein
63	46554	47096	-	Putative tail fiber assembly protein	*Salmonella* phage BP63	100%	1.00E-131	99%	YP_009302964.1	Putative tail fiber assembly protein
64	47106	48440	-	Phage tail fiber protein	*Salmonella* phage BP63	100%	0	99%	YP_009302963.1	Phage tail fiber protein
65	48433	49086	-	Hypothetical protein BP63_43	*Salmonella* phage BP63	100%	2.00E-156	99%	YP_009302962.1	Uncharacterized protein
66	49079	50251	-	Hypothetical protein BP63_42	*Salmonella* phage BP63	100%	0	99%	YP_009302961.1	Putative bacteriophage protein
67	50251	50622	-	Hypothetical protein BP63_41	*Salmonella* phage BP63	100%	5.00E-86	100%	YP_009302960.1	Uncharacterized protein
68	50633	51280	-	Hypothetical protein BP63_40	*Salmonella* phage BP63	100%	2.00E-158	100%	YP_009302959.1	Uncharacterized protein
69	51280	52260	-	Hypothetical protein BP63_39	*Salmonella* phage BP63	97%	0	99%	YP_009302958.1	Uncharacterized protein

Table S2.4 The ORFs characteristics of temperate phage BP96115, functional assignments of coding sequences (cds) for which homologies (e-value< 0.01) to known proteins could be found

ORF#	start	stop	Strand	Predicted functions	Organism, gene or product	E value	identity	Accession no	Predicted domains
1	1	108	+	Orf232	*Salmonella* phage ST64T	7.00E-08	86%	AAL15494.1	serine-rich adhesin for platelets-like
2	246	440	-	Ral	*Salmonella* virus P22	2.00E-37	97%	NP_059603.1	Ral
3	681	1241	+	Superinfection exclusion protein B	*Salmonella* phage SPN9CC	6.00E-113	95%	YP_006383855.1	Superinfection exclusion protein B
4	1254	1556	-	Gp24	Enterobacteria phage ST104	7.00E-69	100%	YP_006377.1	Probable regulatory protein N
5	1920	2123	+	unknown					Uncharacterized protein
6	2162	3205	-	ORF23	Enterobacteria phage ST104	0	100%	YP_006378.1	ORF23
7	3406	4095	-	CI	Enterobacteria phage ST104	2.00E-169	100%	YP_006379.1	CI
8	4206	4421	+	cro	*Escherichia* phage HK639	4.00E-23	74%	YP_004934081.1	cro
9	4532	4813	+	transcriptional activator	*Salmonella* phage ST64T	5.00E-63	100%	NP_720301.1	C1
10	4848	5009	+	Gp53	*Escherichia* virus HK97	1.00E-31	100%	NP_037738.1	Uncharacterized protein
11	4996	5817	+	18, partial	Enterobacteria phage ST104	0	100%	YP_006383.1	DNA replication protein O
12	5814	7190	+	Gp55	Escherichia virus HK97	0	97%	NP_037740.1	DnaB helicase
13	7187	7456	+	ORF30	Enterobacteria phage ST104	6.00E-62	100%	YP_006385.1	Uncharacterized protein
14	7453	7533	+	NinA	Enterobacteria phage UAB_Phi20	8.00E-10	100%	YP_009279781.1	NinA protein
15	7530	7967	+	NinB	*Salmonella* virus P22	6.00E-101	99%	NP_059612.1	NinB protein
16	8283	8624	+	NinX	Enterobacteria phage ST104	3.00E-82	100%	YP_006389.1	NinX protein
17	8617	8793	+	NinF	Enterobacteria phage ST104	2.00E-36	100%	YP_006390.1	NinF protein
18	8786	9397	+	NinG protein	Enterobacteria phage HK446	1.00E-145	99%	YP_007112004.1	NinG protein

19	9394	9618	+	NinY	Enterobacteria phage ST104	4.00E-51	100%	YP_006392.1	NinY protein
20	9799	9978	+	NinZ	Enterobacteria phage ST104	4.00E-37	100%	YP_006394.1	NinZ protein
21	9975	10748	+	late gene regulator Q	Enterobacteria phage HK225	6.00E-90	66%	YP_007112198.1	Antitermination protein
22	11179	11382	+	Gp13	Enterobacteria phage ST104	4.00E-43	100%	YP_006396.1	Class II holin
23	11387	11857	+	Lysozyme	*Salmonella* enterica bacteriophage SE1	5.00E-113	100%	YP_002455881.1	Lysozyme
24	11854	12321	+	endopeptidase	Enterobacteria phage HK620	1.00E-94	92%	NP_112070.1	Rz lysis protein
25	12534	13064	+	ORF45	Enterobacteria phage ST104	2.00E-129	100%	YP_006400.1	Uncharacterized protein
26	13287	13529	+	ORF46	Enterobacteria phage ST104	1.00E-48	100%	YP_006401.1	Uncharacterized protein
27	13533	13922	+	ORF47	Enterobacteria phage ST104	6.00E-95	100%	YP_006402.1	Uncharacterized protein
28	13922	14326	+	ORF48	Enterobacteria phage ST104	1.00E-93	100%	YP_006403.1	Phage Tail Collar Domain
29	14330	14818	+	terminase small subunit	*Salmonella* phage ST64T	2.00E-119	100%	NP_720325.1	Terminase, small subunit
30	14796	16295	+	terminase large subunit	*Salmonella* phage ST64T	0	99%	NP_720326.1	Terminase, large subunit
31	16295	18472	+	portal protein	*Salmonella* phage SEN22	0	99%	YP_009191457.1	Portal protein
32	18486	19397	+	Scaffolding protein	*Salmonella* phage c341	0	99%	YP_003090221.1	Scaffolding protein
33	19397	20689	+	capsid protein	*Salmonella* phage SEN22	0	100%	YP_009191459.1	Major capsid protein
34	20730	21290	+	ORF54	Enterobacteria phage ST104	2.00E-135	100%	YP_006409.1	Uncharacterized protein
35	21274	21774	+	head completion protein	*Salmonella* phage vB_SemP_Emek	8.00E-122	98%	YP_006560557.1	Peptidoglycan hydrolase gp4
36	21734	23152	+	Tail accessory protein	*Salmonella* phage epsilon34	0	100%	YP_002533467.1	Packaged DNA stabilization protein gp10
37	23156	23857	+	26	Enterobacteria phage ST104	1.00E-166	100%	YP_006413.1	Tail needle protein gp26
38	23857	24312	+	14	Enterobacteria phage ST104	1.00E-111	100%	YP_006414.1	Head assembly protein
39	24315	25004	+	7	Enterobacteria phage ST104	7.00E-157	99%	YP_006415.1	DNA transfer protein

40	25015	26451	+	DNA transfer protein	*Salmonella* phage ST64T	5.00E-161	71%	NP_720336.1	DNA transfer protein
41	26451	28427	+	DNA transfer protein	*Salmonella* phage ST64T	0	100%	NP_720337.1	DNA transfer protein
42	28880	29128	-	regulatory protein	*Salmonella* phage ST64T	2.00E-53	100%	NP_720338.1	Transcriptional repressor Mnt
43	29264	31267	+	tail protein	*Salmonella* phage A1	0	100%	AAY43004.1	Tailspike protein
44	31326	32783	-	O-antigen conversion protein C	Enterobacteria phage ST64T	0	100%	NP_720275.1	GtrC
45	32773	33705	-	O-antigen conversion protein B	*Salmonella* phage ST64T	0	100%	NP_720276.1	GtrB
46	33702	34064	-	bactoprenol-linked glucose translocase	*Cronobacter* phage ENT47670	7.00E-58	89%	YP_007237579.1	Bactoprenol-linked glucose translocase
47	34413	35576	-	integrase	Enterobacteria phage UAB_Phi20	0	99%	YP_009279828.1	Integrase
48	35806	35943	-	formate dehydrogenase N alpha subunit	*Salmonella* phage SEN22	3.00E-23	100%	YP_009191477.2	Tetratricopeptide TPR_4
49	36015	36299	-	phage-related protein	*Escherichia* phage TL-2011a	2.00E-62	99%	AEW24508.1	Uncharacterized protein
50	36292	36576	-	hypothetical protein ECRS218_0060	Enterobacteria phage CUS-3	2.00E-61	96%	ABQ88440.1	Uncharacterized protein
51	36576	37367	-	ORF8	Enterobacteria phage ST104	0	100%	YP_006364.1	Uncharacterized protein
52	37437	37946	-	ORF9	Enterobacteria phage ST104	1.00E-121	100%	YP_006365.1	Eae protein
53	37943	38113	-	ORF10	Enterobacteria phage ST104	1.00E-35	100%	YP_006366.1	Uncharacterized protein
54	38124	38417	-	Abc2	Enterobacteria phage ST104	4.00E-66	100%	YP_006367.1	Anti-RecBCD protein 2
55	38464	38748	-	Abc1	*Salmonella* virus P22	8.00E-64	99%	NP_059595.1	Anti-RecBCD protein 1
56	38748	39455	-	Erf	*Salmonella* phage vB_SemP_Emek	2.00E-173	99%	YP_006560590.1	Erf
57	39452	39595	-	Arf protein	*Salmonella* phage vB_SosS_Oslo	5.00E-25	96%	YP_006560849.1	Arf
58	39585	39773	-	Kil	Enterobacteria phage ST104	6.00E-41	100%	YP_006370.1	Kil
59	39997	40311	-	protein gp17	Enterobacteria	1.00E-64	94%	AAD04642.1	Superinfection

					phage H-19B				exclusion protein
60	40462	40587	-	Enterobacter protein of unknown function	*Salmonella* phage c341	7.00E-21	100%	YP_003090253.1	Uncharacterized protein
61	40587	40874	-	ORF18	Enterobacteria phage ST104	2.00E-66	100%	YP_006373.1	Uncharacterized protein
62	40908	41264	-	Orf-232	*Salmonella* phage ST64T	4.00E-73	94%	AAL15494.1	Uncharacterized protein