INVESTIGATING THE EFFECT OF PHAGE THERAPY ON THE GUT MICROBIOME OF GNOTOBIOTIC ASF MICE

INVESTIGATING THE EFFECT OF PHAGE THERAPY ON THE GUT MICROBIOME OF GNOTOBIOTIC ASF MICE

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Lay Abstract

Bacteriophages are viruses that infect bacteria. After their discovery in 1917, bacteriophages were a primary cure against infectious disease for 25 years, before being completely overshadowed by antibiotics. With the rise of antibiotic resistance, bacteriophages are being explored again for their antibacterial activity. One of the critical apprehensions regarding bacteriophage therapy is the possible perturbations to our microbiota. We set out to explore this concern using a simplified microbiome model, namely germ-free mice inoculated with only 8 bacteria plus a mock infection challenged with bacteriophage. We monitored this model for 9 weeks and isolated a collection of phage-resistant bacterial mutants from the mouse gut that developed post phage challenge, maintaining the community of mock infection inside the gut. A single dose of lytic phage challenge effectively decreased the mock infection without causing any extreme long-term perturbations to the gut microbiota.

Abstract

Mounting concerns about drug-resistant pathogenic bacteria have rekindled the interest in bacteriophages (bacterial viruses). As bacteria's natural predators, bacteriophages offer a critical advantage over antibiotics, namely that they can be highly specific. This means that phage therapeutics can be designed to destroy only the infectious agent(s), without causing any harm to our microbiota. However, the potential secondary effects on the balance of microbiota through bacteriophage-induced genome evolution remains as one of the critical apprehensions regarding phage therapy. There exists a significant gap in knowledge regarding the direct and indirect effect of phage therapeutics on the microbiota. The aim of this thesis was to: (1) establish an *in vivo* model for investigation of the evolutionary dynamics and co-evolution of therapeutic phage and its corresponding host bacterium in the gut; (2) determine if phage therapy can affect the composition of the gut microbiota, (3) observe the differences of phage-resistant bacteria mutants evolved *in vivo* in comparison to those evolved *in vitro*. We used germ-free mice colonized with a consortium of eight known bacteria, known as the altered Schaedler flora (ASF). The colonizing strain of choice (mock infection) was a non-pathogenic strain *E. coli* K-12 (JM83) known to co-colonize the ASF model, which was challenged *in vivo* with T7 phage (strictly lytic). We compared the composition of the gut microbiota with that of mice not subject to phage therapy. Furthermore, the resistant mutants evolved *in vivo* and *in vitro* were characterized in terms of growth fitness and motility.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Zeinab Hosseini-Doust, for taking me on as one of her first graduate students and for teaching me what true passion and learning through the ups and downs of scientific research, is about in the bigger picture. Thank you for having me on your team, for giving me the support, mentorship, space and resources to learn and complete my research, challenging me to think harder, read more, do better, and for letting me experience the initial steps in the journey of your laboratory.

I am grateful for my lab mates in the Biohybrids Laboratory for all their advice, company, support, and for sharing this journey with me. It is a blessing to be a part of an inspiring lab group. Together, the flame is always lit in our lab, not the Bunsen burner flame – but the flame of the group's constant desire to learn and to ensure the lab environment is always a fun, loving, and welcoming space.

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King of Plate Pouring. Paul, I thank you for always challenging me to do better, and for all your help with our *in vitro* experiments. Thank you Sina and Paul for your friendship, your support, our shared meals at the most unconventional of times, and all of the fun times we have had together. You are both brilliant young minds and have incredible journeys ahead of you.

To my family and closest friends, thank you. Thank you for your constant support and encouragement to be strong, overcome any challenge, pursue any desire, and remain grounded. Each one of you are my source of strength, and constant reminder that it is not what I have in my life but who I have in my life, and who we help along our journey, that counts. I will never be able to articulate in words my gratitude for being blessed to have such strong and loving individuals in my life. I love you all very much.

"The highest education is that which does not merely give us information but makes our life in harmony with all existence"

Rabindranath Tagore

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List of Abbreviations and Symbols

AREB	Animal Research Ethics Board
ASF	Altered Schaedler Flora
bp	base pairs
c	Cytosine
$CaCO_3$	calcium carbonate
CF	Cystic Fibrosis
CFU	colony forming units
CMCB	Centre for Microbial Chemical Biology
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
EMA	European Medicines Agency
EtOH	Ethanol
FDA	Food and Drug Administration
G1	Group 1
G2	Group 2
G3	Group 3
GI	gastrointestinal
GES	guanidine thiocyanate buffer

h	hours
HIV	Human immunodeficiency virus
IBD	Inflammatory Bowel Disease
IL-6	interleukin
LB	Lysogeny Broth
LTF	long tail fibres
M	molarity
Mac	MacConkey Agar
MacStrep	MacConkey Agar with Streptomycin
MDR	Multidrug resistant
min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
n	number
$NaPO_4$	sodium phosphate
nm	nanometer
nm	nanometer

OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PFU	plaque forming units
phage	Bacteriophage
rcf	relative centrifugal force
RNA	ribonucleic acid
rpm	revolutions per minute
sec	seconds
SEM	scanning electron microscopy
SI	Supporting Information
ssDNA	single stranded deoxyribonucleic acid
ssRNA	single stranded ribonucleic acid
Strep	Streptomycin
T	Thymine
t	time
TEM	transmission electron microscopy
TNF	tumor necrosis factor

TSB	tryptic soy broth
TSB	Tryptic Soy Broth
UC	University of California
U. S	United States
UTI	Urinary tract infection
µL	microliter
um	micrometer
vol	volume
w/v	weight per volume
x g	relative centrifugal force
%	percent
°	degrees

Declaration of Academic Achievement

Sharita Ganeshan completed the majority of experiments, data collection, and analysis presented in this thesis. The first revision of this report was also prepared by Sharita.

Dr. Zeinab Hosseini-Doust (supervisor), provided guidance, direction, and support in the designing of all experiments throughout the project, as well for the compilation of the manuscripts and this thesis. Dr. Michael Surette and Dr. Elena Verdu provided expertise regarding mouse husbandry and handling of ASF mouse samples.

Media preparation, mutant colony picking, and replica pinning for diversification characterization experiments were performed by Undergraduate Research Assistant, Sina Ghasempour.

Heather Galipeau, Dr. Elena Verdu's Laboratory Technician, collected fecal samples from each mice every week in the gnotobiotic facility of the McMaster Central Animal Facility (CAF).

Laura Rossi and Michelle Shah from the Farncombe Institute Surette Lab, DNA from each submitted fecal sample. Samples were then processed for 16S Sequencing in the McMaster Genome Facility.

Mouse inoculum preparations, preparation and processing of fecal samples, endpoint mouse sacrifice, processing, and analysis, *in vitro* anaerobic experiments, and data analysis were performed by Sharita.

Chapter 1- Introduction

1. Introduction to Phage Therapy

1.1. Phage Biology

Bacteriophages (phages) are viruses of bacteria. With an estimated 10^{32} phages, these are the most ubiquitous and diversified biological group residing on Earth.[1] As a result of their obligate requirement of a bacterial host, bacteriophages are abundantly found distributed essentially anywhere their host exists in the biosphere- with ten to hundred million phages in every gram of soil,[2] water,[3] and billions on the human body at any moment.[4] Bacteriophages are diverse in their complexity, structure, genetic material, and are variant in shapes and sizes (tailed, filamentous, and icosahedral, **Figure 1-1**).[5] Phage virions generally consist of a protein envelope (sometimes containing lipids) encapsulating encoded genomes- consisting between two to hundreds of kilo base pairs of single or double stranded DNA or RNA.[6] Phage can be effectively visualized with electron microscopy, specifically, Transmission Electron Microscopy (TEM) (**Figure 1-2 A**).[7]

Phages are more genetically diverse than their bacterial hosts (and prey), however these bacterial viruses only infect a narrow range of bacteria that are closely related due to a combination of various factors. Such limiting factors include the shape and size of the virion, the specificity of the virion's host binding proteins, biochemical interactions during infection, the presence of related prophages or particular plasmids, and bacterial resistance mechanisms to phage (its predator).[8-10]

Bacteriophages are classified via a structural and sequence-based taxonomic system; initially into families, and each family is further categorized in accordance to the capsid structure containing the genome, the structural and chemical composition of the genes and then the mechanism of their mRNA production (**Table 1**).[11,12] These viruses are further broadly categorized in terms of their propagation cycle as lytic, temperate, and chronic phages, regardless of their detailed diversification.[13] Regardless of the nature of propagation cycle, bacteriophages first have to bind to specific sites on the host cell surface (**Figure 1-2 B**). Lytic phages bind and adsorb to the host cell surface, inducing lysis of the host, and further releasing progeny phages (**Figure 1-2 C,D**).[14] Temperate viruses typically do not immediately "kill" the host bacteria; instead, they integrate their genome into the host chromosome, amplifying with every bacteria reproduction cycle; this embedded genome (known as a prophage) can be expelled from the genome host bacteria through the lytic cycle once induced (**Figure 1.2 D**).[14] It is not clear what causes the induction of temperate

phages, but most factors that stress the host cell or cause DNA damage have been shown to induce temperate phage into a lytic cycle.[15,16] This lytic-lysogeny switch (which came into spotlight with phage λ) has been the topic of research for decades.[17,18] There is a third class of phage, known as chronic phage, which do not lyse the cell or integrate their genome into the host genome, but instead use the host a continuous phage-making factory; filamentous phage such as M13 belong to this class.[13,19]

Phage therapy exploits the natural ability of bacteriophages to target and kill host bacteria with high specificity, to treat bacterial infections. For phage therapy, only strictly lytic phage are of immediate of interest.[20] Although, there are strong arguments to be made regarding the use of temperate phage for therapeutic purposes,[21] regulatory agencies have a long way to go before accepting the use of viruses that can transfer their genes directly to bacterial cells in the body.[22]

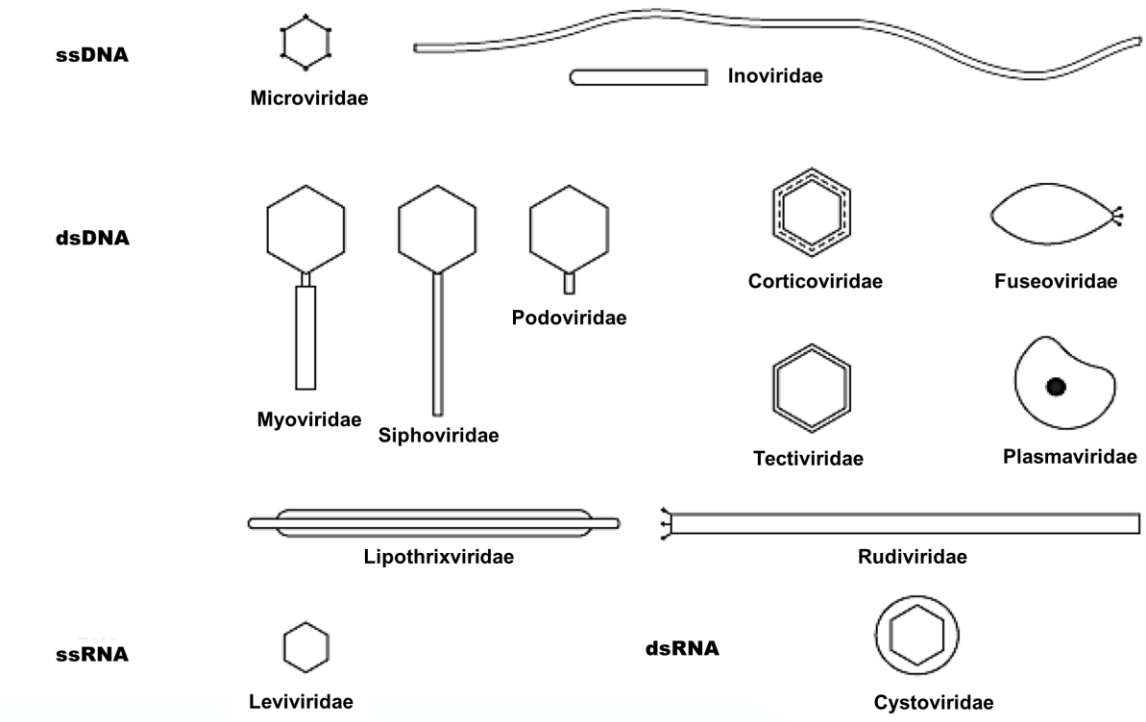

Figure 1-1: *Schematic Representation of Major Groups of Bacteriophages*[23]

Table 1-1: *Classification and Basic Properties of Bacteriophages* [24]

SYMMETRY	NUCLEIC ACID	ORDER AND FAMILIES	GENERA	MEMBERS	PARTICULARS
Binary (tailed)	DNA, ds, L	*Caudovirales*	15	4950	
		Myoviridae	6	1243	Tail contractile
		Siphoviridae	6	3011	Tail long, noncontractile
		Podoviridae	3	696	Tail short
Cubic	DNA, ss, C	*Microviridae*	4	40	
	ds, C, T	*Corticoviridae*	1	3?	Complex capsid, lipids
	ds, L	*Tectiviridae*	1	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	2	39	
	ds, L, S	*Cystoviridae*	1	1	Envelope, lipids
Helical	DNA, ss, C	*Inoviridae*	2	57	Filaments or rods
	ds, L	*Lipothrixviridae*	1	6?	Envelope, lipids
	ds, L	*Rudiviridae*	1	2	Resembles TMV
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	1	6	Envelope, lipids, no capsid
	ds, C, T	*Fuselloviridae*	1	8?	Spindle-shaped, no capsid

In my research, I used bacteriophage T7, a heavily studied bacteriophage belonging to the Podoviridae family that has an icosahedral capsid head approximately 60-61nm in diameter, a short noncontractile tail that is 23nm in length, and contains a dsDNA genome of 39,937 bp (the third phage genome to be ever sequenced).[6] As T7, an Enterobacteria phage, attaches to specific LPS receptors, as well as in specific- OmpA and OmpF proteins on the bacterial cell wall , multiple internal capsid proteins are sent into the host cell wall to construct an ejectosome from the tail of the phage and induce a pore within the bacterial cell wall (**Figure 1.2 E**). This then permits DNA from the capsid of the phage to translocate into the cell, and in turn initiates the process to replicate page DNA within the host.[25]

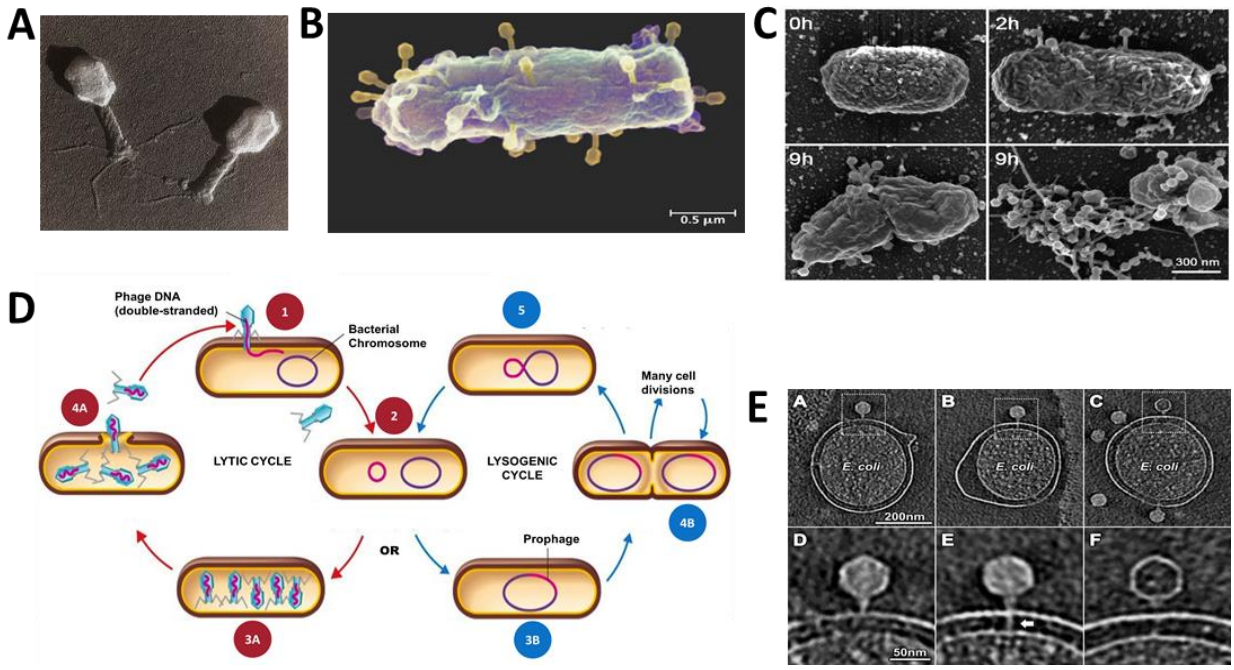

Figure 1-2: An Introduction to Lytic Bacteriophage Biology

(A) *A shadowed Transition Electron Micrograph (TEM) image of T4 phage (Mag 380,000×). This phage, a member in the Myoviridae family of the Caudovirales order, is one of the seven Escherichia coli phages (T1-T7) in this family. This image shows the icosahedral capsid head containing the genetic material, the contractile tail, and the long tail fibers of the phage. T4 head is approximately 90 nm wide and the virion is 200nm in length. This TEM was photographed by M. Wurtzbiozentrum in the University of Basel.*[26,27] ***(B)*** *A colorized scanning electron micrograph (SEM) image of multipleT4 bacteriophage infecting an Escherichia coli bacterium.*[28] ***(C)*** *SEM images at different stages showing the infection of Synechococcus WH8102 by the S-TIM5 phage. 0h- uninfected cells. 2h-phage adsorption. 9h-cell lysis. 9h-viral release. These SEM images were collected from Sabehi. G., from the Israel Institute of Technology.*[29] ***(D)*** *The Lytic and Lysogenic infection cycles. The first two stages are common for both the cycles. Step 1- Attachment of absorption of the phage's tail fibres to a specific receptor site on the bacterial cell wall and injection of viral genome. Step 2- Phage DNA then circularized and enters the lytic cycle or the lysogenic cycle. Lytic cycle: Step 3- Synthesis of new viral proteins within the host. Step 4- Virions are liberated as mature phages upon cell lysis. Lysogenic cycle: Step 3- Phage DNA integrates within the bacterial chromosome by recombination, in turn becoming a prophage. Step 4- Lysogenic bacterium reproduces normally and has the potential to do so over many cell divisions. Step 5- the prophage may be liberated from the bacterial chromosome by another recombination event, resulting in initiation of the lytic cycle.*[14] ***(E)*** *T7 bacteriophage infecting Escherichia coli as seen via cryo electron tomography at ~4nm resolution. A/D- Adsorption of T7 phage into the outer membrane. B /E- Injection of the extended tail into the cell envelope. C/F- DNA ejection. These images are collected from Bo Hu, University of Texas.*[25]

1.2. History of Phage Therapy

Frederick Twort, an English bacteriologist first reported evidence of bacteriophages (lysis of bacterial cultures) in 1915 and suggested the effect might be due to the presence of a virus or an enzyme.[30] In 1917 Felix d'Herelle, a French-Canadian, and a self-taught microbiologist working at Institut Pasteur, independently made the same observations, but he was quick to attribute the effect to a virus and name these microbes "bacteriophages" or bacteria-eaters; he was the first to utilize phages as antimicrobial agents.[31,32] The discovery of phages played a crucial role to the development and understanding of further scientific discoveries, including the initiation of understanding the structure of DNA,[33] and currently in the development of new clusters of regularly interspaces short palindromic repeat and associated genes (CRISPR/Cas) system.[34]

Phage therapy was actively utilized, immediately by d'Herelle and later by others,[35] for antibacterial treatment years prior to the introduction of antibiotics in the 1940s, when infectious diseases were the leading cause of mortality and morbidity within human populations.[36,37] Antibiotics quickly overshadowed the use of phage therapy in western medicine due to the lack of understanding in phage biology, exaggerated claims, a lack of controlled trials, poor documentation, and politics.[38] This in turn sparked scientific controversy about the treatment approach,[39,40] rendering phage therapy essentially obsolete in Western medicine for close to four decades. Due to the occurrence of World War II, certain regions of the Soviet Union and Eastern Europe had limited access to antibiotics, and thus focused on developing phage therapy.[41] The utilization of this therapy within the Soviet Union was well supported and is in fact still heavily practiced in the Eastern European countries for over 80 years, especially within the Eliava Institute in Tbilisi Georgia, co-established by Felix d'Herelle himself.[42,43]

1.3. Antibiotic Therapy versus Phage Therapy

As previously noted, the 1940s brought upon a golden era for the utilization of antibiotics as antimicrobial treatments. Penicillin was not immediately utilized as an antibiotic upon its serendipitous discovery in 1928.[37] However, it was quickly guided into optimization due to the crucial necessity to treat sick or wounded soldiers in the US and Allies' military forces during the war.[44] Antibiotics were discovered at a time where were bacterial infections had high mortality rate; antibiotics were highly attractive due to their broad-spectrum activity allowing them to be used against a wide range of bacteria without necessarily identifying or characterizing the exact infective bacterium. However, this main advantage, is also a great disadvantage. Due to its non-

specific mode of action, antibiotics also destroy the commensal microflora especially within the intestine. This is further associated with side-effects such as intestinal problems, or secondary infection, such as *C. difficile* infection.[45] Furthermore, secondary side-effects are often of occurrence as antibiotics are also mostly needed in repeated administrations.[46] Using lytic phage, however, could circumvent this issue, because bacteriophages are very specific to their host. This means that phage therapeutics could be designed to kill an infection but not harm the microbiota. This specificity could, however, become a challenge because many infections are polymicrobial. This will require phage therapeutics to be cocktails of different phages rather than a pure stock.[47] The non-essential overuse and abuse of antibiotics (in clinics, aquaculture, and agriculture) has fueled the volatile era of antibiotic resistance globally. Antibiotic resistance is currently one of the leading causes of death. It is approximated by the year 2050, we will have had 10 million deaths per year, with more people dying from antibiotic resistance than currently die from cancer.[48] As a consequence of this current dangerous state of infective antibiotics and antibiotic resistance, interest in resurfacing phage therapy as an antimicrobial strategy against lethal pathogens.

1.4. Advantages and Challenges of Phage Therapy

Phage therapy is not a fully established alternative to antibiotics as an antibacterial therapeutic. As phage therapy is in vogue right now as a potential alternative/adjuvant, it is imperative to carefully asses all possible characteristics, parameters, and limitations. Some advantages of phage therapy over antibiotics include:

- Specificity: Bacteriophages are generally very specific in their host range, which means less harm to our microbiota. However, for most clinically relevant infections, a mixture of different phage will have to be used.[47]
- bactericidal versus bacteriostatic: lytic phages infect their target host bacteria and cause cell death, in comparison to certain bacteriostatic antibiotics.[49,50]
- Active on-site propagation: bacteriophages increase in concentration *in situ* as they propagate in the presence of appropriate and enough bacterial densities. Unlike antibiotics which often require frequent doses to efficiently kill the bacteria, only one bacteriophage is theoretically needed to target a single corresponding host bacterium (single-hit kinetics).[51] It is possible to administer a single low- dose of phage, which will then propagate itself given the existing bacterial density as an active therapy, resulting in continued bacterial adsorption and killing.[52]

- Low inherent toxicity: bacteriophages are primarily composed of nucleic acid and proteins that have been studied to be non-toxic with the use of highly purified phage preparations.[53]
- Rapid discovery of phages: isolating and purifying phages necessary to target a known pathogenic bacteria can easily be completed, as phage is found in diverse abundances across the biosphere.[54]
- Formulation and application versatility: Multiple strains of phages can be added together into a "phage cocktail" in order to target multiple bacteria of interest with a broader killing spectrum.[55] Furthermore, mode of administration (liquid, ointment,[56] powder,[57] oral tablet[58]) can also vary in accordance to each unique situation.
- Narrow potential for antibiotic cross-resistance: because of narrow host range for each bacteriophage, the number of bacteria that the phage interacts with is also limited. Therefore, there is a narrower selection of bacteria that are vulnerable to develop both phage and antibiotic resistance. Furthermore, the mechanism of bacterial resistance to antibiotics and phage are entirely different.[48,59] Thus, bacteria that are resistant to antibiotics may be targeted and treated with the use of phage therapy, and *vise-versa*, presenting combination therapies as an attractive strategy.[60]
- Biofilm clearance: phages have been demonstrated to lyse and penetrate through some biofilms that have shown resistance to antibiotics.[61] This is partially attributed to the presence or depolymerases and lysins that can chew through the biofilm extracellular polymeric matrix.[62,63]
- Low environmental impact: bacteriophages are natural components of the environment that can be naturally evolved (as opposed to genetic modification), thus easing public acceptance of Phage therapy. Furthermore, as this natural product is composed primarily of proteins and nucleic acids, unused phage materials can easily be inactivated and discarded.[49]
- Relatively low discovery and production cost: the costs associated with the discovery, isolation, and purification of bacteriophages are significantly decreasing with the progression of screening and sequencing technologies.[64,65]

Regardless of the numerous known advantages of bacteriophages employed for phage therapy thus far, there are still potential limitations to this approach that must be addressed and studied further. In particular, there are currently four primary concerns:

- Phage selection: The criteria for selection of therapeutic phage is not well determined. Most reports to date have focused on phage host range, but the effect of phage ability to infect stationary phase bacteria (most infections in the body are in this mode),[66] phage enzymes, stability to serum inactivation, mutation rate cannot be dismissed and deserve more investigation.[54,67]
- Phage host-range: each phage has a narrow host-range, which limits exactly what strain of bacteria can be infected. Thus it is imperative to employ phage cocktails, curated from a combination of multiple selected phages to develop a broader host spectrum.[68]
- Phages as drivers of evolution: unlike common pharmaceuticals, bacteriophages are DNA/RNA containing protein-based biological viruses, that have the potential to interact with the body's immune system, other cells in the body and the active ability to replicate and evolve within the body. This evolution can, in turn, result in the evolution of the host bacterial communities.[69]
- Public acceptance: there is still associated unfamiliarity and unacceptance with the use of a "virus" as an antibacterial agent. Viruses within the public eye are typically associated with viral pathogens causing harm and thus it is imperative to diminish this notion.[70]

Even though phage therapy was employed in western medicine before antibiotics took over, it currently does not have the approval for human administration from the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) due to an increase in regulatory standards. To obtain this approvals, current research is heavily exploring the roadblocks, including numerous animal studies and clinical trials.[71-73] The FDA has, however, approved the use of phages for food decontamination,[74] dietary supplements,[75] and environmental prophylaxis. Phage is allowed only on a compassionate care basis for human therapeutic use.[76,77]

1.5. Clinical Trials and Current Phage Therapy

The Eliava Institute in Tbilisi Georgia remains a leading expert clinic for active practice of phage therapy, with hundreds of local, as well as international patients that have received lifesaving phage treatment.[42] Moreover, there have been several notable clinical cases trials of phage therapy within recent time. PhagoBurn, funded by the European Commission, is the first phage therapy clinical

trial utilizing manufacturing practices.[78] This phage cocktail was curated as a treatment for *Escherichia coli* and *Pseudomonas aeruginosa* burn wound infections. The development and validation of PhagoBurn served as an eye-opening experience for the community in terms of designing an efficient manufacturing process, with an active phage cocktail. This clinical trial was also met with significant challenges. Thus PhagoBurn also provided a major understanding of the challenged in design of clinical trials for human phage therapy, and other potential limitations that occur with the manufacturing and administration of a phage treatment.[78]

As mentioned above, phage therapeutic products are currently on the market in certain parts of the world. Sextaphage is one such commercial pharmaceutical phage composition from Russian company Microgen (**Figure 1-3**).[79] This phage therapeutic contains phages against six specific pathogens, with the intent of treating urinary tract infections in pregnant women. Pregnant women are prone to urinary tract infections, however they cannot consume most of antibiotics because of the threat posed to the developing fetus.[80] Thus, phage therapeutics are favourable to cases of patient intolerance to antibiotic consumption. Microgen offers multiple other phage products for other infections.

Figure 1-3: *Sextaphage Pharmaceutical Product from Microgen* [81]

Another successful notable clinical attempt of phage therapy is reported from scientists and physicians at the University of California San Diego School of Medicine, who had worked alongside colleagues from the U.S. Navy Medical Research Centre- Biological Defence Research Directorate. As a first attempt in United States, they successfully utilized intravenous phage therapy to treat a patient in the United States with a severe systemic infection caused by multidrug resistant organisms. Due to the utilization of a specially curated phage therapeutic on a compassionate care level, this patient, a professor in the Department of Psychiatry at UC San Diego School of Medicine, was saved from an end stage comatose condition.[77]

Table 1-2: Recent Phage Therapy Clinical Trials

TRIAL TITLE	CONDITION/ INFECTION	INTERVENTION	STATUS	COUNTRY	REF.
Standard Treatment Associated with Phage Therapy Versus Placebo for Diabetic Foot Ulcers Infected by *S. aureus*	Diabetic Foot, Staphylococcal Infections	PhagoPied: Topical anti-Staphylococcus bacteriophage therapy	Not Yet Recruiting	France	82
Analysis of changes in inflammatory markers in patients treated with bacterial viruses	Wide-range, non-healing postoperative wounds or bone, upper respiratory tract, genital or urinary tract infections whom extensive antibiotic therapy failed	oral, rectal and/or topical bacteriophage lysates/purified phage formulations/phage cocktails	Completed	Poland	83
Evaluation of Phage Therapy for the Treatment of *Escherichia coli* and *Pseudomonas aeruginosa* Wound infections in Burned Patients	Wound infection	PhagoBurn: *E. coli* phages cocktail (15-phage cocktail), Aeruginosa Phages cocktail (13-phage cocktail)	Completed	Belgium, France, Switzerland	78
Bacteriophage Effects on *Pseudomonas aeruginosa*	Cystic Fibrosis (CF)	Mucophages (10-phage cocktail)	Completed	France	84
Therapeutic bacteriophage preparation in chronic otits due to antibiotic-resistant *Pseudomonas aeruginosa*	Antibiotic-resistant Pseudomonas aeruginosa in chronic otitis	Biophage-PA	Completed	United Kingdom	85
Antibacterial Treatment against Diarrhea in Oral Rehydration Solution	ETEC and EPEC Diarrhea	Oral T4 phage cocktail	Completed	Bangladesh	71
Bacteriophages for treating Urinary Tract Infections in Patients Undergoing Transurethral Resection of the Prostate	Urinary Tract Infections (UTI)	Intravesical instillation-PYO phage	Completed	Georgia	86
Individual Patient Expanded Access for AB-SA01, an Investigational Anti- *S. aureus* Bacteriophage Therapeutic	MDR *Staphylococcus aureus* infections	AB-SA01 (3- phage cocktail)	In Progress	United States of America	87
Individual Patient Expanded Access for AB-PA01, an Investigational Anti-Pseudomonas Aeruginosa Bacteriophage Therapeutic	*Pseudomonas aeruginosa* infections (incl. MDR stains)	AB-PA01 (4-phage cocktail)	In Progress	United States of America	88
A Prospective, Randomized, Double-Blind Controlled Study of WPP-201 for the Safety and Efficacy of Treatment of Venous Leg Ulcers	Venous Leg Ulcers	WPP-201 (8-phage cocktail)	Completed	United States of America	89

2. Gut Microbiota and Human Health

2.1. Introduction to the Gut Microbiota

The human microbiota harbors a rich and complex community of microbial cells. This plethora of cells come from all three domains of life (archaea, bacteria, eukarya), with the number of bacteria in the body approximately the same order as that of eukaryotic human cells, and phage outnumbering bacterial population by another factor of 10.[90,91] A symbiotic relationship is established directly between the composition of the human gut microbiome and the human host, the disturbance of which could lead to initiation or progression of many chronic and degenerative diseases and syndromes, including Chron's disease,[92] ulcerative colitis,[93] rheumatoid arthritis,[94] inflammatory bowel disease,[95] among others. In addition, recent research has also associated the human microbiota with non-intestinal diseases, such as diabetes,[96] obesity,[97] certain forms of cancer,[98] various neurological degenerative diseases,[99] and various mental disorders.[100] Furthermore, evidence has been found linking the use of a wide range of drugs to alterations in the human gut microbiome, which in turn impact the body's susceptibility and progression towards chronic and degenerative diseases.[101-104]

2.2. Microbiome Therapy with Bacteriophages

Microbiome therapy aims to modulate the human gut microbiome as a therapeutic strategy to combat many of the chronic or degenerative diseases.[105] In specific, due to bacteriophage's naturally profound ability to for highly specific bacteria targeting, the exploitation of lytic or temperate phage as a tool for microbiome therapy has the potential to manipulate and engineer the gut microbiota to achieve desirable effects for healthy balanced microflora.[106,107]

2.3. Bacteriophages in the Gut Microbiota

Bacteriophages are naturally present within the human gut microbiome in collection with other bacteria, fungi, protozoa and fungi. Bacteriophage predation in the body (especially the lytic cycle) is a key selective pressure on their corresponding bacterial hosts, in turn influencing and shaping the quality of the symbiotic relationship and homeostasis of the gut microbiome.[108] Furthermore, induction of prophage (abundantly present in the human microbiome) under different conditions can led to preserving the balance of the microbiome, or initiating dysbiosis.[109,110]

2.4. Possible Interactions of Therapeutic Phage and Gut Microbiota

Despite the presence of commensal bacteriophage naturally present within the human gut microbiota, the introduction of therapeutic bacteriophage to the gut microbiota will still act as an external agent.[111] Moreover, an immune response of the human host could be triggered as bacteriophages do have the potential to cause rapid and massive bacterial lysis, subsequently releasing components from the cell wall into the blood stream.[112] Hence, the potential of an immune response that may induce the production of antibodies against phage action is of concern.[111] Furthermore, it is important to prove the administered therapeutic phage (1) does not have the ability to integrate its genome into the genome of the target bacteria or any of the components of the niche microbiome setting of further indirect effects within the microbiome, (2) will not indirectly affect the microbiome through selective pressure on various non-target commensal bacteria or the infection, to impact the balance surrounding beneficial bacteria in the niche, as well as the effects of antagonistic phage coevolution on the gut microbiota.[113]

Furthermore, influence of external factors may impact the successful administration and persistence of therapeutic phage within the gut microbiome- in specific, external physical and chemical factors, such as acidity and presence of various ions. The utilization of certain therapeutic phages in acidic gastrointestinal environments may significantly reduce phage stability and phage titer.[114-116] In a fasting human, the median gastric pH is 1.7, in comparison to a pH over 6 in certain regions of the digestive tract.[117,118] To overcome this concern regarding decreased stability from acidity, an antacid can be simultaneously consumed upon the administration of oral phage therapy, or the therapeutic phage is encapsulated in protective matrices.[119,120]

3. Phage-Host Evolutionary Dynamics

3.1. Phage-Bacteria Interactions and the Evolutionary Arms-Race

Bacteria-phage interactions have remained central to the evolution and ecology of microbial communities, as they alter competition of bacterial species and result in evolution of new strains.[121] In specific, bacteria develop resistance, and consequently, bacteriophage will counter-adapt and evolve from this resistance.[122-124] As a result of this co-evolution, phages have acquired counter bacterial defense mechanisms, including anti CRISPRs.[125] Bacteria, in turn, evolve to develop

resistance mechanisms;[126] it is partially due to this co-evolution that effective phage infection is not always observed regardless of phage adsorption.[127]

3.2. Phage Resistance and the Effect of Mutants

Bacterial resistance to therapeutic phages is unique in comparison to bacterial resistance to antibiotics. In specific, as bacterial populations evolve to resist phage predation via random mutations, the phage for this specific host range will also shadow the bacteria and mutate at a similar rate to combat the resistant bacteria, consequently increasing genomic diversity of both partners.[122]

Due to differences in mutational supply of both the bacteriophage and corresponding bacterial host and levels of resource supply for bacterial resistant mutations and phage infectivity mutations, there is a further difference of the strength of selection, population divergence, trajectory, and adaptation patterns.[128,129] Moreover, lytic phages typically have larger population sizes and a shorter generation time in comparison to their corresponding bacterial host, which impacts the evolutionary race between bacteria and phage.[130] The strategic exploitation of multiple phages in form of a therapeutic phage cocktail (and thus multiple selective pressures) has the potential to limit the bacteria's ability to evolve resistance against a single phage species.[131]

3.3. Phage-Host Evolutionary Dynamics in the Gut

It is important to study if phenotypic and genetic diversity within the gut microbiota, and if the overall community environment structure can be maintained during the coevolutionary processes underlying interactions between bacteria and lytic therapeutic phages. As the coevolutionary arms race of phage and their corresponding host bacteria progresses, evolution of the microbiome may also be observed.[132] The evolutionary dynamics of therapeutic phage with its host in the gut is further complicated by competition between the host and the entire gut microbiota for colonization and resources, nutrient availability, and special heterogeneity of the gut microenvironment, all of which could affect the co-evolution, leading to an outcome other than that observed in a typical lab culture.[133-136] There are currently very few investigations on phage-host co-evolution in the gut environment. One of the few notable recent attempt is the work from Institut Pasteur on the investigation of phage evolving to infect different hosts in the gut.[113]

4. Aim and hypotheses

There is a significant gap knowledge with the community dynamics of therapeutic phage and host populations in the gut environment. The purpose of this study was to address this gap in knowledge by investigating the effect of phage therapy on the gut microbiome. We investigated two major hypotheses:

(i) Resistant mutants formed in the process of phage challenge of a bacterial infection may affect the balance of the gut microbiota.

(ii) Diversification of host community will significantly differ *in vivo*, compared to an *in vitro* lab culture.

5. Scientific Contributions

This thesis reports investigation into the effect of phage challenge (using a single dose of T7 phage) on a community of *Escherichia coli* K12, colonized in gut of germ-free mice along with 8 commensal strains. Our results will be reported in the form of two manuscripts (in preparation):

1- Effect of phage therapy on gut microbiome and diversification of host communities in the context of ASF microbiota (**Chapter 2**)

2- *In vivo* and *in vitro* diversification of an *E. coli* host under phage predation (**Chapter 3**)

The developed insight and knowledge can be applied for designing effective phage therapeutics that not only do not disturb the microbiome but can work in concert with the microbiome.

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Chapter 2 – ASF Mouse Model of Lytic Phage-Host Dynamics Within the Gut Microbiota

1. Abstract

As bacteria's natural predators, bacteriophages offer a critical advantage over antibiotics, namely specificity. This means phage therapeutics can be designed to destroy the infectious agent(s) without causing harm to our microbiota. There exists, however, a significant gap in knowledge regarding the direct and indirect effect of phage therapeutics on the microbiota. This investigation is aimed at addressing that knowledge gap by investigating the effect of phage-host community dynamics on mouse gut microbiota for germ-free mice, inoculated with the Altered Schaedler Flora (ASF). The community dynamics between *Escherichia coli* K-12 and the virulent phage T7 (inoculation multiplicity of infection, MOI~1) was monitored in the context of an ASF microbiota for 6 weeks. 16S ribosomal DNA sequencing and differential culture techniques showed a significant reduction in host concentration (based on both CFU count (5 logs) and relative abundance) immediately post phage challenge, which increased and stabilized by week 6. The phage-host community dynamics appeared to affect the *Enterococcus* population in the gut, keeping it consistently higher than the control groups, with minimal effects on the other components of the gut microbiota. All host colonies isolated within 24hrs of phage challenge were resistant to the evolved phage, also isolated from the mice fecal samples. However, by week 6, there was only a small fraction of resistant colonies in feces or the intestines, even though T7 persisted at high titers, suggesting a stable community of susceptible host and virulent phage co-existing in the gut.

2. Introduction

Mounting concerns about drug-resistant pathogenic bacteria has rekindled interest in bacteriophages for therapeutic use. Bacteriophages (phages) are naturally occurring viruses of bacteria and are the most ubiquitous and diversified biological group, with an estimated 10^{32} phages residing on Earth.[1] Phage therapy (the use of bacteriophages for infection control) was actively employed immediately after discovery of phage by d'Herelle and later by others,[2] for treating infections, years prior to the introduction of antibiotics in the 1940s, when infectious diseases were the leading cause of mortality and morbidity within human populations.[3,4] A lack of

understanding in phage biology in the early days, combined with exaggerated claims, a lack of controlled trials, and poor documentation, among others, all led to phage therapy being overshadowed by antibiotics in Western medicine.[5]

Antibiotics have since been highly attractive due to their broad-spectrum activity allowing them to be used against a wide range of bacteria without necessarily identifying or characterizing the exact infective agent(s). With increased understanding of the role of human microbiota in our overall well-being, this main advantage, is increasingly presenting itself as a great disadvantage. Due to their non-specific mode of action, antibiotics also destroy the commensal microflora of the gut.[6] A symbiotic relationship is established directly between the composition of the human gut microbiome and the human host, the disturbance of which could lead to initiation or progression of many chronic and degenerative diseases, ranging from ulcerative colitis,[7] and rheumatoid arthritis,[8] to certain forms of cancer,[9] and various mental disorders.[10] As bacteria's natural predators, bacteriophages offer a powerful advantage over antibiotics, namely that they can be highly specific, targeting only the population of their host bacteria. This means that phage therapeutics could, in theory, be designed to kill an infectious agent but not harm the microbiota.

Learning from past mistakes, the scientific community and regulatory agencies are being extra vigilant, demanding detailed mechanistic investigation of interaction of new antimicrobials (including but not limited to phage) with the microbiota. Although, there are strong arguments to be made regarding the use of temperate phage for therapeutic purposes,[11] currently, only strictly lytic or virulent phage are of immediate of interest for phage therpay.[12] One of the critical apprehensions concerning phage therapy is the potential for perturbations towards the niche microbiota as a result of the strong selective pressure exerted by lytic phage on their host communities. Unfortunately, there exists a significant gap in knowledge when it comes to understanding primary and secondary effects of phage therapy on the niche microbiota. To add to the complexity, little is known about phage-host interaction, both in terms of the biology and the community dynamics and community evolution *in vivo*, with most of our knowledge on the subject being rooted in experiments performed under idealized lab settings *in vitro*. Multiple animal studies and pre-clinical trials with less than ideal outcomes have proven that the knowledge about phage-bacteria community dynamics *in vitro* cannot be directly extended to predict the outcome of such dynamics *in vivo*, where these interactions take place in a chemically and physically

heterogeneous environment, in a complex landscape of selective pressures, on both on both phage and its host bacteria.[13-15]

To help bridge this gap in knowledge, we developed a model for investigating the community dynamics of a mock infection with virulent phage in the context of the gut microbiota. This model consisted of a gnotobiotic mouse model colonized with an Altered Schaedler Flora (ASF), a consortium of eight known bacterial species, providing a simplified defined and traceable model of the gut microbiota. The ASF community was co-colonized with the non-pathogenic bacterium *Escherichia coli* K-12 (JM83), which was then challenged with the lytic bacteriophage T7. This model allowed us to investigate (i) the effect of phage-host community dynamics on composition of the gastrointestinal microbiota, (ii) evolution of phage-resistant bacterial mutants and diversification of host community i*n vivo*.

3. Methods

All experiments involving mice were conducted and performed according to guidelines set by the Canadian Council on Animal Care and approved by McMaster University Animal Research Ethics Board (AREB). All experiments involving mice were performed with protocols approved by the McMaster University Central Animal Facility. Procedures for gnotobiotic mouse husbandry, bacterial ASF cultivation, *E. coli* K-12 (JM83) inoculation preparation, T7 Phage inoculum preparation, collection and sampling of fecal microbiota from gnotobiotic mice, isolation of total DNA from mouse feces and intestinal tissues, *in vitro* assays for bacterial host and phage tropism, are described in the following.

3.1. Gnotobiotic Mouse Husbandry

Germ-Free mice belonging to the C57BL/6 inbred strain were housed in plastic, gnotobiotic, individually vented isolators and fed a regular, autoclaved, low-fat chow diet with a constant supply of autoclaved water in the cage. Each therapy group of mice (n=3) were age-matched and specifically consisted of two male mice housed together in one cage isolator, and one female mouse housed in a separate cage isolator. At 8 weeks of age, all mice were colonized with a single 100uL oral gavage of the prepared ASF culture. Mice were categorized into three groups; mice from group one and group two were also immediately inoculated with *E. coli* K-12 (JM83) post ASF gavage. Three weeks post initial inoculation, mice from group one and group three were inoculated

with T7 phage and 0.25% $CaCO_3$ as antacid. Each week, mice were weighed and inspected for signs of disease, then fecal samples were collected form mice and immediately transferred into anaerobic jars, transferred to anaerobic chamber and processed. Mice were sacrificed after 9 weeks by cardiac bleed and cervical dislocation and the organs were removed aseptically. The process is demonstrated in **Figure 2-1**.

3.2. Inoculation of ASF into Germ-Free Mice

The eight microorganisms composing the ASF consortium are all derived from mice and have been stably passed through multiple generations in gnotobiotic mice continually bred in isolators. Contents from the cecum of a healthy ASF mice was collected using sterile scissors and forceps. Caecal contents were added to a 15mL sterile conical tube containing 10mL sterile PBS and 0.5g/L sterile filtered L-cysteine. This was then stored in a prepared anaerobic container. Each mouse was gavaged with 100 μL of the diluted caecal contents at 8 weeks of age, using a sterile syringe and gavage needle.

3.3. Preparation of *E. coli* Inoculum

E. coli strain K-12 (JM83), containing a streptomycin resistance cassette, inoculated from frozen glycerol stock, was anaerobically growth in 50 mL fresh Tryptic Soy Broth (TSB, equilibrated for 12 hrs in the anaerobic chamber prior to inoculation) to exponential phase (OD_{600} ~ 0.3). This was then aliquoted into 10 mL conical tubes and washed 3 times with PBS (Centrifuged at 4℃, 7000rpm for 5 mins. Supernatant was discarded, and pellet was resuspended in 3mL of fresh sterile PBS to wash). A 100 μL aliquot of washed bacteria (~ 10^9 CFU/mL) was then transferred into 1.5 mL sterile microcentrifuge tubes and placed into sterile 50 mL conical tube and transferred in an icebox to mouse inoculation site. For infection, each mouse of group 1 and group 2 were orally gavaged using 100 μL of the sterile bacterial inoculum at 8 weeks of age, immediately after ASF inoculation, using a sterile syringe and gavage needle.

3.4. Preparation of Phage Challenge

A 1:100 overnight culture of *E. coli* strain K-12 (JM83) was added to an autoclaved baffled flask containing with 150 mL fresh TSB and grown aerobically to mid exponential phase (OD_{600} ~0.4) in a shaking incubator (210 rpm, 37°C). A 10 μL aliquot of a 10^9 PFU/mL stock of T7 phage was then added to the host culture and incubated aerobically overnight. The propagated crude lystate was aliquoted into sterile 50 mL conical tubes and centrifuged (4°C, 5000×g, 10 min) to pellet bacteria and subsequently purified as described by Sambrook et al.[16] Briefly, the supernatant was

sterile-filtered using a 0.2µm filters, then mixed with 1/6th volume of 20% polyethylene glycol/2.5M NaCl and incubated overnight at 4°C. Post-incubation, phage was pelleted (4°C, 5000×g, 45 min) and supernatant was discarded. Phage pellet was resuspended in 10 mL SM buffer (100mM NaCl, 8mM $MgSO_4$, 0.01% Gelatin, 50mM Tris-HCl) and rocked for 3 hrs at 4°C to resuspend pellet. To remove residual bacteria debris, phage was centrifuged again for 10 min and supernatant was collected. A 250 μL aliquot of purified phage were then mixed with 0.25% w/v, final concentration, of $CaCO_3$ as antacid). This was then added to 750 μL purified phage and thoroughly vortexed. A 100 μL of phage-antacid inoculum (~10^9 PFU/mL) was administered to each mouse in groups one and three via oral gavage, 3 weeks post ASF/ASF-*E.coli* inoculation.

3.5. Sampling and Quantifying Bacteria from Fecal samples and Intestinal Contents

Approximately 2-4 fecal pellets from each mouse were collected at every timepoint and transferred anaerobically to an anaerobic chamber. Contents were then immediately homogenized with 500 μL of fresh TSB. Small intestine sections and the cecum were aseptically extracted from the mouse after sacrifice. Contents were immediately transferred into 2 mL sterile plastic screw top tubes containing 0.2 g of 4.8 mm ceramic beads to extract contents.

Aliquots of 300 μL of the homogenized fecal content (per mouse) or homogenized intestinal content was added into a 2 mL sterile plastic screw top tube containing 800 μL of 200 mM of monobasic $NaPO_4$ (pH 8), 100 μL of guanidine thiocyanate (GES) buffer, and 0.2 g of 2.8 mm ceramic beads (Mo Bio Laboratories, #13114-50). All contents were vortexed and sent to Macmaster MOBIX facilities for DNA isolation and amplification. Aliquots of the homogenized fecal content (100 μL per mouse) were then serially diluted and plated, using autoclaved glass beads, on MacConkey agar plates, MacConkey agar plates supplemented with 50 μg/mL of streptomycin, and BHI (Brain Heart Infusion) agar plates for preparation of stocks (see 3.9). All steps post fecal collection was performed in an anaerobic chamber using pre-equilibrated media in the anaerobic chamber. All BHI plates were incubated anaerobically. Bacterial colonies from the BHI plates were pooled together after 48 hrs of incubation by flooding the plates with BHI media. The pooled bacterial mixture was mixed with 20% skim milk solution (1:1), loaded in a 1.5 mL cryopreservation tight screw tubes and stored at -80°C.

MacConkey plates (with or without antibiotic) were incubated aerobically and used for counting colonies of *E. coli* K-12 (JM83) to determine bacterial concentration per gram of fecal matter (CFU/g). These plates were further used for isolating *E. coli* colonies for phenotypic analysis.

3.6. Phage Enumeration

Aliquots of 100 μL of homogenized fecal content or homogenized intestine contents were suspended in 900 mL of fresh sterile TSB, vortexed to mix, and then centrifuged (5 min, 4℃, 7000 rpm) to separate bacteria from supernatant. The supernatant was then collected and stored as *in vivo* evolved T7 phage, which was quantified using agar overlay technique as describes elsewhere, using 100 μL of *E. coli* K-12 (JM83), parental strain.[17] Plaques were then counted to quantify phage titer per gram of fecal matter (PFU/g).

3.7. DNA extraction, 16S rRNA Gene Sequencing, and Sequence Processing

Genomic DNA was extracted from samples as described by Whelan et al., with some modifications.[18] Briefly samples were transferred to screw cap tubes containing 2.8mm ceramic beads, 0.1 mm glass beads, GES and sodium phosphate buffer. Samples were bead beat and centrifuged and the supernatant was further processed using the MagMAX Express 96-Deep Well Magnetic Particle Processor from Applied Biosystems with the multi sample kit (Life Technologies#4413022). Purified DNA was used to amplify the variable region 3 of the 16S rRNA gene with PCR using Illumina adapted primers as described in Whelan *et al.* Resulting PCR products were normalized using the SequalPrep normalization kit (ThermoFisher#A1051001) and sequenced with the Illumina MiSeq platform at the McMaster Genomics Facility (Hamilton, Canada) was performed as described previously.[18] Resulting sequences were run through the bioinformatic pipeline sl1p as described in the literature.[19] In specific, as per the library stat output provided by the McMaster Genomics Facility, 642 observations were made from 127 samples. Resulting in a total count of 12 027 142. Min counts/sample was 8207.000, Max counts/sample was 232 000.000. The median being 89 313.000 and mean of 94 701.906, with a standard deviation of 42 824.925 from taxonomic observed metadata. Paired-end sequences of the v3 region of the 16S rRNA gene were processed through a standardized workflow.[19] A negative genomic extraction and sequencing controls were conducted to ensure that sequencing contamination was not an issue for low-biomass samples.

3.8. Data Analysis

Data analysis was performed as described by Lamarche et al.[20] Briefly, the α and β-diversity estimates were generated in R (R Core Team 2016)[21] using the 'phyloseq' package.[22] β-diversity was calculated on the proportionally normalized operational taxonomic unit (OTU; *i.e.*, a proxy for bacterial 'species') table excluding singletons, and OTUs classified as non-bacterial. The α-diversity was calculated using the OTU table excluding only non-bacterial OTUs and rarefied to 2800 reads per sample 100 times, and the mean value of the α-diversity measurements was used. Three α-diversity metrics were included in this investigation; the Shannon and Simpson diversity indices account for richness (*i.e.*, number of taxa) and evenness (*i.e.*, taxa relative abundances), while the metric Observed Species accounts only for richness.[23] The within-body site distance-to-centroid was calculated on a Bray-Curtis distance matrix using the function betadis-per from the R package 'Vegan'.[24] The UPGMA consensus tree was generated in QIIME with support established using jackknife.[25] The correlation analysis between patients' metadata and α-diversity measurements was performed using the R packages 'Hmisc' and 'corrplot', using the Spearman's rank correlation coefficient.[26,27] The Kaplan-Meier estimate was generated using the R packages 'survival' and 'survminer'.[28,29]

3.9. Inflammatory Response Assay

To determine presence of inflammation, at time of sacrifice blood samples were collected via cardiac bleed. Serum was immediately removed from corresponding blood samples by centrifuging collected sample at 1000- 2000×g for 10 mins. Collected serum was then stored in a sterile polypropylene tube at -20℃. Samples were analyzed for TNF-α and IL-6 inflammation, using the MCYTOMAG-70K MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic bead Panel Immunology Multiplex Assay from Millipore Sigma.

3.10. Isolation of *E. coli* colonies

Bacterial colonies were isolated from samples of fecal matter from each time point and endpoint intestinal content that plated on MacConkey and MacConkey-Streptomycin agar plates (30-50 colonies per mouse, per media, per time point). Each collected colony was set to a specific identification number for record keeping. Initial colony morphology was recorded per colony. Each colony was then cultured in TSB aerobically and the overnight culture of each isolate was

mixed with 50% glycerol solution (1:1) and stored as 200 μL aliquots in microtiter plates and at -80℃.

3.11. Phage Susceptibility and Fitness Characterization of Isolates

Phage susceptibility. Bacterial isolates were analyzed for susceptibility against both parental strain of T7 bacteriophage, as well as the evolved bacteriophage isolated from fecal/endpoint organ samples, using the streak test, as described elsewhere.[17] Briefly, 10 μL droplets of phage (parental T7 = 1×10^9 PFU/mL, experimental evolved = stock diluted 1:10 in SM buffer) were ripped down a straight-line top to bottom, on LB agar plates and left to dry. Overnight cultures of collected *E. coli* colonies were then streaked across the dried phage teardrop using multichannel pipette (5 mutants per plate) and incubated at 37 ℃. Results were recorded after 24 hrs. of aerobic incubation at 37℃. Susceptibility was determined based on inhibition of growth of the streaked *E. coli* line.

Growth Rate/Yield. Anaerobic growth curves were prepared for each bacterial isolate using the kinetic cycle on BioTek Synergy Neo Plate Reader (OD_{600}, 16 hrs., 37℃, shaking, absorbance reading every 10 min). Growth Rate and growth yield was calculated based on the slope of this curve at logarithmic phase. Growth yield was determined as OD_{600} at stationary phase.

Motility. Swim agar was made with 0.3% BD Bacto Agar (214010), 1% tryptone (VWR J859), 0.5% Yeast Extract (VWR J850), and 0.5%NaCl, autoclaved and used fresh. Swarm Agar was made with 0.5% agar-BD Bacto Agar, 1% tryptone, 0.5% Yeast Extract, 0.5% NaCl, 0.5% Dextrose (VWR 0188), autoclaved and used fresh. Overnight cultures of E. *coli* isolates were stamped slightly under the surface of the agar for swim, and on top of the surface for swarm, using custom-made replica stamp pin. Samples were also collected from the swarm edge for resistant isolates from 48 hr. swarm plates.

3.12. Statistical Analysis

All data presented is the average of at least three independent mouse models per treatment group, or three independent experiment replicates, presented along with the standard deviation between values obtained for the independent experiments. Statistical significance of differences was tested using t-test and P values lower than 0.05 were chosen as the cutoff for significant difference. P values less than 0.05 are annotated with one asterisk, P values less than 0.01 are annotated with two asterisks, P values less than 0.001 are annotated with three asterisks, and P values less than 0.0001 are annotated with four asterisks.

4. Results and Discussion

4.1. Single dose lytic phage can effectively thrive for long term within the gut microbiome

To investigate the dynamics of lytic phage and a host bacterial population in the context of the gut microbiota, we developed a model utilizing a non-commensal strain of *E. coli* K-12, and the well-studied T7 lytic bacteriophage. In specific, the experimental design consisted of three groups of germ-free C57BL/6 mice (G1, G2, G3, n=3 per group). Each group of mice was caged separately in gnotobiotic isolators. All mice were orally gavaged with the ASF consortium consisting of eight known bacteria at eight weeks of age. Mice from the G1 and G2 were also gavaged with 100 µL of 10^9 CFU/mL *E. coli* K-12 (JM83). Three weeks later, mice from G1 and G3 were each inoculated with 100µL of 10^9 PFU/mL T7 phage. Fecal samples were collected from each mouse within each treatment group at frequent intervals, at least once per week. We collected intestinal contents from the duodenum, the ileum of the small intestine, and the cecum, as well as a fecal sample from each mouse in each treatment group at the time of their sacrifice, 9 weeks after initial inoculation (**Figure 2-1**). The phage were delivered to the stomach via oral gavage and protected against stomach acid (pH~3)[30] using $CaCO_3$, which acts to decrease the free protons in the medium and thus increase the pH (**Figure S2-9**).

16 S ribosomal RNA sequencing of fecal samples from all germ-free mice prior to bacterial inoculation disclosed that the mice did not have any pre-established bacterial taxa or bacterial spores that would cause initial inaccuracies. Neither the bacterial gavage nor the phage inoculum contained components that appeared to compromise the gut barrier/immune function or perturb the overall health status. Throughout the duration of the challenge or at the time of sacrifice, none of the treatment groups exhibited any significant decrease in total body weight (**Figure S2-10 A**). The weight of the fecal pellets collected from each mouse also remained relatively stable throughout the study (**Figure S2-10 B**).

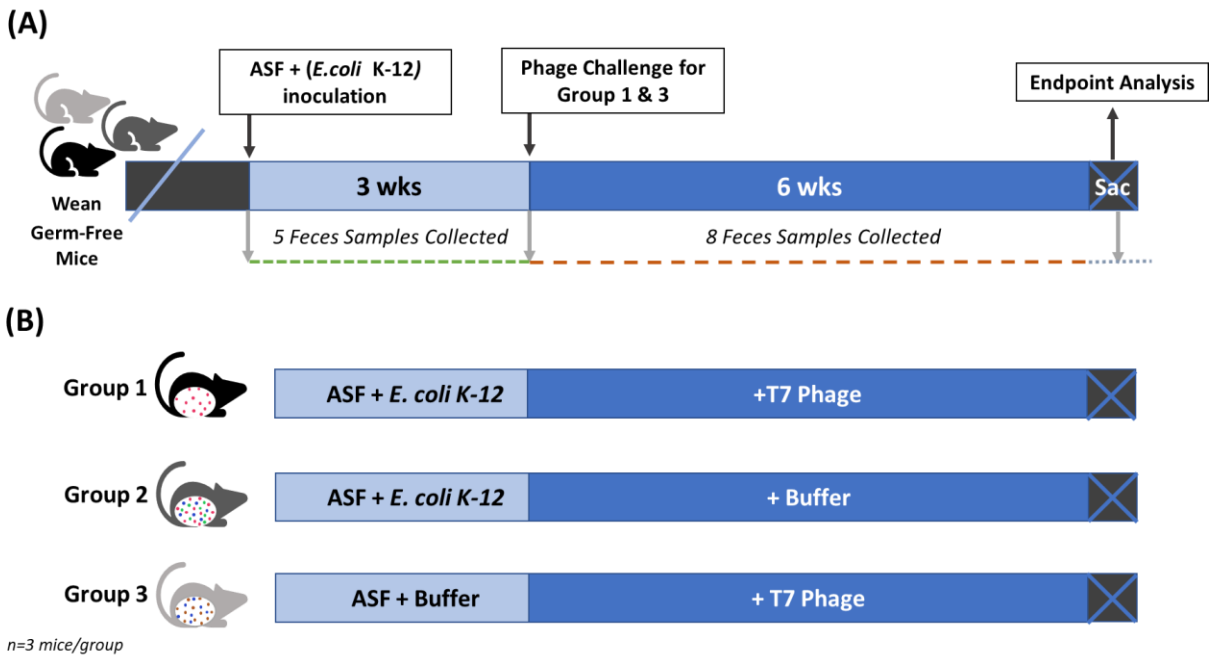

Figure 2-1: Schematic Diagram of Experimental Design

(A) *Three groups of 8-week-old C57BL/6 mice (n=3 per treatment group) were gavaged with ASF culture (+E. coli K-12 (JM83)). Day 0 refers to the initial inoculation of ASF culture (+JM83 E. coli) to the Germ-Free mice. Phage challenge started 3 weeks after inculcation and the mice were sacrificed 9 weeks after initial inoculation. Fecal samples were collected frequently all through the duration of the study.* ***(B)*** *All mice were inoculated with the ASF culture. Mice from G1 and G2 were also gavaged with 100µL of E. coli K-12 (JM83) (10^9 CFU/mL) prepared from an exponential phase culture and washed with PBS. At the three-week mark (day 23), mice from G1 and G3 were inoculated with 100µL of T7 phage (10^9 PFU/mL) suspended in SM buffer and 0.25% $CaCO_3$ as antacid. All mice were sacrificed, and tissues harvested for further sampling on day 63.*

Selective culturing of fecal samples (to isolate only *E. coli*) as well as phage plaque assays confirmed that a single dose of T7 lytic phage can persist and propagate in the mouse gut (**Figure 2-2A**). Phage added to G3 mice, where the host did not exist, was cleared almost immediately, where 24 hrs after inoculation, the titer in feces dropped to ~10^3 CFU/gr and was undetectable after a week. G2 mice were not inoculated with T7 and the *E. coli* concentration remains stable ~2×10^9 CFU/gr for the duration of the study. G1 initially had *E. coli* concentrations similar to G2 but showed a drastic drop of ~6.5 Logs in *E. coli* concentration 24 hrs after an aggressive phage challenge. The *E. coli* concentration recovered slowly, but consistently remained ~1-2 logs less than the *E. coli* concentration in G2, not challenged with phage. Despite the presence of a stable

community of *E. coli* in fecal samples, T7 phage also persisted in fecal samples from G1 mice at an average concentration of 3×10^7 PFU/gr, indicating the presence of a community of susceptible host cells in the gut at any time point. The community of *E.coli* in the mouse gut is expected to be predominantly at stationary phase by the time it is challenged with phage. T7 is known to infect bacteria at stationary phase with a large burst size,[31] explaining its ability to persist inside the GI tract of mice where a phage like T4 that is not efficient at infecting stationary phase host cells, has been shown unable to persist.[14]

Examining the fraction of phage-resistant *E. coli* colonies reveals a more complex picture. Even though immediately after starting the phage challenge, near to 100% of the isolated colonies were resistant to evolved phage (**Figure 2-2B**) and close to 80% were resistant to the parental T7 phage (**Figure S2-11A**), the resistant fraction drops drastically with time, showing a diversified population of susceptible and resistant isolates (to both the evolved and the parental T7 populations), before stabilizing at ~30% resistance at the endpoint, 6 weeks after starting the phage challenge. Interestingly, the T7 population maintained a stable concentration through the duration of the experiment, despite the significant change in the population of susceptible bacteria.

The contents of the intestines and the cecum at the endpoint (6 weeks after T7 challenge) showed *E. coli* concentrations, for G1 that were 2-2.5 Logs lower than G2, with cecum showing the smallest difference of 1 Log (**Figure 2-2C**). Phage concentrations in the intestines was lower than that of the fecal samples and varied, with the highest concentration of $\sim10^6$ PFU/gr in the cecum, where the concentration of host bacteria is the highest (and very close to that of the G2, no phage control), again demonstrating communities of host bacteria and virulent phage co-existing in the intestines and the cecum. A very small fraction the bacterial isolates collected from the organs after 6 weeks of challenge were resistant to the evolved (**Figure 2-2D**) or parental strains of T7 (**Figure S2-11B**).

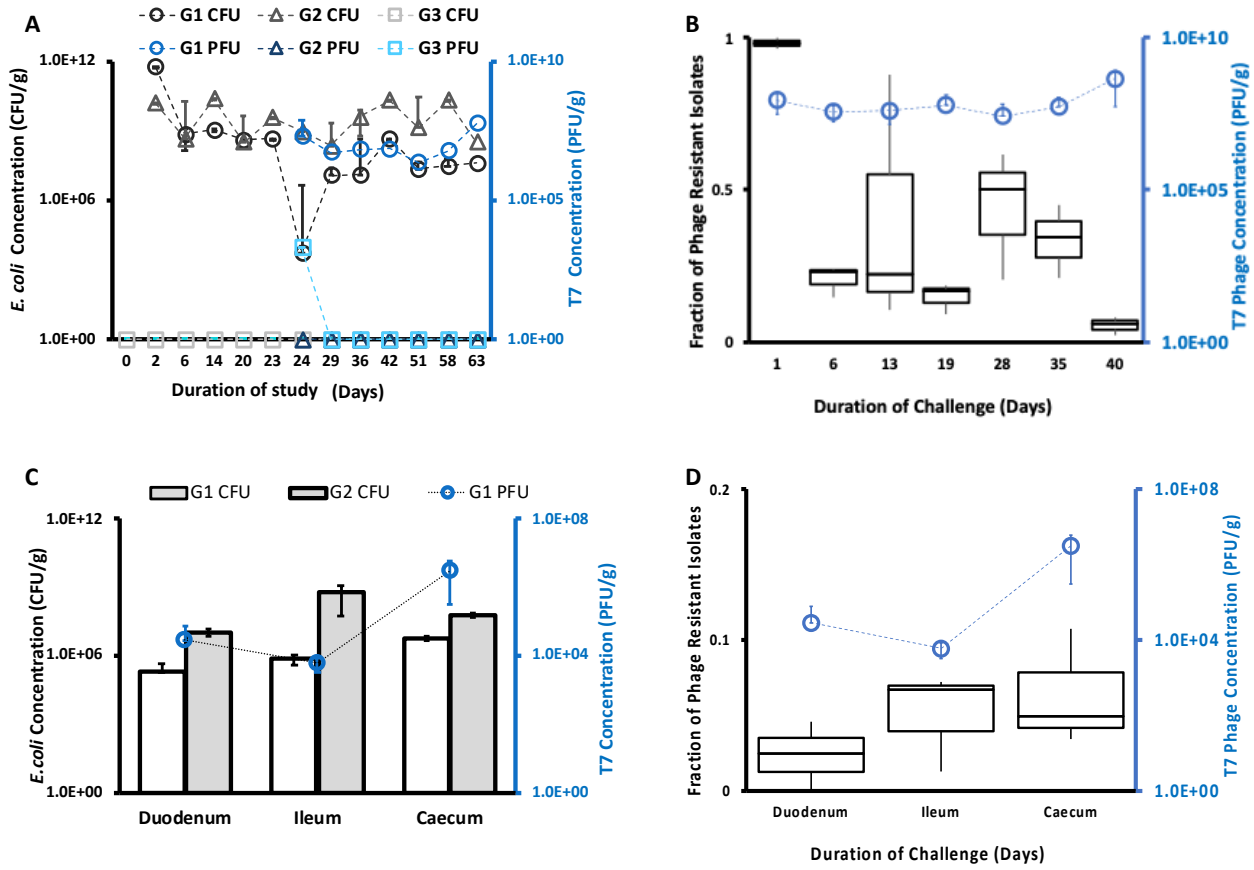

Figure 2-2: Long term coexistence of T7 and susceptible host in the gut

(A) *Change in the concentration of E. coli K-12 JM83 (CFU/g) and T7 Phage (PFU/g) of the three groups of mice over the course of 9 weeks. Each data point presents the average of three mice in each group with the standard deviations presented as error bars. The highlighted time point (day 24) is the first sampling point after phage challenge.* ***(B)*** *Resistance profile of E. coli isolates to evolved T7 strain over the course of the 6-week T7 challenge. For comparison, the concentration of T7 in fecal samples is shown for each time point on the second axis. Day1 on the x-axis refers to the initiation of the phage challenge (day 23 of experiment).* ***(C)*** *E. coli K-12 JM83 bacterial concentration and T7 Phage concentration for organs harvested at endpoint. For clarity, only E. coli concentrations in G1 and G2 and phage concentrations in G1 are shown. Other values were zero, as expected.* ***(D)*** *Resistance profile of collected E. coli isolates from organs to the evolved T7 community isolated from the same sample. For comparison, the concentration of T7 in each sample is shown on the second axis.*

4.2. Lytic phage challenge effects on the composition of gnotobiotic microbiota

The composition of the ASF community as well as the relative abundance of *E. coli* in the community was quantified using 16S rRNA sequencing. As shown in **Figure 2-3 A-C and Figure S2-12 A-I**, *Lactobacilli* and *Parabacteroides* are the dominant species in the ASF mice, regardless

of the presence or absence of *E. coli* or phage challenge. Groups 1 and 2 show a larger variation of composition with time, likely because of the presence of a non-commensal species. Upon the start for the phage challenge, the relative abundance of *E. coli* in the gut is kept consistently lower in G1 than G2 (no-phage control). However, one of the major differences between G1 and G2 appears to be the low abundance of *Clostridium* in G1 compared to G2 after the phage challenge. *Enterococcus*, present before phage challenge at very low abundance, persists in the community after phage challenge. The presence of *Enterococcus* is evident also in the endpoint data for G1 in the ilium, only (**Figure 2-3 D**). Another clear trend in the endpoint data is the consistently low abundance of *Lactobacilli* compared to *Parabacteroides*. Bacterial relative abundances were too low for detection in the duodenum of group one and group two mice, as expected due to the anatomy and orientation of the folds of the small intestine. There is a high abundance of lactobacillus within the duodenum and ileum of all three mice groups, as opposed to the caecum. *Lactobacillus* is a gram-positive, facultative anaerobic bacteria, which is commensal to the digestive tract and was also a part of the ASF consortium. In comparison, the caecum has a significantly higher abundance of *Parabacteroides* in comparison to other bacterial strains.

It is important to note that the location within the gut presented significant variability in concentration of both phage and its host. (**Figure 2-3 D**), suggesting that the trend observed in **Figure 2-2D**, namely the change in abundance of both the host bacteria and phage in different locations of the intestines, is likely influenced by the selective pressures of the gut microbiome and the physical environment.

To determine the temporal stability of mice throughout the duration of the phage challenge, we performed a longitudinal analysis by determining the Bray-Curtis dissimilarity between timepoints. As expected, G2 and G3 were fairly similar with time, with slight variation of dissimilarity (**Figure 2-4 B,C**). However, in Group 1 we can evidently see a larger variation in dissimilarity compared to that of G2 and G3 (**Figure 2-4 A**). Furthermore, we compared the temporal stability of each timepoint to a baseline to determine how the samples collected from each group change in comparison to our baseline, namely day 23 (a day prior to initiation of phage challenge and also when we see stabilization of the *E. coli* community within the gut as per our determined CFU counts). In G1 (**Figure 2-4 D**), we can see a large variance in dissimilarities especially within the first 36 days of after phage challenge. Within G2 (**Figure 2-4 E**), there is less of a dissimilarity between timepoints in comparison to G1, and in G3 (**Figure 2-4 F**) we see the

least amount of variance in dissimilarities between the time points and the baseline timepoint as expected.

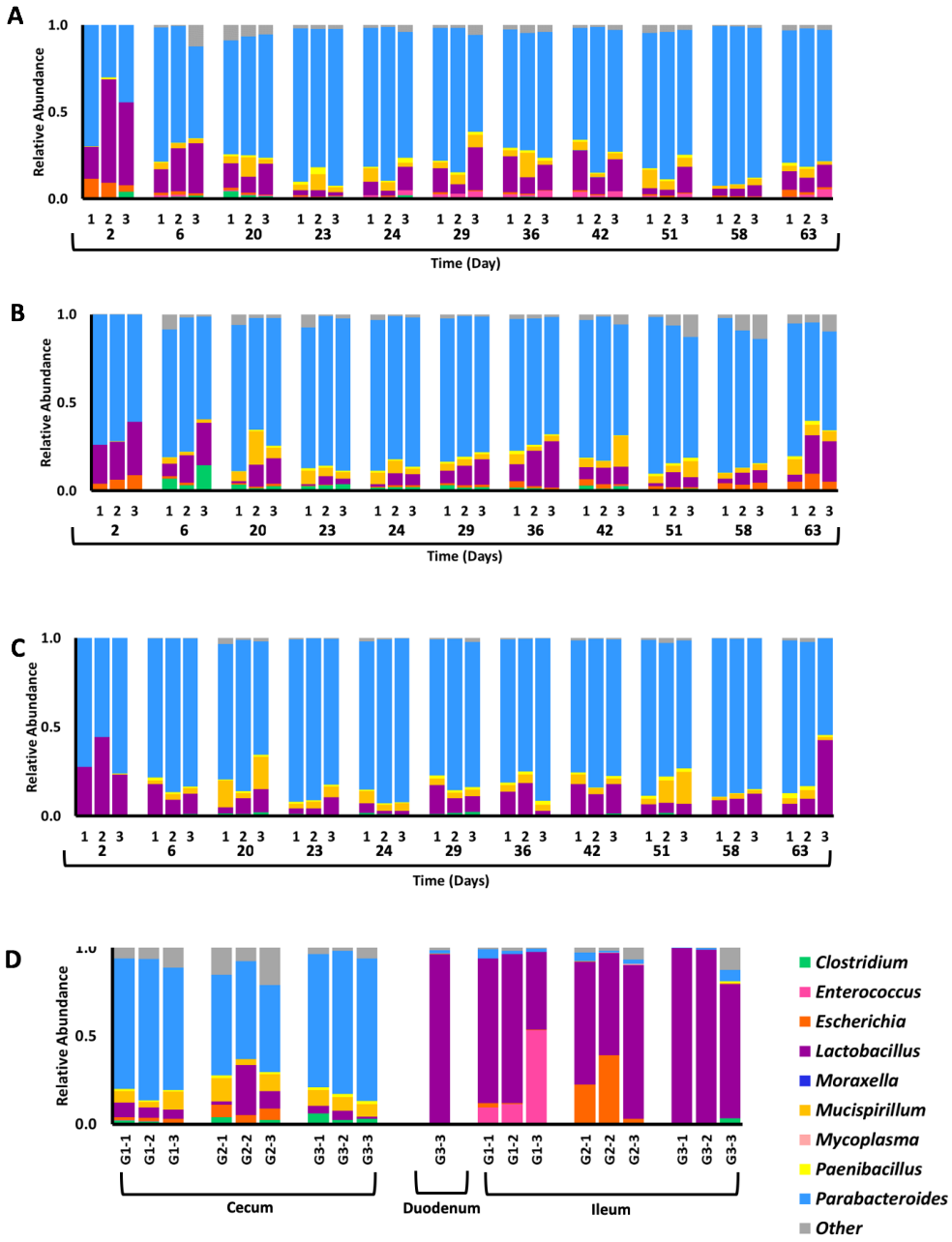

Figure 2-3: Relative abundance plots for the composition of fecal microbial communities of the mice at the genus level

(A) *Group 1: ASF + E. coli K-12 (JM83) + T7 Phage (added on day 23);* ***(B)*** *Group 2: ASF + E. coli K-12 (JM83);* ***(C)*** *Group 3: ASF + T7 Phage challenge (added on day 23);* ***(D)*** *Endpoint organs. The color key indicates the identity of each bacterial species.*

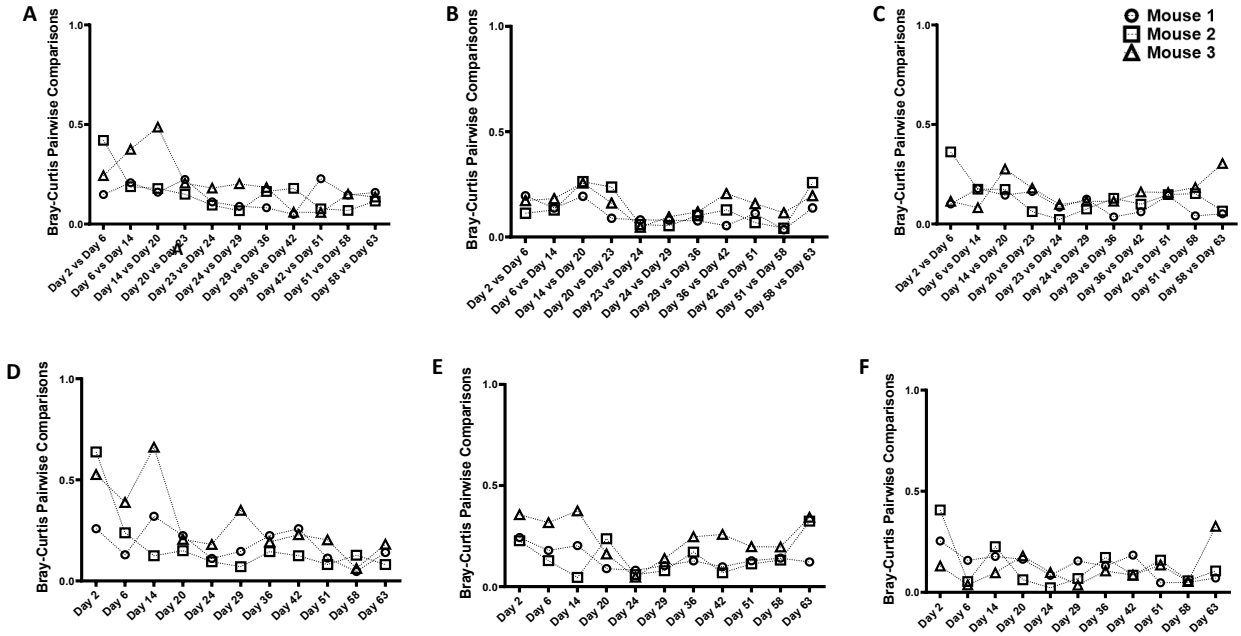

Figure 2-4*: *Temporal Stability of mice

*Longitudinal Analysis (**A**) Group 1 (**B**) Group 2 (**C**) Group 3. Comparison to baseline (**D**) Group 1 (**E**) Group 2 (**F**) Group 3.*

Principal coordinate analysis (PCoA) ordination utilizing the Bray-Curtis dissimilarity metric between the different treatment groups endpoint samples and between timepoints is shown in **Figure 2-5**. To investigate the homogeneity of the microbial composition within each group and to track any change in biogeography within the collected fecal samples between all 3 groups, we compared the microbial community structures of the mice from key timepoints to day 23, day prior to phage challenge (**Figure 2-5 A)**. The β-diversity was measured utilizing the Bray-Curtis dissimilarity metric. Difference in microbial community segregation is evident by group. G1 and G2 present a lack of distinct microbial clustering, suggesting higher dissimilarities within the timepoints as opposed to between timepoints, whereas G3 presents clear clustering. Furthermore,

the ordination plots for the endpoint (**Figure 2-5 B)** demonstrate that samples collected from the endpoint (feces, duodenum, ileum, cecum) lack microbial community homogeneity within different anatomical sites. The ordination plots demonstrate that the fecal samples collected from the key timepoints in relation to day 23 lack distinct microbial clustering, suggesting higher diversity and dissimilarities within the timepoints itself as opposed to between timepoints.

As expected, the presence of the non-commensal *E. coli* strain or T7 phage did not cause any inflammatory response, as presented by the concentration of IL6 and TNF in plasma at the endpoint (**Figure S2-13**). Signals were detected from IL6 in one mouse from each of the 3 groups, which is suggestive of a random variation in immune response in different mice rather than a clear immune response to the presence of *E. coli* of T7 phage. Therefore, the changes in microbiota composition in the presence of *E. coli* and/or phage cannot be attributed to any kind of immune response. To determine if phage therapy has a direct effect on the tissue composition of the gut, histology of duodenum, ileum, and caecum should be performed. However, other studies with T7 phage have shown no inflammation effects in tissue histology.[32]

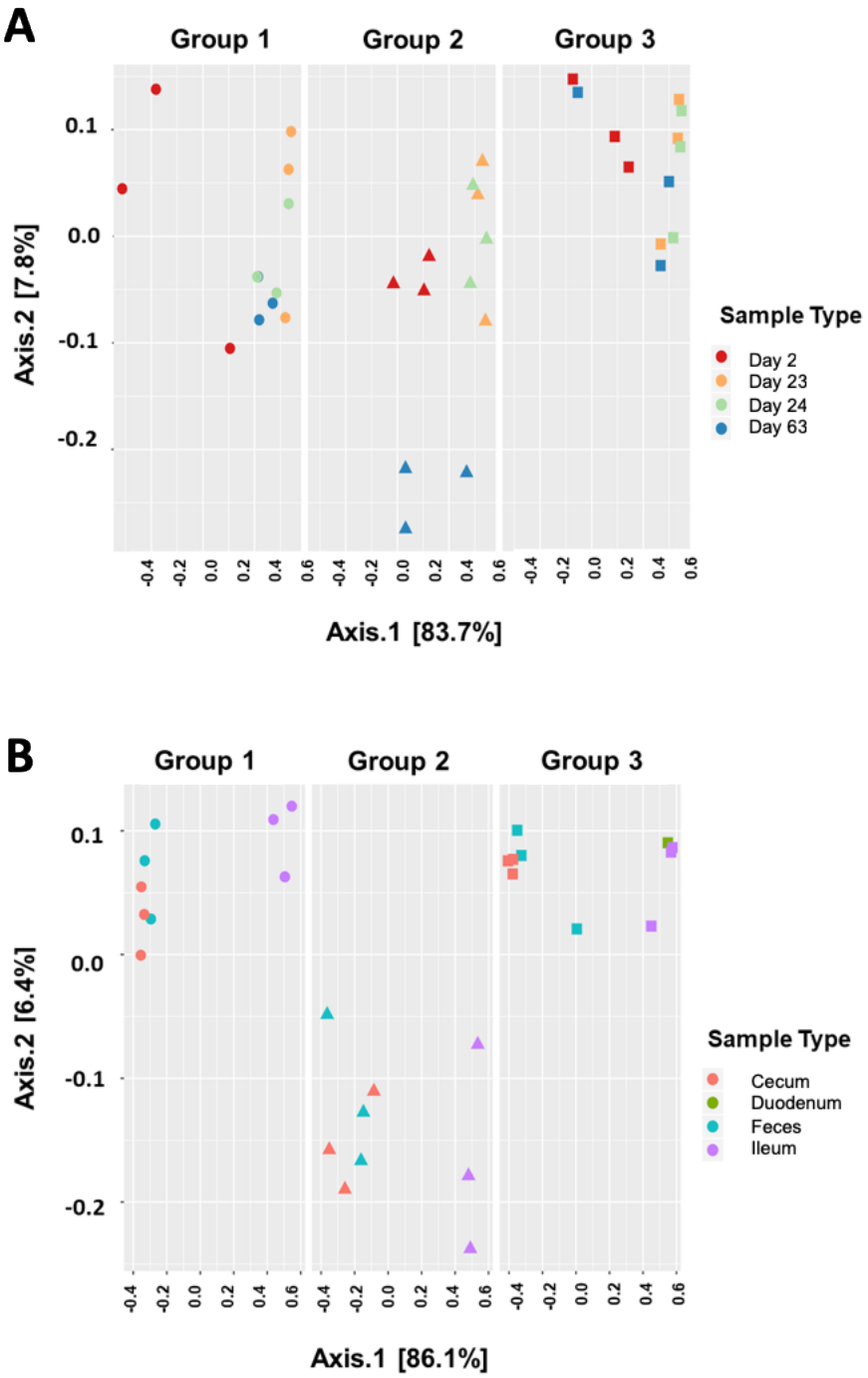

Figure 2-5: PCoA ordination utilizing the Bray-Curtis dissimilarity metric between the different treatment groups

(A) between timepoints and between (B) endpoint samples.

4.3. Diversification of Host Population *In Vivo*

We hypothesized that the coexistence of susceptible host bacteria and lytic phage in the mouse fecal samples is a result of the presence of subpopulations of host bacteria within the mouse intestines. The *E. coli* isolates from G1 and G2 where examined for fitness markers, namely growth (rate and yield) and motility (swimming and swarming). **Figure 2-6 A-D** shows growth and motility of isolated *E. coli* colonies compared to the control, a lab culture of the parental strain of *E. coli* K-12 (JM83) from the same frozen stock that was used to prepare the original inoculum for *in vivo* work. **Figures 2-6 A,B** show a clear diversification in terms of generation time and growth yield, compared to the control. In addition, the phage resistant and phage susceptible isolates are separated along the y-axis, with the phage-resistant isolates exhibiting slower growth (longer anaerobic generation times) and lower growth yields. The majority of very low growing isolates, in particular, correlate strongly with the low yield isolates. These quantitative results confirm the qualitative observations of colonies on MacConkey plates where the resistant colonies typically exhibited a small colony phenotype (**Figure 2-6 C**). A similar trend is observed for bacterial swarming motility where the T7-resistant isolates swarm faster than T7-suceptible ones (**Figure 2-6 D,F**). Swimming motility of the isolates, however, does not show a clear divide between the resistant and susceptible isolates, although the population is evidently diversified in terms of its ability to swim (**Figure 2-6 E**).

Phage predation is not the only driver of diversification in the mouse gut. Phenotypically homogeneous *E.coli* lab cultures have been reported to diversify in the mouse gut, in the absence of lytic phage predation, as a result of nutritional competition with the microbiota and acquisition of niche-specific traits.[33] To evaluate if the effect of phage predation was significant compared to niche-specific selective pressure, we compared diversification among G1 isolated colonies to that among G2 isolated colonies in terms of growth and motility. Even though signs of diversification in generation time (**Figure 2-7 A**), growth yield (**Figure 2-7 B**),, swarming (**Figure 2-7 C**), and (to a higher extent) swimming motility (**Figure S2-14 A**), could be observed in the data for G2 mice, not challenged with phage, which may be attributed to competition and niche driven diversification, when compared to G1 isolates 24 hrs after phage challenge the scatter in phenotype was significantly smaller (**Figure 2-7 D,E,F**). In addition, the G1 isolate population (T7-susceptible and resistant) were found to have higher generation times, lower yields and higher swarming motility diameters than the G2 isolates that were not challenged by T7 predation. This

confirms observations reported in **Figure 2-6** and suggests a combination of loss of growth speed and increase in swarming motility to be evolutionary beneficial for the G1 isolates (challenged by a combination of selective pressures from T7 predation and microbiota competition). The reversion rate of these mutants and the specific mutations of the close to 1500 G1 isolates, is the subject of further study in our lab.

To investigate if the origin of the observed swarming phenotype and its relation to the phenotypes with increased generation time, we looked at the aggregation plots of swarm diameter-generation time (**Figure 2-8 A-C**) and swarm diameter-swim diameter (**Figure 2-8 D-F**) for all isolates. Even though not all T7-resistant isolates are associated with high swarm diameters, evidently, certain isolates have evolved that combine both increased generation time and increased swarm diameter (**Figure 2-8 A**). These phenotypes cannot be observed in an isogenic culture (**Figure 2-8 B**) but can be seen (albeit to a much lesser extent) in the population of no-phage control isolates (**Figure 2-8 C**). Likewise, phenotypes have evolved in the T7-resistant community that combine high swarm diameter with low swim diameter (**Figure 2-8 D**); these phenotypes are not present in the isogenic host culture (**Figure 2-8 E**) and to a much lesser extent in the isolates from the no-phage control (**Figure 2-8 F**). The presence of a small number of higher swarming isolates in the no-phage control led us to investigate whether the phenotypic swarm cells were in fact resistant to T7. This experiment is currently in progress in our lab.

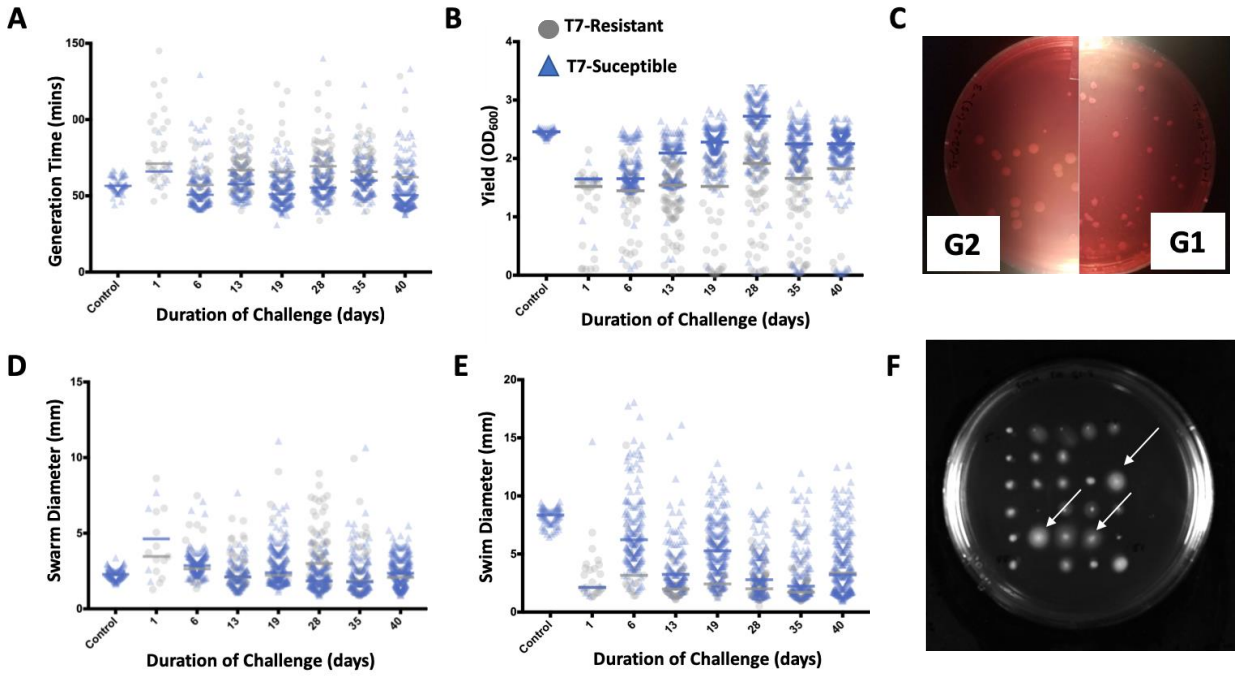

Figure 2-6: Diversification of host population into subpopulations with various fitness in G1 mice

*(**A**) Bacterial generation time for the E. coli colonies isolated at each time point from G1 mice. (**B**) Growth yield for the E. coli colonies isolated at each time point from G1 mice. (**C**) representative MacConkey plate with E. coli isolates from G2 mice compared to the colonies from G1 mice. (**D**) Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from G1 mice. (**E**) Bacterial swimming motility diameter for the E. coli colonies isolated at each time point from G1 mice. (**F**) representative swarming agar plate showing colonies of E. coli isolates from G1 mice; select resistant colonies are shown with a white arrow.*

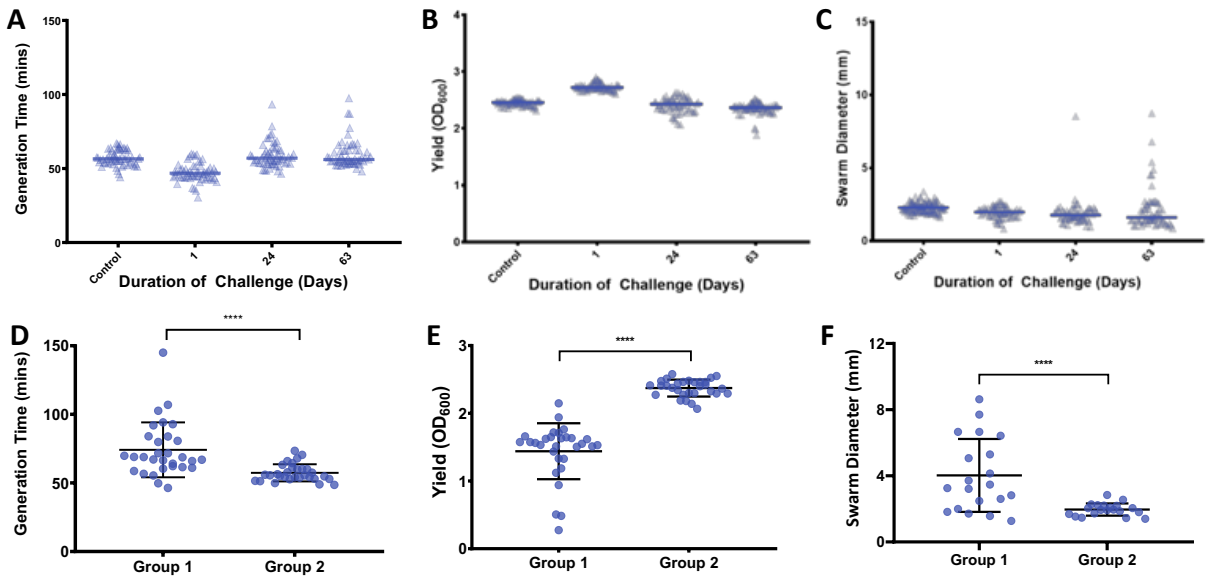

Figure 2-7: Niche vs predation-driven diversification

(A) *Bacterial generation time for the E. coli colonies isolated at each time point from G2 mice.* ***(B)*** *Growth yield for the E. coli colonies isolated at each time point from G2 mice.* ***(C)*** *Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from G2 mice. Fitness determinants related to E. coli colonies isolated from G1 and G2 mice 24 hrs after the phage challenge (for G1 mice):* ***(D)*** *Generation time,* ***(E)*** *growth yield, and* ***(F)*** *swarming motility. (*** *p-value < 0.001, **** *p-value <0.0001).*

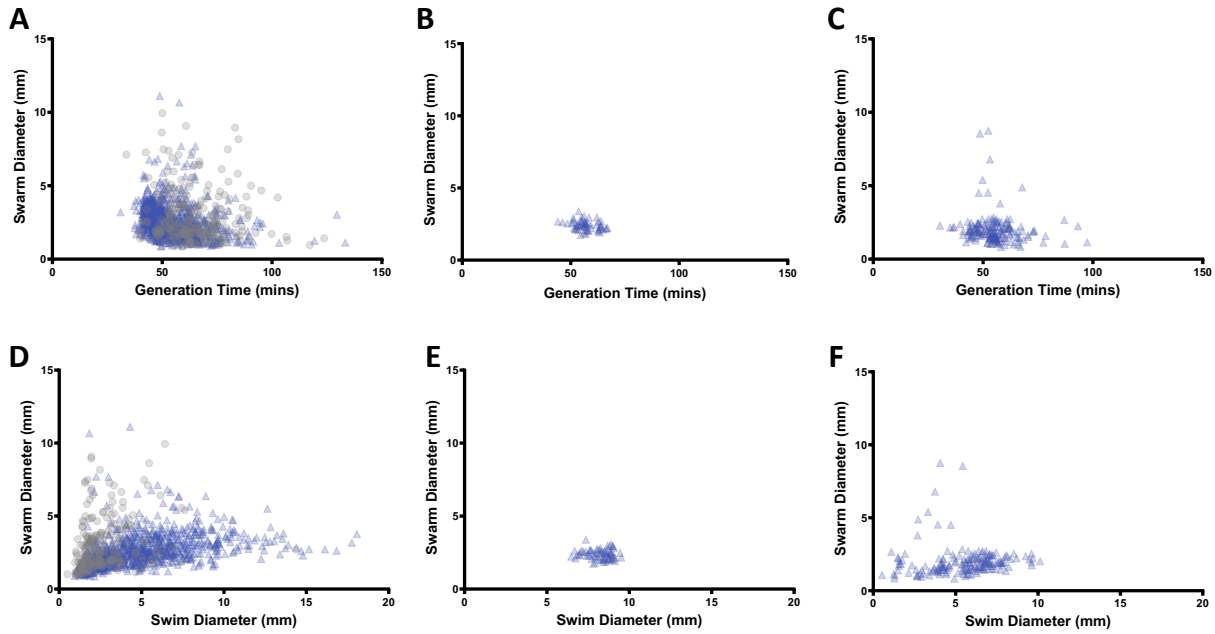

Figure 2-8: Origin of swarming phenotype

*Bacterial swarming diameter vs generation time for **(A)** control; **(B)** all E. coli colonies isolated from G1 mice; **(C)** all E. coli colonies isolated from G2 mice. Bacterial swarming diameter vs swimming diameter for **(A)** control; **(B)** all E. coli colonies isolated from G1 mice; **(C)** all E. coli colonies isolated from G2 mice.*

5. Prospectus

Our work introduces an effective model for the investigation of phage-microbiome interactions. Gnotobiotic mice colonized with an ASF consortium provide a defined and tractable system to observe and identify therapeutic interaction of lytic phage T7 with its *E. coli* host. bacteria host dynamics within the gut microbiome. Upon observation and analysis of three groups of ASF mice, evidence suggests: (i) a single dose of T7 lytic phage could effectively thrive within the gut microbiome and managed significantly decrease the population of bacterial community in the short term. (ii) Long term, phage-host arms race led to the diversification of the host population into subpopulations susceptible to evolved phage that maintained the phage population in the intestines and a T7-resistant sub population with significantly lower growth rate and yield, likely to decrease the rate of phage propagation, that maintained the host population in the intestines. In the long term these subpopulations coexisted in the mouse gut. (iii) Population and evolutionary host-phage dynamics, impacted the relative abundance of certain species in the mouse microbiota, leading to a different composition than the no-phage control. The significance of this effect on the gut symbiosis and overall mouse health, however, is unknown. What is evident is that during the length of this study, the mice did not lose weight or show signs of stress.

The knowledge generated in this investigation is the initial step in bridging the gap regarding the direct and indirect effect of bacteriophage therapeutics on the microbiota and will in turn help develop bacteriophage therapeutics as alternatives/adjuvants to antibiotics for treating infectious disease and also as agents for microbiome therapy for treating dysbiosis-related ailments. This is the first investigation of its kind and has ignited the path for future investigations regarding investigating and elucidating interactions between phage therapy and the microbiota. A follow up investigation is currently underway in our lab, investigating the genetic diversity of the bacterial isolates and the evolved T7 phage. This follow up study will shed more light into the nature of host-phage dynamics in the context of the less complex ASF microbiota.

With FDA approval of phage-based products for use in environmental prophylaxis and food preservation, regulatory approval for therapeutic use in humans is not far off. Completing these investigations is necessary to understand the potential for phage-induced population/genome evolution in host bacteria and its possible effect on the human microbiome, as the current major hurdle in obtaining regulatory approval for phage therapy. With the current attention focused on bacteriophage therapy by governmental and private organizations in North America and around the world, more studies of this kind are required to pave the way for the effective re-employment of bacteriophages within Western medicine.

6. Acknowledgements

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7. Supporting Information

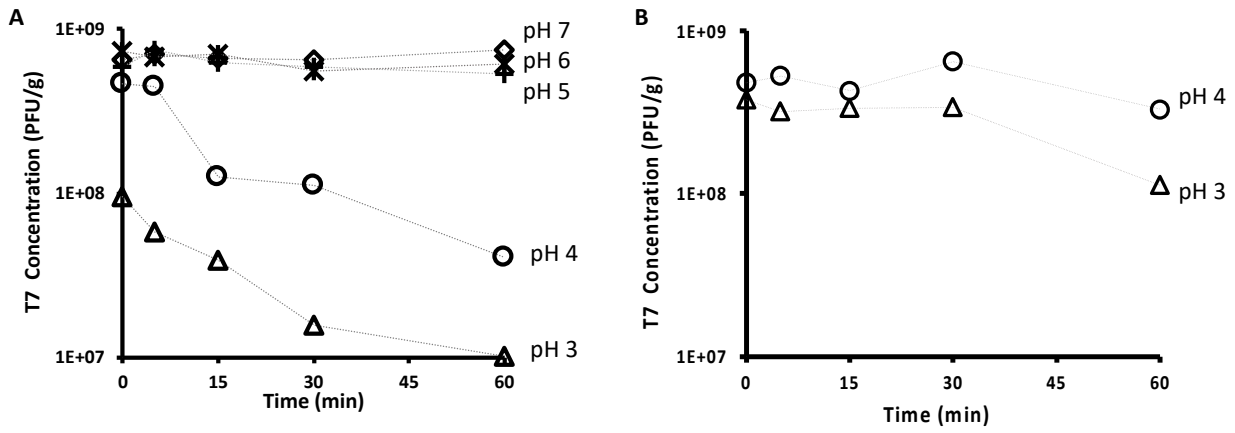

Figure S2-9: Susceptibility of T7 bacteriophage at different pH levels

(A) *Susceptibility of T7 bacteriophage at pH levels 1-7 in vitro without the addition of 0.25% $CaCO_3$ antacid, over the duration of 1 hour.* ***(B)*** *Susceptibility of T7 bacteriophage at pH levels 3 and 4 in vitro, expected pH mouse stomach, with the addition of 0.25% $CaCO_3$ antacid, over the duration of 1 hour.*

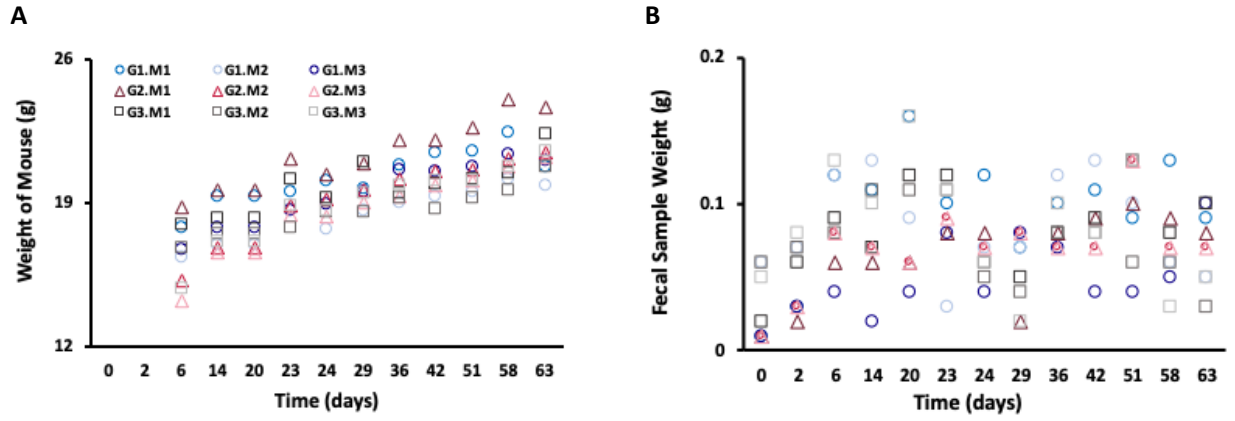

Figure S2-10: Recorded weight of each mouse and of each collected fecal sample per mouse

(A) *Recorded weight of each mouse per treatment group throughout the duration of the experiment. There were no major fluctuations in weight indicating the overall health of the mice for a premature termination.* ***(B)*** *Recorded fecal weight of each collected sample per mouse in treatment group. Fecal weights did fluctuate each week despite constant supply of low-fat diet and water. Mice were not dehydrated and did not indicate any major signs of distress during fecal sample collection.*

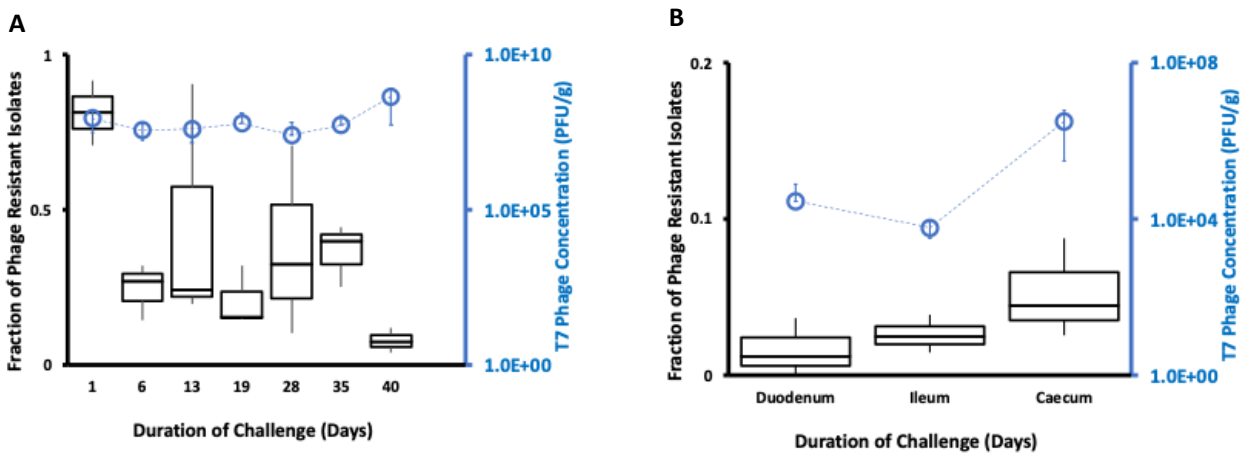

Figure S2-11: Resistance profiles of E. coli isolates

(A) *Resistance profile of E. coli isolates to parental T7 strain over the course of the 6-week T7 challenge. For comparison, the concentration of T7 in fecal samples is shown for each time point on the second axis. Day1 on the x-axis refers to the initiation of the phage challenge (day 23 of experiment).* ***(B)*** *Resistance profile of collected E. coli isolates from organs to the parental T7 strain. For comparison, the concentration of T7 in each sample is shown on the second axis.*

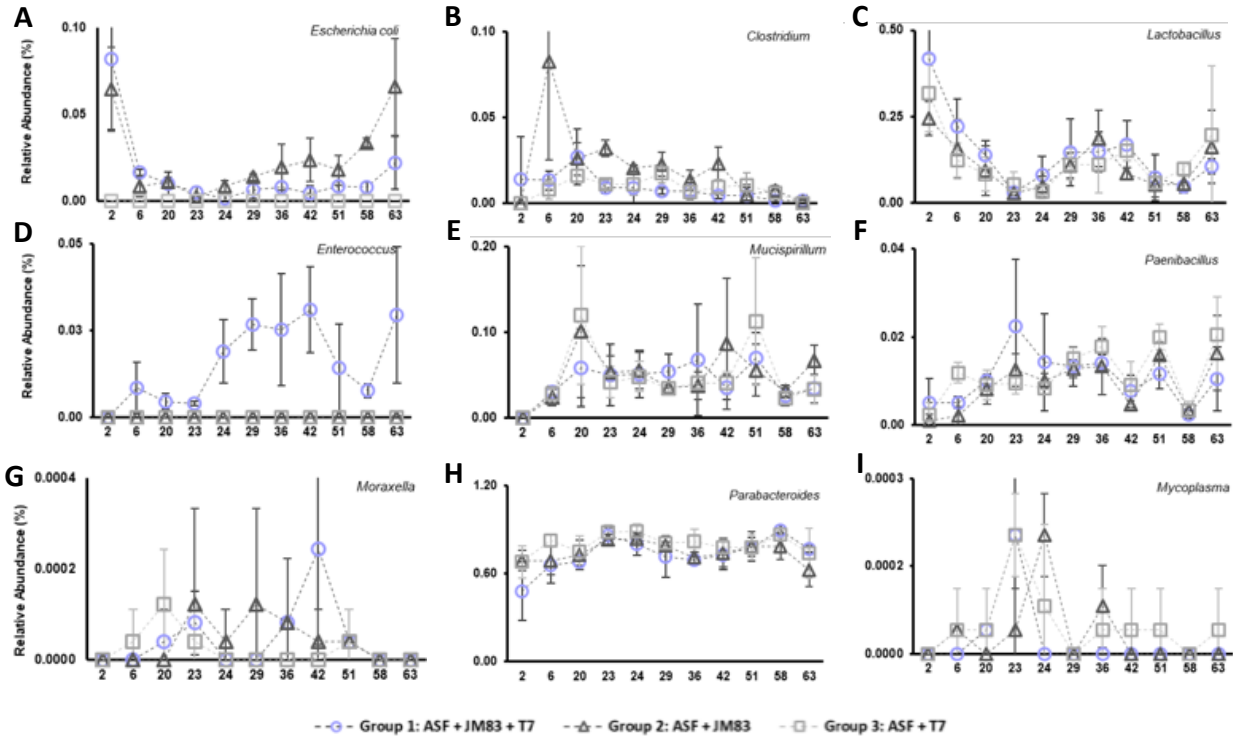

Figure S2-12: Relative abundance of 9 species (8 ASF and E. coli) in fecal samples collected from G1, G2 and G3 mice at every time point

(A) *E. coli,* ***(B)*** *Clostridium,* ***(C)*** *Parabacteroides,* ***(D)*** *Lactobacillus,* ***(E)*** *Mucispirillum,* ***(F)*** *Enterococcus,* ***(G)*** *Paenibacillus,* ***(H)*** *Moraxella,* ***(I)*** *Mycoplasma.*

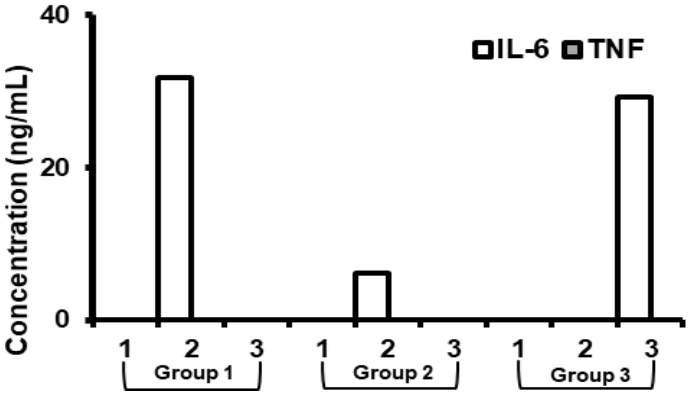

Figure S2-13: Endpoint inflammation indicating concentrations of IL-6 and TNF in each mouse from all three groups upon endpoint

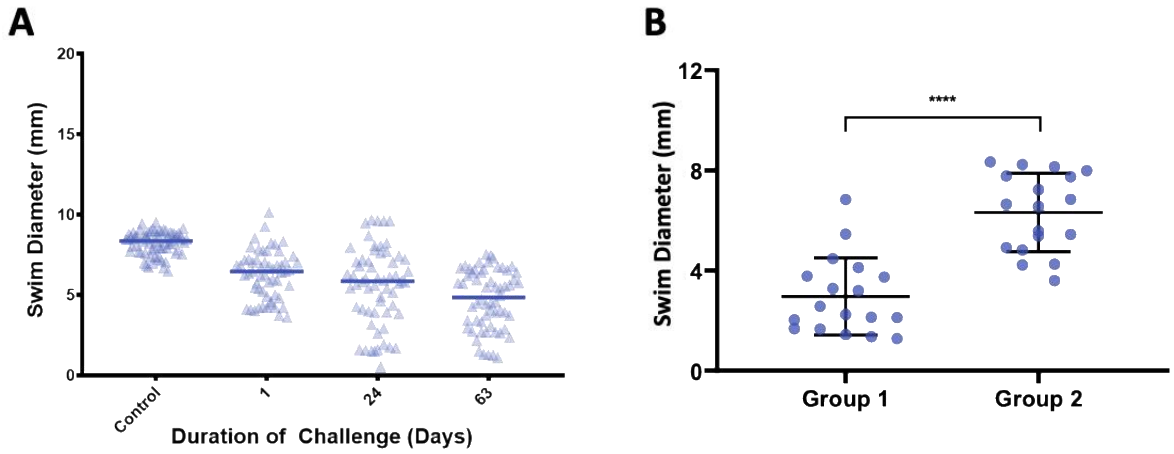

Figure S2-14- Bacterial swimming and swarming motility diameter for isolated E. coli colonies from G1 and G2 mice

*(**A**) Bacterial swimming motility diameter for the E. coli colonies isolated at each time point from G2 mice.* ***(B)*** *Swarming motility for E. coli colonies isolated from G1 and G2 mice 24 hrs after the phage challenge, (**** p-value <0.0001).*

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Chapter 3 – *In vivo* and *In vitro* Diversification of *E. coli* host Under Predation from Lytic Phage

1. Abstract

The phage-host arms race is a driver for diversity across microbial communities. However, most of what we know about this arms race, the associated community dynamics, and the resulting diversification of phage and host communities is based on insights from *in vitro* lab cultures. However, this evolutionary arms-race is played out in a complex context of physical, chemical, and physical heterogeneity, of which culture flasks in the lab are not representative. One of the complex environments of relevance to phage-host interactions is the mammalian gut, which is not only physically complex, but colonized by hundreds of commensal species, competing for resources. Phage-host interaction in the gut context is not only of interest for therapeutic applications of phage (phage therapy), but also critical for understanding the role of commensal phage in the gut microbiome. In this work, we use a model we developed in a previous report for long-term investigation of phage-host community dynamics in the context of mouse gut microbiota, germ-free mice co-colonized with the Altered Schaedler Flora (ASF) and *E. coli* K-12 (JM83), to compare community dynamics and diversification of host community to that *in vitro*. Our results demonstrate that *E. coli* K-12 communities underwent a more severe diversification *in vivo* compared to *in vitro*, highlighting the importance of evaluating phage-host community dynamics under realistic conditions, especially in the context of utilizing bacteriophages for therapeutic purposes.

2. Introduction

Bacteria-phage interactions have remained central to the evolution and ecology of microbial communities. Bacteriophages play an important role in the transfer of genetic material between bacteria and play a central role in the evolution of bacterial communities.[1] Mounting evidence points to the high incidence of phage-related sequences in bacterial genome,[2] some of which are thought to contribute to pathogenicity,[3,4] form the origin of bacteriocins,[5] or bacterial surface appendages.[6] Aside from transfer of genetic material, bacteriophages can drive evolution of the

host population by exerting selective pressure on host communities. This selective pressure is especially strong for lytic phage. In *in vitro* monoculture studies, phage resistance can evolve on the order of hours to days. Rates of bacterial phage-resistance can be as high as 10^{-3} per cell, but usually average 10^{-7}. Phage, unlike antibiotics, could also mutate to produce phage strains that are lytic towards these mutant bacteria.[7] When the bacterial host mutates, selection will favor phages that are capable of killing and replicating in these bacteria and thus evolved phages have a selective advantage.[8]

Bacteria and their corresponding lytic phages coevolve and establish equilibrium between their respective populations. Natural communities of bacteria and virulent phage will commonly exhibit phage-mediated coexistence of resistant and sensitive bacterial clones.[9] From a population-dynamic perspective, the interactions between phages and bacteria are analogous to those of a predator and a prey; communities of phage and bacteria can be remarkably stable.[10] Coexistence of phage T4 and *E. coli* has been reported for periods as long as 52 weeks.[11] These communities are often more stable than predicted by theory. Various hypotheses have been suggested to explain this stability, including coevolutionary "arms" race,[12] theory of numerical refuges,[13] physiological refuges,[14] spatial refuges,[15] or merely productivity of the environment (oscillations in nutrient supply)[16].

It appears that phage-resistant bacteria are inevitable. The interactions of lytic phage and its bacterial host will determine the success or failure of therapeutic use of lytic phage for control of infectious diseases. However, most of what we know about host-phage interactions and community dynamics is rooted in *in vitro* investigations performed in an ideal lab setting. Host-phage interactions in their natural habitat (human body or the natural environment), however, will be far from that in an idealized lab setting, and will likely be in a chemically and physically heterogenous context with multiple selective pressures from the competing microbes, cells, and the niche.

The mammalian gut is one such complex landscape, with hundreds of microbial species competing or resources in a complex chemical, physical and mechanical setting, which could affect the co-evolution, leading to an outcome other than that observed in a typical lab culture.[17-20] There are currently very few investigations comparing phage-host co-evolution and resulting host diversification in the gut environment with that *in vitro*.

In this work, we used a model we had developed previously for investigating the community dynamics of a mock infection with virulent phage in the context of the gut microbiota, to investigate and compare diversification of host populations in the mouse gut, compared to that *in vitro*. Our *in vivo* model consisted of a gnotobiotic mouse model colonized with an Altered Schaedler Flora (ASF), a consortium of eight known bacterial species, providing a simplified defined and traceable model of the gut microbiota. The ASF community was co-colonized with the non-pathogenic bacterium *Escherichia coli* K-12 (JM83), which was then challenged with the lytic bacteriophage T7. Our *in vivo* model was a liquid culture co-incubated with phage undergoing 3 weeks of experimental evolution.

3. Methods

3.1. Gnotobiotic Mouse Husbandry

Germ-Free mice belonging to the C57BL/6 inbred strain were housed in plastic, gnotobiotic, individually vented isolators and fed a regular, autoclaved, low-fat chow diet with a constant supply of autoclaved water in the cage. Each therapy group of mice (n=3) were age-matched and specifically consisted of two male mice housed together in one cage isolator, and one female mouse housed in a separate cage isolator. At 8 weeks of age, all mice were colonized with a single 100uL oral gavage of the prepared ASF culture. Mice were categorized into three groups; mice from group one and group two were also immediately inoculated with *E. coli* K-12 (JM83) post ASF gavage. Three weeks post initial inoculation, mice from group one and group three were inoculated with T7 phage and 0.25% $CaCO_3$ as antacid. Each week, mice were weighed and inspected for signs of disease, then fecal samples were collected form mice and immediately transferred into anaerobic jars, transferred to anaerobic chamber and processed. Mice were sacrificed after 9 weeks by cardiac bleed and cervical dislocation and the organs were removed aseptically. The process is demonstrated in **Figure 3.1**.

3.2. Inoculation of ASF into Germ-Free Mice

The eight microorganisms composing the ASF consortium are all derived from mice and have been stably passed through multiple generations in gnotobiotic mice continually bred in isolators. Contents from the cecum of a healthy ASF mice was collected using sterile scissors and forceps. Caecal contents were added to a 15mL sterile conical tube containing 10mL sterile PBS and 0.5g/L sterile filtered L-cysteine. This was then stored in a prepared anaerobic container. Each mouse was

gavaged with 100 μL of the diluted caecal contents at 8 weeks of age, using a sterile syringe and gavage needle.

3.3. Preparation of *E. coli* Inoculum

E. coli strain K-12 (JM83), containing a streptomycin resistance cassette, inoculated from frozen glycerol stock, was anaerobically growth in 50 mL fresh Tryptic Soy Broth (TSB, equilibrated for 12 hrs in the anaerobic chamber prior to inoculation) to exponential phase (OD_{600} ~ 0.3). This was then aliquoted into 10 mL conical tubes and washed 3 times with PBS (Centrifuged at 4℃, 7000rpm for 5 mins. Supernatant was discarded, and pellet was resuspended in 3mL of fresh sterile PBS to wash). A 100 μL aliquot of washed bacteria (~ 10^9 CFU/mL) was then transferred into 1.5 mL sterile microcentrifuge tubes and placed into sterile 50 mL conical tube and transferred in an icebox to mouse inoculation site. For infection, each mouse of group 1 and group 2 were orally gavaged using 100 μL of the sterile bacterial inoculum at 8 weeks of age, immediately after ASF inoculation, using a sterile syringe and gavage needle.

3.4. Preparation of Phage Challenge

A 1:100 overnight culture of *E. coli* strain K-12 (JM83) was added to an autoclaved baffled flask containing with 150 mL fresh TSB and grown aerobically to mid exponential phase (OD_{600} ~0.4) in a shaking incubator (210 rpm, 37°C). A 10 μL aliquot of a 10^9 PFU/mL stock of T7 phage was then added to the host culture and incubated aerobically overnight. The propagated crude lystate was aliquoted into sterile 50 mL conical tubes and centrifuged (4°C, 5000×g, 10 min) to pellet bacteria and subsequently purified as described by Sambrook et al.[21] Briefly, the supernatant was sterile-filtered using a 0.2µm filters, then mixed with 1/6th volume of 20% polyethylene glycol/2.5M NaCl and incubated overnight at 4°C. Post-incubation, phage was pelleted (4°C, 5000×g, 45 min) and supernatant was discarded. Phage pellet was resuspended in 10 mL SM buffer (100mM NaCl, 8mM $MgSO_4$, 0.01% Gelatin, 50mM Tris-HCl) and rocked for 3 hrs at 4°C to resuspend pellet. To remove residual bacteria debris, phage was centrifuged again for 10 min and supernatant was collected. A 250 μL aliquot of purified phage was then mixed with 0.25% w/v, final concentration, of $CaCO_3$ as antacid). This was then added to 750 μL purified phage and thoroughly vortexed. A 100 μL of phage-antacid inoculum was administered to each mouse in groups one and three via oral gavage, 3 weeks post ASF/ASF-*E.coli* inoculation.

3.5. Sampling and Quantifying Bacteria from Fecal samples and Intestinal Contents

Approximately 2-4 fecal pellets from each mouse were collected at every timepoint and transferred anaerobically to an anaerobic chamber. Contents were then immediately homogenized with 500 μL of fresh TSB. Small intestine sections and the cecum were aseptically extracted from the mouse after sacrifice. Contents were immediately transferred into 2 mL sterile plastic screw top tubes containing 0.2 g of 4.8 mm ceramic beads to extract contents.

Aliquots of 300 μL of the homogenized fecal content (per mouse) or homogenized intestinal content was added into a 2 mL sterile plastic screw top tube containing 800 μL of 200 mM of monobasic $NaPO_4$ (pH 8), 100 μL of guanidine thiocyanate (GES) buffer, and 0.2 g of 2.8 mm ceramic beads (Mo Bio Laboratories, #13114-50). All contents were vortexed and sent to Macmaster MOBIX facilities for DNA isolation and amplification. Aliquots of the homogenized fecal content (100 μL per mouse) were then serially diluted and plated, using autoclaved glass beads, on MacConkey agar plates, MacConkey agar plates supplemented with 50 μg/mL of streptomycin, and BHI (Brain Heart Infusion) agar plates for preparation of stocks (see 3.9). All steps post fecal collection was performed in an anaerobic chamber using pre-equilibrated media in the anaerobic chamber. All BHI plates were incubated anaerobically. Bacterial colonies from the BHI plates were pooled together after 48 hrs of incubation by flooding the plates with BHI media. The pooled bacterial mixture was mixed with 20% skim milk solution (1:1), loaded in a 1.5 mL cryopreservation tight screw tubes and stored at -80℃.

MacConkey plates (with or without antibiotic) were incubated aerobically and used for counting colonies of *E. coli* K-12 (JM83) to determine bacterial concentration per gram of fecal matter (CFU/g). These plates were further used for isolating *E. coli* colonies for phenotypic analysis.

3.6. Phage Enumeration

Aliquots of 100 μL of homogenized fecal content or homogenized intestine contents were suspended in 900 mL of fresh sterile TSB, vortexed to mix, and then centrifuged (5 min, 4℃, 7000 rpm) to separate bacteria from supernatant. The supernatant was then collected and stored as *in vivo* evolved T7 phage, which was quantified using agar overlay technique as describes elsewhere, using 100 μL of *E. coli* K-12 (JM83), parental strain.[22] Plaques were then counted to quantify phage titer per gram of fecal matter (PFU/g).

3.7. Isolation of *E. coli* colonies

Bacterial colonies were isolated from samples of fecal matter from each time point and endpoint intestinal content that plated on MacConkey and MacConkey-Streptomycin agar plates (30-50 colonies per mouse, per media, per time point). Each collected colony was set to a specific identification number for record keeping. Initial colony morphology was recorded per colony. Each colony was then cultured in TSB aerobically and the overnight culture of each isolate was mixed with 50% glycerol solution (1:1) and stored as 200 μL aliquots in microtiter plates and at -80℃.

3.8. Experimental Evolution

An *in vitro* system was utilized for 3 weeks under anaerobic conditions to mimic the anaerobic condition of the gut microbiome. A MOI of 0.1 was used in this system to obtain a similar MOI to that of the *in vivo* system after stabilizing. To achieve this, 10mL of *E. coli* K-12 JM83 was grown anaerobically to 10^8 CFU/mL, and then 100µL of polyethylene glycol (PEG) purified 10^9 PFU/mL, T7 bacteriophage was added to the system. Simultaneously a no-phage control was also in placed in the incubator. This system was monitored and sampled anaerobically. Every 48hrs, a third of the media was supplemented with fresh deoxygenated TSB. Samples was collected every 48 hrs for selective culturing, phage and colony isolation, and further controlled experiments.

3.9. Phage Susceptibility and Fitness Characterization of Isolates

Phage susceptibility. Bacterial isolates were analyzed for susceptibility against both the parental strain of T7 bacteriophage, as well as the evolved bacteriophage isolated from fecal/endpoint organ samples, using the streak test, as described elsewhere.[22] Briefly, 10 μL droplets of phage (parental T7 = 1×10^9 PFU/mL, experimental evolved = stock diluted 1:10 in SM buffer) were dripped down a straight-line top to bottom, on LB agar plates and left to dry. Overnight cultures of collected *E. coli* colonies were then streaked across the dried phage teardrop using multichannel pipette (5 mutants per plate) and incubated at 37 ℃. Results were recorded after 24 hrs of aerobic incubation at 37℃. Susceptibility was determined based on inhibition of growth of the streaked *E. coli* line.

Growth Rate/Yield. Anaerobic growth curves were prepared for each bacterial isolate using the kinetic cycle on BioTek Synergy Neo Plate Reader (OD_{600}, 16 hrs, 37℃, shaking, absorbance reading every 10 min). Growth Rate and growth yield was calculated based on the slope of this curve at logarithmic phase. Growth yield was determined as OD_{600} at stationary phase.

Motility. Swim agar was made with 0.3% BD Bacto Agar (214010), 1% tryptone (VWR J859), 0.5% Yeast Extract (VWR J850), and 0.5%NaCl, autoclaved and used fresh. Swarm Agar was made with 0.5% agar-BD Bacto Agar, 1% tryptone, 0.5% Yeast Extract, 0.5% NaCl, 0.5% Dextrose (VWR 0188), autoclaved and used fresh. Overnight cultures of E. *coli* isolates were stamped slightly under the surface of the agar for swim, and on top of the surface for swarm, using custom-made replica stamp pin.

3.10. Determination of T7 Latent Period, Burst Size, and Mutation Rate

One-Step Growth Curve. To investigate and analyze the relationship between lytic phage T7 and host bacteria *E. coli* JM83, a one-step growth curve was completed as per Ellis and Delbruck. Analyzing this single-step growth experiment phage, we were able to determine the number of infective phage particles released from the bacteria at various intervals via plaque assay. Upon plotting the released bacteriophages from the host *E. coli* cells versus time shows distinct phases, including the latent period, rise period, and plateau which is used to determine the burst size. In specific, the latent period is immediately following phage addition, which is then followed by the rise period (burst) – the phase when the host cells rapidly lyse, in turn releasing infective phages. As the plateau is reached, the total number of phages released is used to calculate the burst size- the number of viruses produced per infected cell. **Mutation Rate.** The Mutation rate of exponential phase *E. coli* JM83 with T7 bacteriophage was calculated utilizing a MOI of 0.1. Colonies were counted 24 hours after incubation to determine mutation rate in aerobic conditions, and 48 hours for anaerobic conditions.

3.11. Statistical Analysis

All data presented is the average of at least three independent mouse models per treatment group, or three independent experiment replicates, presented along with the standard deviation between values obtained for the independent experiments. Statistical significance of differences was tested using t-test and P values lower than 0.05 were chosen as the cutoff for significant difference. P values less than 0.05 are annotated with one asterisk, P values less than 0.01 are annotated with two asterisks, *P* values less than 0.001 are annotated with three asterisks, and P values less than 0.0001 are annotated with four asterisks.

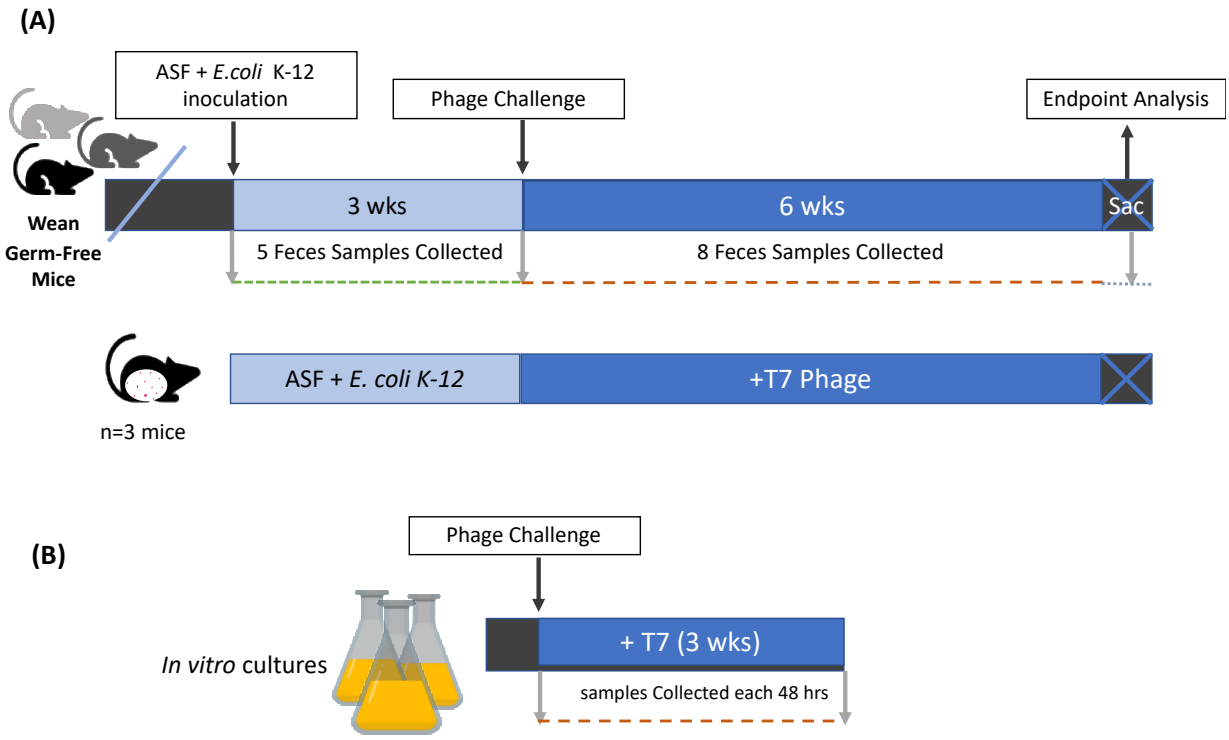

Figure 3-1: Schematic Diagram of Experimental Design

(A) *two groups of 8-week-old C57BL/6 mice (each mouse indicates n=3 per treatment group) were gavaged with ASF culture (+E. coli K-12 (JM83)). Day 0 refers to the initial inoculation of ASF culture (+JM83 E. coli) to the Germ-Free mice. Phage challenge started 3 weeks after inculcation and the mice were sacrificed 9 weeks after initial inoculation. Fecal samples were collected frequently all through the duration of the study. All mice were inoculated with the ASF culture and 100µL of E. coli K-12 (JM83) (10^9 CFU/mL) prepared from an exponential phase culture and washed with PBS. At the three-week mark (day 23), mice from G1 were inoculated with 100µL of T7 phage (10^9 PFU/mL) suspended in SM buffer and 0.25% $CaCO_3$ as antacid. All mice were sacrificed, and tissues harvested for further sampling on day 63.* ***(B)*** *Experimental evolution was conducted in vitro at a MOI of 0.1 Samples were collected and processed every 48 hours for 3 weeks.*

4. Results and Discussion

4.1. *In Vitro* vs *In Vivo* Phage-Host Community Dynamics

The experimental design consisted of three groups of germ-free C57BL/6 mice (n=3 per group.) Each group of mice was caged separately in gnotobiotic isolators. All mice were orally gavaged with the Altered Schaedler Flora (ASF) consortium consisting of eight known bacteria (SI-Dataset1) at eight weeks of age. Mice from the first group and second group were also gavaged with 100µL of 10^9 CFU/mL *E. coli* K-12 (JM83) prepared from exponential phase and washed.

An *in vitro* system was utilized for 3 weeks of experimental evolution under anaerobic conditions to mimic the anaerobic condition of the gut microbiome. A MOI of 0.1 was used in this system to obtain a similar MOI to that of the *in vi*vo system. To achieve this, 10mL of JM83 Escherichia coli grown anaerobically to 10^8, and then 100µL of polyethylene glycol (PEG) purified 10^9 T7 bacteriophage was added. Simultaneously a similar system for a JM83 control was also in place without the addition of T7 bacteriophage. This system was monitored anaerobically, and a third of the media was supplemented with fresh anaerobic Tryptic Soy Broth (TSB) every 42 hours. A sample was collected every 42 hours for selective culturing and further controlled experiments.

Figure 3-2 A shows the change in *E. coli* and T7 concentration *in vivo* with time. G2 (no-phage control) exhibited a stable *E. coli* concentration throughout the 9 weeks. G1 mice initially had *E. coli* concentrations similar to G2 but showed a drastic drop of ~6.5 Logs in *E. coli* concentration 24 hrs after an aggressive phage challenge. The *E. coli* concentration recovered slowly, but consistently remained ~1-2 logs less than the *E. coli* concentration in G2, not challenged with phage. Despite the presence of a stable community of *E. coli* in fecal samples, T7 phage also persisted in fecal samples from G1 mice at an average concentration of 3×10^7 PFU/gr, indicating the presence of a community of susceptible host cells in the gut at any time point. (As per Chapter 2 manuscript) The *in vitro* culture shows a similar trend (**Figure 3-2 B**), with the no phage control maintaining a stable community during the course of three weeks, but the E. coli challenged with phage dropping 5 Logs of titer immediately, before stabilizing slowly to whiting 2 Logs of the control population. All the while, T7 phage persisted in this culture at a relatively high titer, albeit experiencing a drop in the first 5 days, likely due to formation of T7-toelrant sub-communities in the liquid culture.

Examining the fraction of phage-resistant *E. coli* colonies reveals a more complex picture. Even though immediately after starting the phage challenge, near to 100% of the isolated colonies were resistant to evolved phage (**Figure 3-2 C**), the resistant fraction drops drastically with time, showing a diversified population of susceptible and resistant isolates (to both the evolved and the parental T7 populations), before stabilizing at ~30% resistance at the endpoint, 6 weeks after starting the phage challenge. Interestingly, the T7 population maintained a stable concentration through the duration of the experiment, despite the significant change in the population of susceptible bacteria. Interestingly, in the *in vitro* culture, the fraction of isolates resistant to the

parental or evolved T7 increases consistently with time, with the initial time points showing a clear trend towards resistance to evolved T7, indicating a fierce arms-race in the liquid culture.

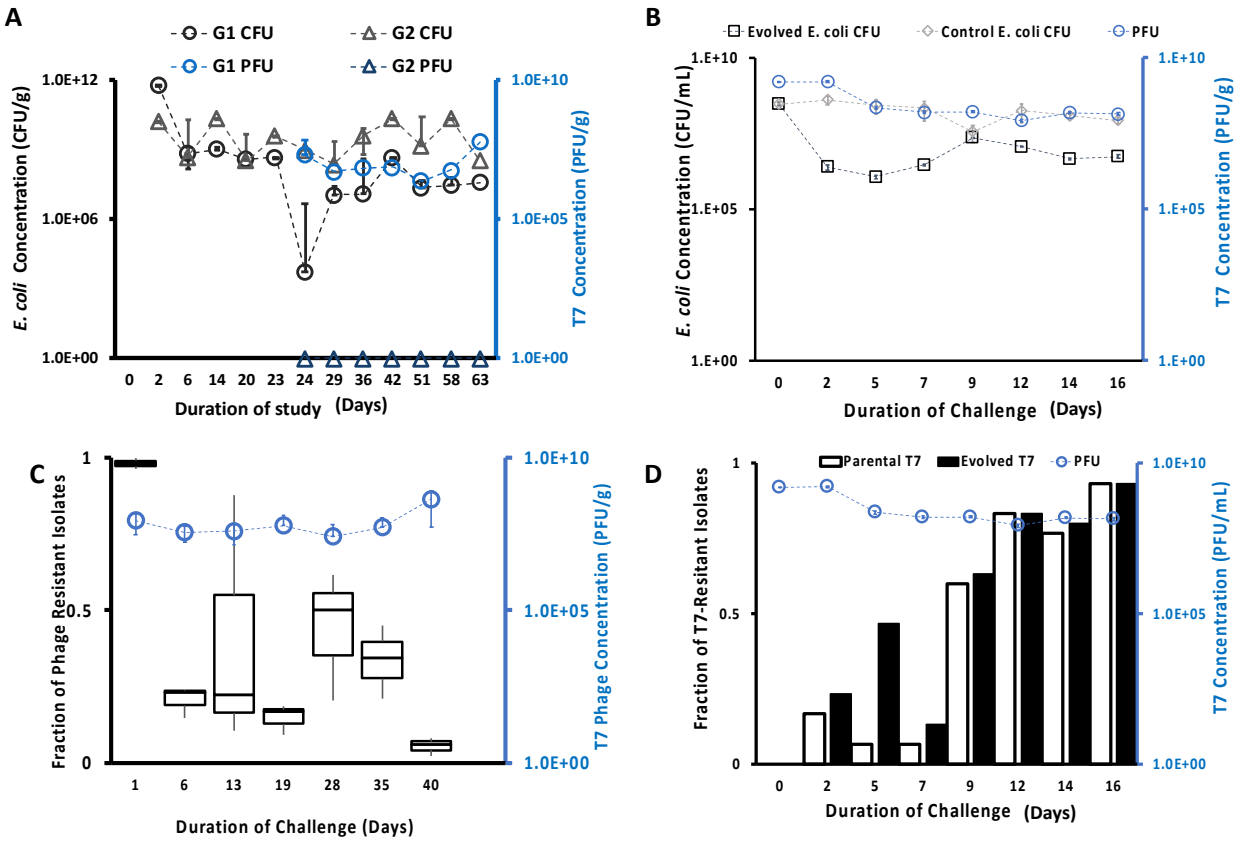

Figure 3-2: Bacterial Host-phage Community Dynamics in vivo and in vitro

(A) *Change in the concentration of E. coli K-12 JM83 (CFU/g) and T7 Phage (PFU/g) of the three groups of mice over the course of 9 weeks. Each data point presents the average of three mice in each group with the standard deviations presented as error bars. The highlighted time point (day 24) is the first sampling point after phage challenge.* ***(B)*** *The concentration of bacteria and phage in an in vitro evolutionary experiment at a MOI of 0.1.* ***(C)*** *Resistance profile of E. coli isolates to evolved T7 strain over the course of the 6-week T7 challenge. For comparison, the concentration of T7 in fecal samples is shown for each time point on the second axis. Day1 on the x-axis refers to the initiation of the phage challenge (day 23 of experiment).* ***(C)*** *Resistance profile of E. coli isolates to evolved and parental T7 strain over the course of the 3-week T7 in vitro challenge. For comparison, the concentration of T7 in the flask is shown for each time point on the second axis.*

4.2. Diversification *In Vitro* and *In Vivo*

The *E. coli* isolates from G1 and G2 where examined for fitness markers, namely growth (rate and yield) and motility (swimming and swarming). **Figure 3-3 A-C** and **Figure S3.6A** show growth and motility for isolated *E. coli* colonies compared to the control, a lab culture of the parental strain of *E. coli* K-12 (JM83) from the same frozen stock that was used to prepare the original inoculum for *in vivo* work. **Figures 3-3 A,B** show a clear diversification in terms of generation time and growth yield, compared to the control. In addition, the phage resistant and phage susceptible isolates are separated along the y-axis, with the phage-resistant isolates exhibiting slower growth (longer anaerobic generation times) and lower growth yields. The very slow growing isolates, in particular, correlate strongly with the low yield isolates. A similar trend is observed for bacterial swarming motility where the T7-resistant isolates swarm faster than T7-suceptible ones (**Figure 3-3 C**). Swimming motility of the isolates, however, does not show a clear divide between the resistant and susceptible isolates, although the population is evidently diversified in terms of its ability to swim (**Figure S3-6 A**).

The *in vitro* population, interestingly, exhibits the same trends but shows much less diversification in terms of its phenotypes. Towards day 9, post challenge, isolates from the *in vitro* culture became predominantly resistant. Thus, the generation time of the population increased (**Figure 3-3 D**) and the growth yield decreased (**Figure 3-3 E**). In addition, swarming motility increased after day 9 with increase in population of T7-resistant colonies (**Figure 3-3 F**). Although the *in vitro* community was evidently diversified compared to the control, compared to the *in vivo* isolates, the diversification was minimal.

To evaluate if the effect of phage predation was significant compared to niche-specific selective pressure, we compared diversification among G1 isolated colonies to that among G2 isolated colonies in terms of growth and motility. Even though signs of diversification in generation time (**Figure 3-4 A**), growth yield (**Figure 3-4 B**), swarming (**Figure 3-4 C**), and (to a higher extent) swimming motility (**Figure S3-7 A**), could be observed in the data for G2 mice, not challenged with phage, which may be attributed to competition and niche driven diversification, when compared to G1 isolates 24 hrs after phage challenge, The scatter in phenotype was significantly smaller. The *in vitro* cultures exhibit a similar trend of low diversification and phenotypic characteristics that are bundled tightly around a community average (**Figure 3-4 D,E,F**), with a

similar level of scatter to the *in vivo* isolates. This observation suggests that predation from lytic phage is the driving factor for the observed *in vitro* and *in vivo* diversification in **Figure 3-3**.

Figure 3-5 further highlights these trends. When comparing *in vitro* and *in vivo* populations challenged with phage for the same amount of time (in this case one week), *in vivo* cultures are significantly more diversified than *in vitro* cultures in terms of growth rate (**Figure 3-5A)**, and swarming motility (**Figure 3-5 C**), and slightly more diversified in terms of growth yield (**Figure 3-5 B**). However, when comparing no-phage controls, in vivo and in vitro, no significant difference can be observed in terms of diversification of growth or motility (**Figure 3-5 D, E, F**).

In addition, we looked at the aggregation plots of swarm diameter-generation time (**Figure S3-8 A-C**) and swarm diameter-swim diameter (**Figure S3-8 D-F**) for all isolates. Evidently, certain isolates evolved *in vivo* that combine both increased generation time and increased swarm diameter (**Figure S3-8 B**). These phenotypes cannot be observed in an isogenic culture (**Figure S3-8 A**), but can be seen (albeit to a much lesser extent) in the population of *in vitro* T7-resistant isolates (**Figure S3-8 C**). Likewise, phenotypes have evolved in the T7-resistant community that combine high swarm diameter with low swim diameter (**Figure S3-8 E**); these phenotypes are not present in the isogenic host culture (**Figure S3-8 D**) and to a much lesser extent in the isolates from the *in vitro* culture (**Figure S3-8 F**). The presence of a small number of higher swarming isolates in the no-phage control led us to investigate whether the phenotypic swarm cells were in fact resistant to T7. This experiment is currently in progress in our lab.

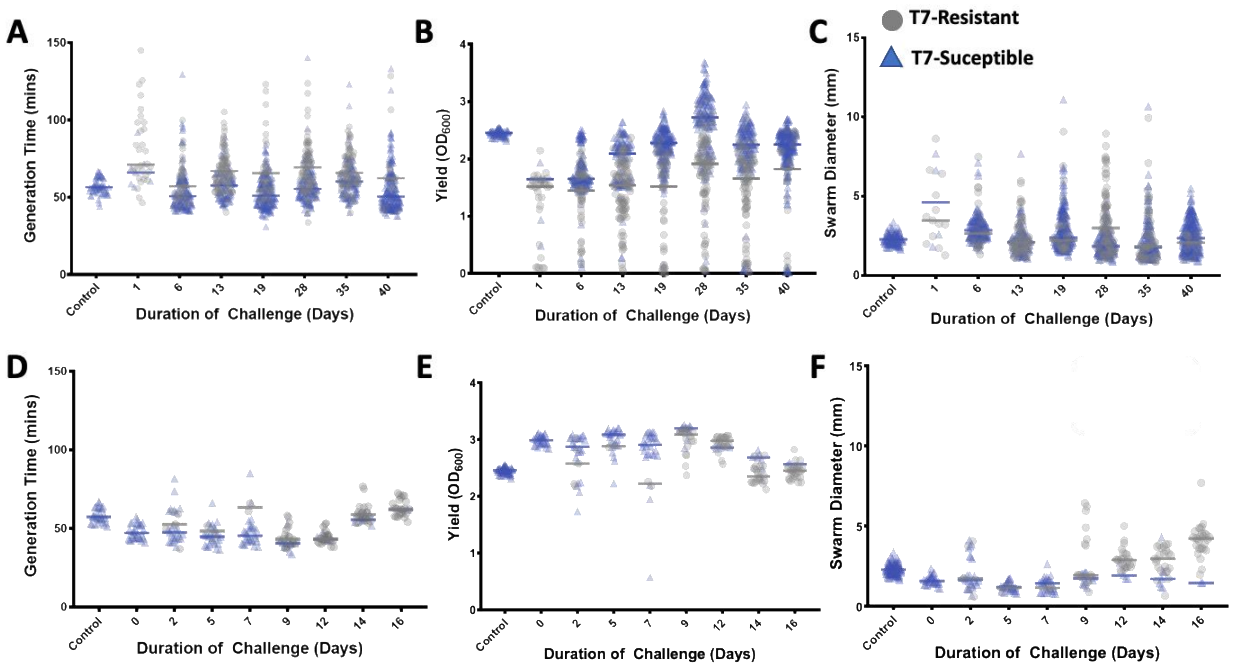

Figure 3-3: Diversification of host population into subpopulations with various fitness in vitro vs in vivo

(A) *Bacterial generation time for the E. coli colonies isolated at each time point from G1 mice.* ***(B)*** *Growth yield for the E. coli colonies isolated at each time point from G1 mice.* ***(C)*** *Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from G1 mice.* ***(D)*** *Bacterial generation time for the E. coli colonies isolated at each time point from in vitro culture challenged with phage.* ***(E)*** *Bacterial growth yield for the E. coli colonies isolated at each time point from in vitro culture challenged with phage.* ***(F)*** *Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from in vitro culture challenged with phage.*

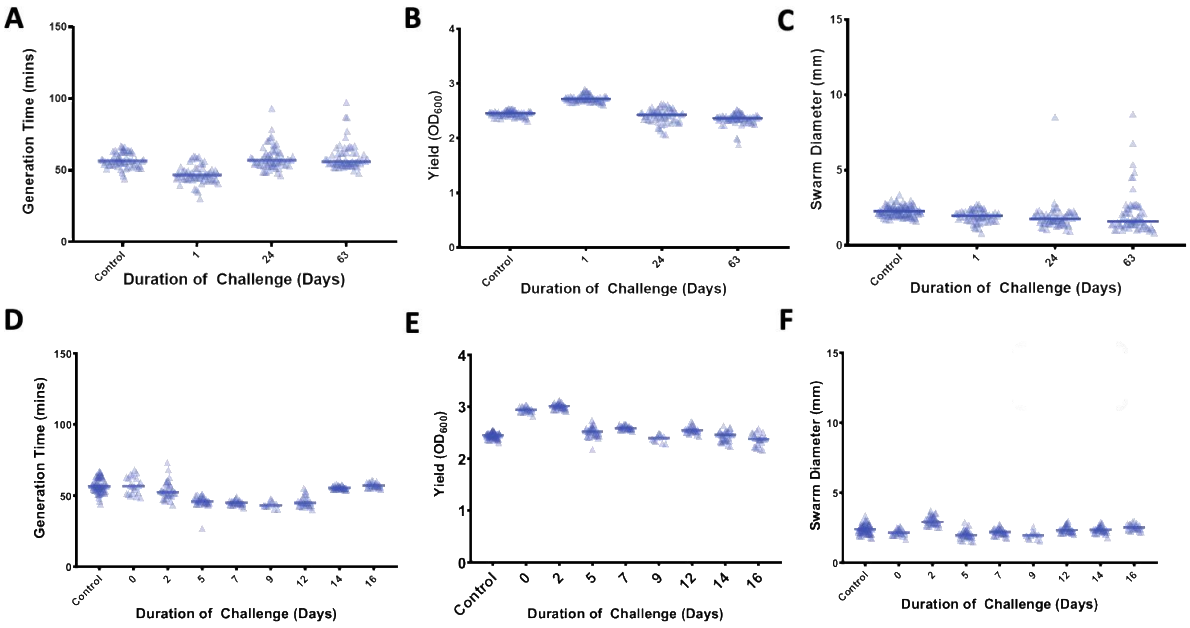

Figure 3-4: Diversification of no-phage controls

(A) *Bacterial generation time for the E. coli colonies isolated at each time point from G2 mice.* ***(B)*** *Growth yield for the E. coli colonies isolated at each time point from G2 mice.* ***(C)*** *Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from G2 mice.* ***(D)*** *Bacterial generation time for the E. coli colonies isolated at each time point from in vitro culture no phage controls.* ***(E)*** *Growth yield for the E. coli colonies isolated at each time point from in vitro culture no phage controls.* ***(F)*** *Bacterial swarming motility diameter for the E. coli colonies isolated at each time point from in vitro no phage controls.*

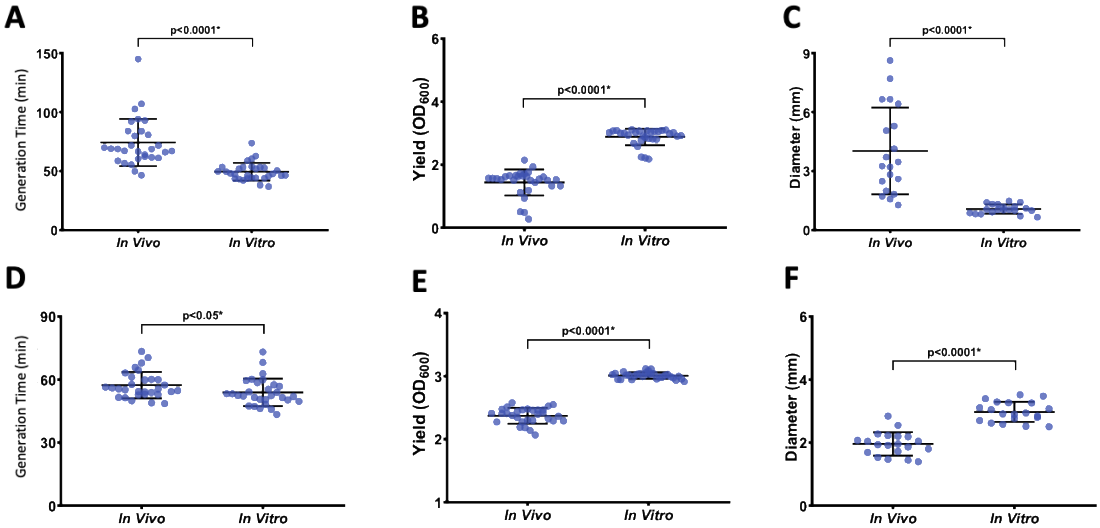

Figure 3-5: Fitness determinants related to E. coli colonies isolated from G1 mice (in vivo) and in vitro lab culture a week after the phage challenge

***(A)** Generation time, **(B)** growth yield, and **(C)** swarming motility. Fitness determinants related to E. coli colonies isolated from G2 mice (in vivo) and in vitro lab culture without phage: **(D)** Generation time, **(E)** growth yield, and **(F)** swarming motility.*

5. Prospectus

Investigating community dynamics of T7 lytic phage with its bacterial *E. coli* K-12 (JM83) host in both an *in vivo* as well as an *in vitro* system, we can conclude that the outcome of the two systems is significantly different, with the *in vitro* model providing a more toned-down picture of both phage-host dynamics, diversification, and coexistence. In specific, although we saw similar a similar dynamic relationship between the host *E. coli* K-12 JM83 bacteria and T7 phage in the sense that mutants had diversified as a result of phage predation, the extent of diversification *in vivo* in terms of growth and motility of the isolates was significantly higher than that *in vitro*. The *in vivo* gut environment, being highly heterogeneous in terms of both chemistry, biology and physical landscape, allows for a more sophisticated dynamic with enough heterogeneity to act as a driving force for a much longer time.

A gnotobiotic *in vivo* model is effective and beneficial to study the phage-bacterial dynamics in a more mechanistic manner. Whereas, we can utilize this *in vitro* system for cost effective fast-paced testing. A good applicable example of this is for determining a necessary phage needed as a therapeutic against a pathogenic bacterium residing within a patient. It is well known that *in vitro* cultures fail at predicting the required therapeutic does of lytic phage. Hence an *in vivo* model could be very useful in that context. An in interesting, but complicated problem that can be tackled with this model is the competition and co-existence of multiple lytic phage against the same host or the coexistence of a mixed bacterial community with lytic phage predators. Such investigations will contribute to an in-depth understanding and profiling of how lytic phage will behave inside the body, thus allowing us to predict and design phage therapeutics that can act in synergy with the human microbiota and immune system to fight infections.

6. Acknowledgments

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7. Supporting Information

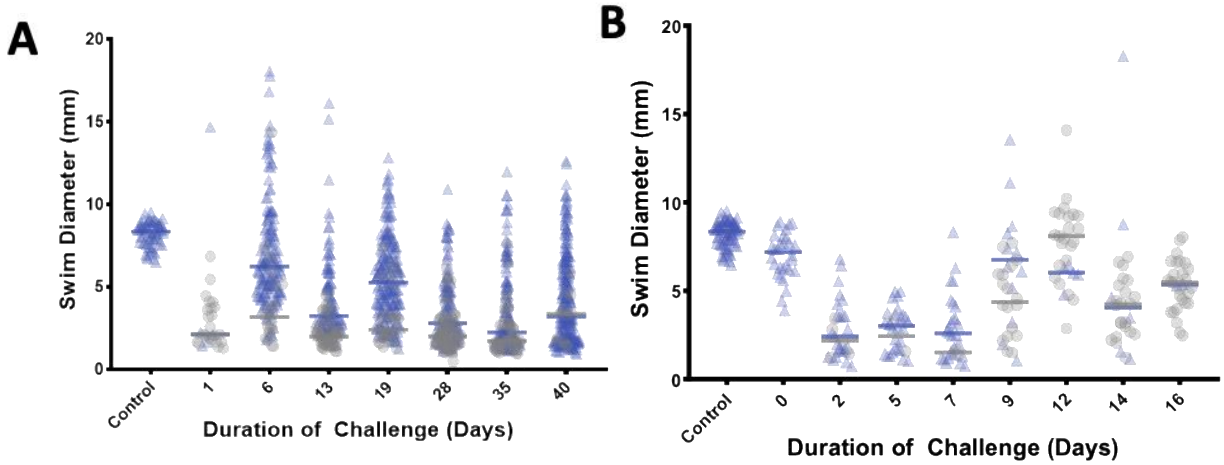

Figure S3-6: Bacterial swimming motility diameter for E. coli colonies isolated from G1 mice and in vitro samples co-evolved with phage

*(**A**) Bacterial swimming motility diameter for the E. coli colonies isolated at each time point from G1 mice. **(B)** Bacterial swimming motility diameter for the E. coli colonies isolated in vitro from samples co-evolved with phage.*

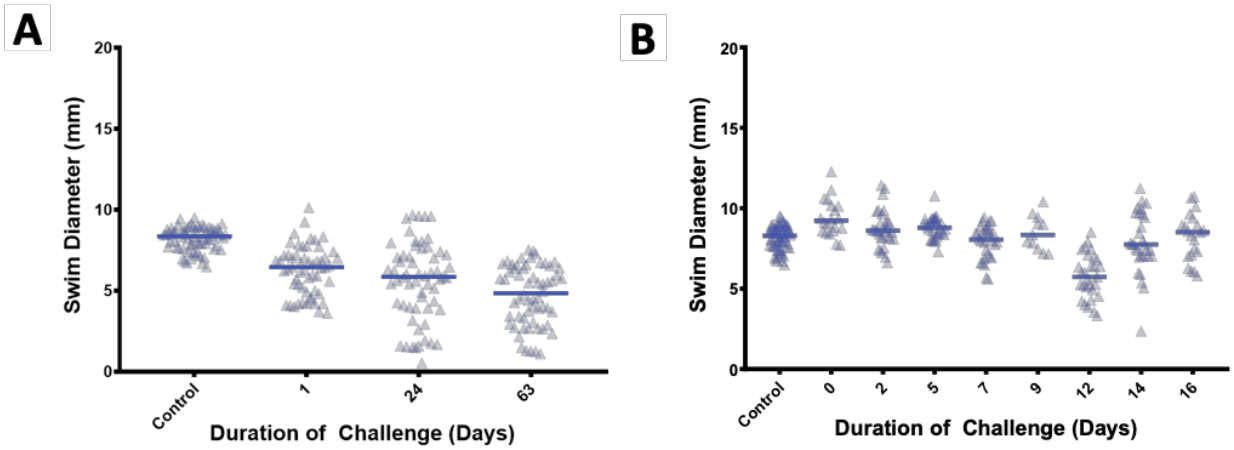

Figure S3-7: Bacterial swimming motility diameter for E. coli colonies isolated from G2 mice and in vitro no-phage control

(A) *Bacterial swimming motility diameter for the E. coli colonies isolated at each time point from G2 mice.* ***(B)*** *Bacterial swimming motility diameter for the E. coli colonies isolated in vitro from no-phage control.*

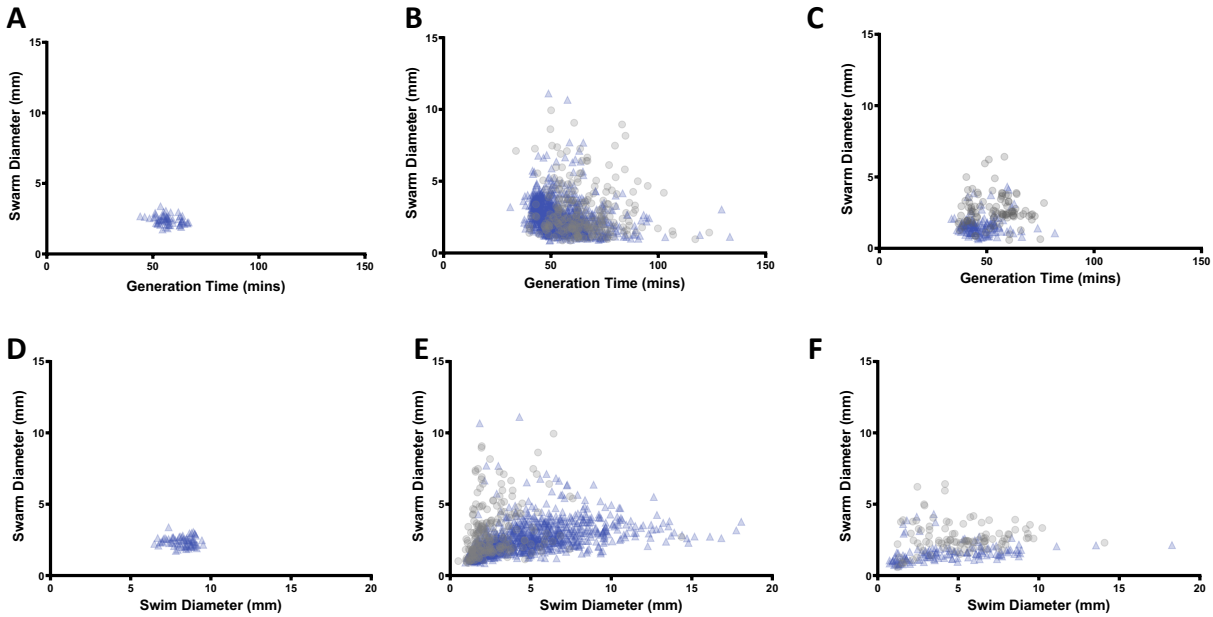

Figure S3-8: Aggregate plots

Bacterial swarming diameter vs generation time for ***(A)*** *control;* ***(B)*** *all E. coli colonies isolated from G1 mice;* ***(C)*** *all E. coli colonies isolated from in vitro culture. Bacterial swarming diameter vs swimming diameter for* ***(A)*** *control;* ***(B)*** *all E. coli colonies isolated from G1 mice;* ***(C)*** *all E. coli colonies isolated from in vitro culture.*

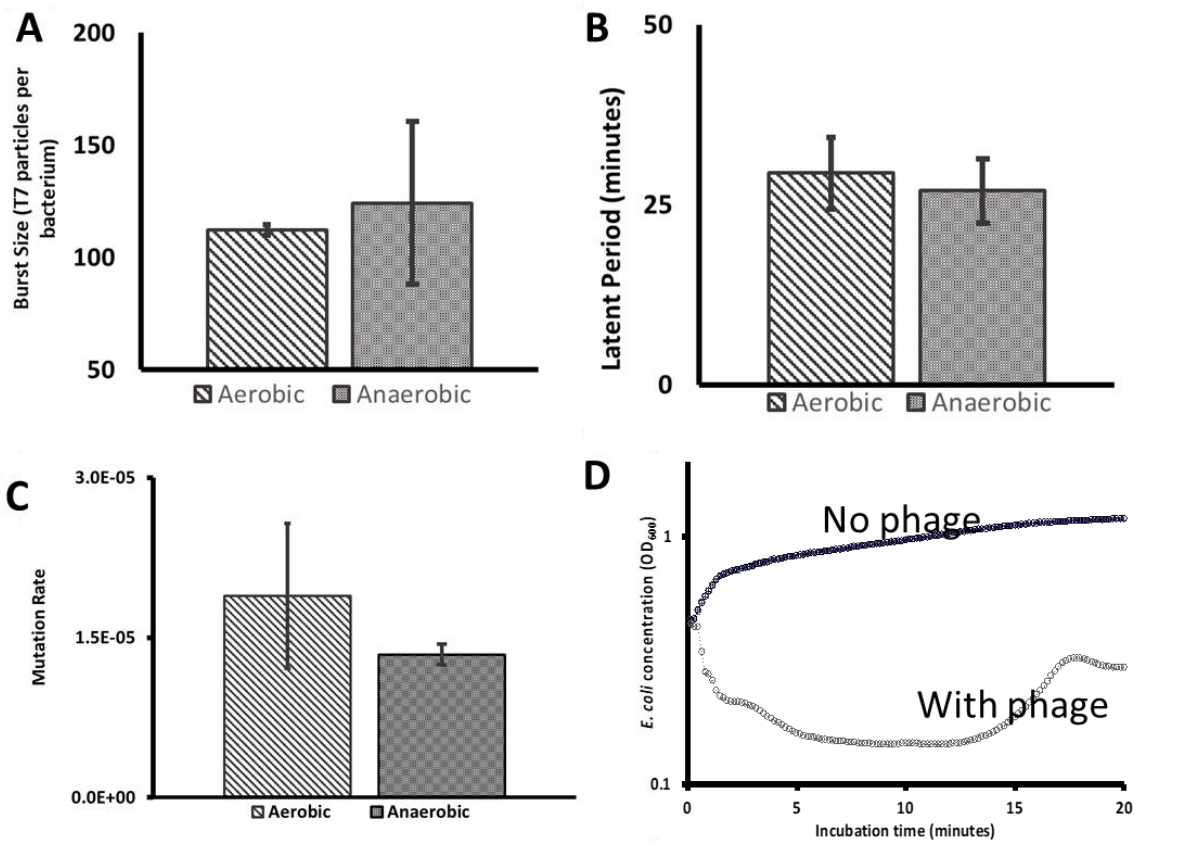

Figure S3-9: Coexistence Dynamics does differ in vitro within aerobic and anaerobic conditions

(A) *Burst Size of Escherichia coli K-12 (JM83) calculated from the completion of a 1-step growth curve in both aerobic and anaerobic conditions.* ***(B)*** *Latent Period of Escherichia coli K-12 (JM83) calculated from the completion of a 1-step growth curve in both aerobic and anaerobic conditions.* ***(C)*** *Mutation Rate of Escherichia coli K-12 (JM83) calculated from the completion of a 1-step growth curve in both aerobic and anaerobic conditions.* ***(D)*** *Growth Curves of Escherichia coli K-12 (JM83) in vitro with and without the addition of lytic phage T7 (MOI = 0.1).*

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Chapter 4 – Concluding Remarks and Future Directions

The investigation presented in this thesis provides an insight into the dynamics of lytic T7 bacteriophage and a non-pathogenic commensal *E. coli* K-12 (JM83) host, with the intent of determining effect of phage therapy on the gut microbiome, as well as the overall effectiveness and feasibility of this antibacterial therapy. Although phage therapy has previously been used in western medicine as an antibacterial therapy, it does not have FDA approval for current use in modern day. Our work sought to delve into the details of how phage therapy can interact with the gut microbiome in order to provide more information and progress the research needed to understand phage therapy further and obtain FDA approval in this era of antibiotic resistance.

Upon observation and analysis of our designed *in vivo* system, we can conclude: (i) a single dose of therapeutic lytic phage can effectively thrive within the gut microbiome and will significantly decrease a targeted bacterial community if it is the appropriate host to the phage. (ii) Phage therapy does not significantly affect the composition of the bacterial composition of the gut microbiome (iii) there is co-evolution of the therapeutic phage-bacterial host, which leads to a diversification of the host population with various fitness (iv) gnotobiotic mice colonized with an ASF consortium provide a defined and tractable system to observe and identify therapeutic T7 phage –*E. coli* K-12 (JM83) host dynamics within the gut microbiome.

Furthermore, the analysis of regulatory evolution and distinguishing of neutral stochastic change from functional divergence is particularly challenging for the collected bacterial host mutant isolates. To rigorously evaluate and determine the extent at which the dynamics further, whole genome sequencing of the collected fecal samples should be performed.

Selective pressures of the gut microbiome do have an influence on the abundance of both the host bacteria and the ASF consortium, as well as the concentration of bacteriophage. In specific, the location within the gut presented significant variability in concentration availabilities. Furthermore, phage therapy does not significantly alter the community dynamics of the ASF consortia within the microbiota. To determine if phage therapy as a direct effect on the tissue composition of the gut, histology of duodenum, ileum, and caecum should be performed.

Studying the therapeutic T7 lytic phage-bacterial *E. coli* K-12 (JM83) host dynamics in both an *in vivo* as well as an in vitro system, we can conclude that both system models are effective to study dynamics with variable limitations to each. In specific, although we saw similar a similar dynamic relationship between the host *E. coli* K-12 (JM83) bacteria and therapeutic T7 phage in the sense that mutants had diversified to become resistant to the phage. This had occurred at a faster pace in the *in vitro* system. We can deduce that we see a slower dynamic in the gut environment due to selective pressures such as the anatomy and folds within the gut and its microbiome, as well as pressures including body temperature, pH level, the surrounding bacterial microbiota, supplemented food.

A gnotobiotic *in vivo* model is effective and beneficial to study the phage-bacterial dynamics in an in-depth purpose. We can utilize this *in vitro* system for cost effective fast-paced testing. A good applicable example of this is for finding an appropriate phage to utilize as a therapeutic against a pathogenic bacterium residing within a patient.

This project does provide insight into the effectiveness of single dose phage challenge; we can further experiment the dynamics of multiple dose phage therapy (administered respectively at multiple time points) with their bacterial host. Furthermore, upon investigating the fitness diversification and virulence of phage-resistant mutants, within the microbiota, we propose the use a more complex animal model, as well as further exploring carefully designed phage cocktails from a selection of different phages that target similar hosts, ensuring that it is evolutionarily robust and will not harm the microbiota. With this antibacterial therapeutic now targeting a larger host range, the possibility of decreasing and diminishing the pathogenic host is much higher. However, it is also important to keep into consideration the aggressiveness of the phage cocktail as well as the frequency of administration, to avoid triggering an immunological shock within the host body. Future experiments can also include comparing phage therapy-host bacteria dynamics *in vivo* and verify it in a designed *in vitro* microfluidic device to resemble the gut microbiome in order to obtain a closer representation of the selective pressures present within the gut microbiome.

Our proposed project objective to investigate the direct and indirect effect off bacteriophage therapeutics on the gastrointestinal (GI) microbiota and the associated cascade of immune response, was successfully fulfilled. The results obtained in this experiment were expected and retained activity similar to current available literature and the current phage therapy use in Europe.

This project is a successful initial step to bridge the gap in knowledge regarding the direct and indirect effect of bacteriophage therapeutics on the microbiota, and in turn will help develop bacteriophage therapeutics as alternatives/adjuvants to antibiotics for treating infectious disease and also as agents for microbiome therapy for treating dysbiosis-related ailments. This is the first investigation of its kind and has ignited the path for future investigations regarding investigating and elucidating interactions between phage therapy and the microbiota. With FDA approval of phage-based products for use in environmental prophylaxis and food preservation, regulatory approval for therapeutic use in humans is not far off. Completing these investigations is necessary to understand the potential for phage-induced genome evolution in host bacteria and its possible effect on the human microbiome, as the current major hurdle in obtaining regulatory approval for phage therapy. With the current attention focused on bacteriophage therapy by governmental and private organizations in Canada and around the world, this work will advance the status of Canada and McMaster as the Canadian leader in basic translational research on infectious disease and microbiome research, and the current process to regulate phage therapy within Western medicine.