Investigation of Prophages and Lysogenic Bacteria in the Human Gut Microbiota

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Abstract

Bacteriophages are abundantly found in the human gut, both extracellularly, as well as integrated as prophages in bacterial genomes. Communities of bacteria and bacteriophages stably coexist in the gut of healthy adults and are altered with disease and medication exposure. It is unclear whether prophages found in lysogenic bacteria contribute to this stability. We hypothesize that lysogenic bacteria are highly abundant in the human gut, as seen in mice, and that their prophages contribute to the populations of extracellular bacteriophages observed in the gut through prophage induction. Xenobiotics, substances foreign to the body, including some dietary compounds, have been shown to trigger prophage induction and alter the phage community. We present data showing that other xenobiotics, such as host-targeted medications and antibiotics, are capable of triggering prophage induction of human gut bacterial isolates (Chapter 2). In the gut of a healthy individual, in the absence of exposure to antibiotics, triggered prophage induction is rare over 2.4 years. Instead, prophages maintain their presence in the gut through continuous spontaneous prophage induction (Chapter 3). When healthy individuals are exposed to antibiotics and prophage induction is triggered, its magnitude and effects on gut and phage communities seems minor (Chapter 4). These results highlight the importance of lysogeny for the stable coexistence of bacteria and bacteriophages in the adult gut, even in the presence of external triggers of prophage induction.

Résumé

Les bactériophages sont très abondants dans l'intestin humain, extracellulaires ou intégrés dans les génomes bactériens sous forme de prophages. Ces communautés bactériennes et de bactériophages coexistent de manière stable dans l'intestin d'adultes en bonne santé, mais sont altérées lors de maladies ou d'expositions à des médicaments. Le rôle des prophages dans le maintien de cette stabilité n'est pas connu. Nous faisons l'hypothèse que les bactéries lysogènes sont très abondantes dans l'intestin humain, et que lorsque leurs prophages sont induits, ils contribuent à la population de bactériophages extracellulaires présents dans l'intestin. Une exposition à des substances étrangères au corps humain, des xénobiotiques, qui comprennent aussi certains composés alimentaires, peut induire ces prophages et modifier la communauté de phages intestinaux. Dans cette thèse, nous démontrons que des médicaments et des antibiotiques sont capables d'induire des prophages présents dans des isolats bactériens intestinaux (Chapitre 2). En l'absence d'une exposition à des antibiotiques, une induction provoquée de prophages est rare dans l'intestin d'un adulte sur une période de 2,4 ans. Nous démontrons que les prophages maintiennent leur présence dans les intestins grâce à des évènements d'induction spontanée, qui maintiennent une population continue de prophages (Chapitre 3). Lorsque des individus en bonne santé sont exposés à des antibiotiques et que l'induction de prophages est provoquée, son ampleur et ses effets sur le microbiote intestinal semblent mineurs (Chapitre 4). Ces résultats mettent en évidence l'importance de la lysogénie pour la coexistence stable des bactéries et des bactériophages dans l'intestin adulte, même en présence de stimuli externes provoquant l'induction de prophages.

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Chapter 3: Bacteriophages Playing Nice: Lysogenic Bacteriophage Replication Increases Stability of the Human Gut Microbiota

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Chapter 5 Discussion

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List of Acronyms

16S	16S rRNA marker gene sequencing
AUC	Area Under the Curve
FCIC	Frequency of Chemically Inducible Cells
GI	Gastrointestinal
HMP	Human Microbiome Project
IBD	Inflammatory Bowel Diseases
MAG	Metagenome Assembled Genome
MC	Mitomycin C
MDA	Multiple Displacement Amplification
META-HIT	European Commission's METAgenomics of the Human Intestinal Tract
NIH	National Institutes of Health
OTU	Operational Taxonomic Unit
PFU	Plaque Forming Units
RefSeq	Reference Sequence database
SCFA	Short-Chain Fatty Acids
VLP	Virus-Like-Particle

Glossary

Active Lysogeny	Prophage integration that disrupts the coding sequence or promoters of the bacterial genome
Active Prophages	Prophages capable of switching to lytic replication by prophage induction.
Burst Size	Number of phages released after lysing bacteria from lytic replication
Carrier State	Form of phage replication. Occurs post-phage production, but before phage release. Phage particles are produced, but bacteria continue to replicate
Chronic Infection	Form of phage replication. The host cell is not lysed post-phage production. Instead, bacteria release phage particles from the cell through continuous release through the membrane
Gut	Luminal section of the colon
Latency	The time it takes for phage replication to occur
Lysogenic Conversion	Lysogenic replication when prophages encode virulence factors
Lysogenic Replication	Form of phage replication. Phages integrate into bacterial genome or as plasmid (prophage) rather than going to phage production. Prophages are replicated alongside bacterial genome until prophage induction occurs.
Lytic Replication	Form of phage replication. After phage production, phages are released by lysing the bacteria, and degrading bacterial genome. Synonymous with lytic infection.
Microbiome	The gene profiles of microorganisms
Microbiota	The taxonomic profiles of microorganisms
Non-Active Prophages	Prophages that are no longer capable of switching to lytic replication. Synonymous with degraded prophage, or cryptic prophage.
Obligate Lytic Phages	Phages with no intermediary replication steps between adsorption, and phage production, and release their phage progeny. Phages are released by lysing bacteria, and the bacterial chromosome is degraded in the process of replication. Also, referred to as strictly lytic phages
Perturbation	An external event that causes a distinct selective pressure on an ecosystem. Also, referred to as a disturbance

Phage Replication	Replication of phage genome post-phage adsorption. Synonymous with phage infection, and phage lifestyle.
Phageome	The phage fraction of the virome
Prophages	The integrated stage of lysogenic replication.
Pseudo-lysogeny	Form of phage replication. Phage production is interrupted, and phages replicate alongside the host pre-production. Differs from lysogenic replication in that the phage genome does not integrate
Spontaneous Prophage Induction	Form of prophage induction that occurs in the absence of an external trigger, and leads to only a small portion of the lysogen population switching to lytic replication
Superinfection Exclusion	Prophage mediated resistance to phage infection by interfering with phage attachment (e.g., receptor modification)
Superinfection Immunity	Prophage mediated resistance to phage infection by interfering with phage post-adsorption (e.g., repressor mediated immunity)
Temperate Phages	Phages capable of lytic and lysogenic replication.
Triggered Prophage Induction	Form of prophage induction that occurs by an external trigger, and results in the induction of the majority of the inducible population of bacteria.
Virome	The viral fraction of the microbiota

Chapter 1. Introduction

1.1. The Gut: Gastrointestinal Tract Microbiota

The study of the 'microbiome' is a truly 21st century scientific pursuit, coined by Lederberg and McCray in 2001 [1]. The microbiota of the human gut spans all domains of life and viruses, and its diversity and functions are central to human health [2]. Early on, Breitbart *at al*. (2003) showed that viruses were present in the human gut and consisted mostly of bacteriophages (abbreviated as phages throughout this thesis) [3]. Of all the microorganisms of the microbiota, bacteria have been the focus; due to their abundance; role in human health and disease; technological advances in high throughput sequencing of the 16S rRNA marker gene; and two major international consortiums investigating the gut microbiota: the National Institutes of Health's (NIH) Human Microbiome Project (HMP) [4], and the European Commission's METAgenomics of the Human Intestinal Tract (META-HIT) [5]. In contrast to bacteria, the viral fraction or gut virome, has lagged because of additional costs and technical challenges in sequencing, and an absence of direct observable effects on human health. Two decades of gut virome studies have shown that phages' primary role is modulating the bacterial fraction [6].

The site of the gut microbiota, the human gastrointestinal (GI) tract or gut, is approximately 10 metres long [7], with distinct anatomical sections from the mouth to the anus, lumen to the mucosa. Gastric acids, bile, pancreatic secretions, and flow, limit bacteria from establishing in the stomach and proximal small intestine. Bacterial density increases along the GI-tract in direction of the anus, reaching the highest concentrations in the distal colon and the site of peristaltic mixing [8, 9]. The gut microbiota is almost

exclusively sampled through stool collection, due to its non-invasive nature. The downside of relying on stool samples is that they are more representative of the lumen of the colon than the mucosal section, tissue, or small intestine [10], yet the benefits and ease of stool sample collection outweigh these limitations. The term 'gut' will be used in this thesis in reference to the luminal microbial communities of the colon represented by stool samples.

1.1.1. Bacterial Communities in Human Health

The gut contains a dense community of bacteria, mainly anaerobes, that ferment non-metabolized polysaccharides from the small intestine [10]. The most important by-products of this fermentation are short-chain fatty acids (SCFA) [11]. The most abundant SCFAs in the gut are acetic acid, propionic acid, and butyric acid [12]. Butyrate is used by colonocytes as an energy source [13], and can strengthen the epithelium barrier [14]. A healthy epithelial barrier is extremely important as the large intestine presents a surface area of 1.9 m^2 [15] and is the only physical line of defence against potential intestinal pathogens [14]. Commensal bacteria in the gut provide additional protection against pathogenic bacteria by competing with them, producing antimicrobial peptides, and/or enhancing the innate and adaptive immune responses [16].

In healthy adults, most gut bacteria belong to two phyla: *Bacteroidetes* and *Firmicutes*, with the other phyla (*Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*)[i]

[i] International Code of Nomenclature for Prokaryotes (ICNP) recently voted to include phyla which led to a renaming of commonly used prokaryotic phyla names. We will use the familiar naming in this thesis.

17. Oren, A.; Garrity, G. M., Valid publication of the names of forty-two phyla of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology* **2021,** *71*, (10).

representing a minor fraction of the whole community [18]. At the operational taxonomic unit (OTU) level, individuals show a high amount of inter-individual variation and thousands of 'species-level' bacterial phylotypes, but low intra-individual variation [18]. When considering the microbial genes found in the gut, or microbiome [19], functional gene profiles are shared amongst individuals [18]. The overlap of bacterial functional niches in the gut signals an essential ecological role in human health [20].

1.1.2. Viral Community Composition

Viral diversity surpasses bacterial diversity as viruses span a variety of hosts (animal, plant, fungi, protist, bacteria, and archaea), genome structures (DNA, RNA, double or single stranded, and translational polarity) [21], and have high rates of genetic recombination and mosaicism [22]. As many viral hosts coexist in the gut, it is not surprising that the diversity of the viral world is also found [23]. Viruses are not simply diverse, but extraordinarily abundant (global abundance is estimated at 10^{31} particles, with an average 5-10 virus-like particles (VLP) per bacterium [24]), and approximately $\sim 1.2 \times 10^9$ to 5.58×10^9 RNA/DNA VLP per gram of feces, close to or below 1:1 VLP per bacterium [25].

The magnitude of viral diversity to characterize is daunting, and by nature of being obligate parasites, isolation depends on first cultivating their hosts. Progress has been made in culturing bacteria from the gut [26] but in 'culturomic' studies sequencing is usually the end goal [27]. Culturing gut bacteria for isolating phages is an extra challenge (e.g., gut bacteria can be extremely oxygen-sensitive, halophiles or only form microcolonies, not the bacterial lawns needed for plaque assays) [28]. Researchers are

now increasingly relying on sequencing uncultivated viruses [29]. Viral sequencing is still a challenge as viruses do not have a universal viral marker-gene (e.g., 16S for bacteria and 18S for eukaryotes) [30]. Characterization of uncultivated viruses is dramatically outpacing viral isolates in all ecosystems, including the gut [29, 31, 32].

Despite the extensive viral diversity characterized in the gut [23], viral diversity is still underrepresented, because the focus is largely on DNA viruses [31, 32]. This comes from the fact that DNA eukaryotic viruses are either not found by metagenomics [33] or are in low abundance [31, 34-36] and phages are responsible for shaping gut bacterial communities [36], not eukaryotic viruses. Of the eukaryotic DNA viruses that are found, they appear to be taxonomically diverse and mostly within the ssDNA families (*Anelloviridae*, *Genomoviridae*, and *Circoviridae*) [31]. RNA viruses have also been largely ignored in the gut as they are thought to typically infect eukaryotes and plants [37], originate from our diet, are transient [38], and require different experimental approaches to characterize. As such, RNA viruses only represent 0.1% of the gut virome database [31]. However, efforts are being made to characterize more RNA bacteriophages [39]. As a result, in this thesis and in associated publications [31], gut viruses are synonymous with dsDNA and ssDNA bacteriophages.

The dsDNA and ssDNA bacteriophages are extremely important to the function of microbial ecosystems, including the mammalian gut [3]. Early efforts to identify phage diversity used sequence homology to genomes from databases like National Center for Biotechnology Information (NCBI) Reference Sequence Database (RefSeq) where only 4-17% of nucleotide sequences matched [33]. Relaxed searches showed only minor improvements (19% matching) [34] or with supplemental prophage databases [40].

Databases can bias results, as not all phage taxonomic groups are equally represented. Of the 12,403 characterized gut phage genomes available, they are known to infect only 234 different bacterial hosts and mostly belong to four genera: *Mycobacterium spp.*, *Escherichia spp.*, *Streptococcus spp.*, and *Pseudomonas* [41]. As most phages in the gut are uncharacterized, researchers are tackling the problem by creating databases of uncultivated gut phages [31, 32] with bioinformatic host prediction [32]. In addition to improving reference databases, efforts have been made to move beyond nucleotide sequence homology and leverage gene-sharing networks instead and assign taxonomic identity with viral clusters [42]. Even with viral cluster-based approaches, only 1% of phages in the gut at the genus-level overlapped with RefSeq, and only 20% at the family-level [32].

Of the phages we can taxonomically identify, it appears the gut virome is populated mostly by the order *Caudovirales* (tailed dsDNA phages: *Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Ackermannviridae* families), the ssDNA family *Microviridae*, and the filamentous family *Inoviridae* [31, 32]. ssDNA phage-abundance is likely over-represented due to multiple displacement amplification (MDA) bias often used in gut virome studies [43]. The CrAss-like-phages, which likely represent a new family of phages, have also been shown to be highly abundant in the gut and widespread [40, 44]. It is also important to highlight that viral taxonomy is currently going through a shift to move from morphological classifications to ones that consider genetic phylogeny [45]. For example, the phage families within the *Caudovirales* order appear to show little homology [45], leading to a current taxonomic reorganization of the *Caudovirales* order

[46]. Therefore, all phage taxonomic identification should be taken with a grain of salt; yet remains useful when comparing current results to previously published ones.

1.2. Healthy Adult Microbiota

1.2.1. Factors Shaping the Human Gut Microbiota

In the gut of a healthy adult, both bacterial and viral communities are stable over time, in the absence of perturbations [35], and these two communities are strongly correlated [36]. Broadly speaking, factors shaping the gut microbiota apply to both viral and bacterial communities. The first years of life are important in establishing the stable state of the adult gut microbiota for bacteria [47, 48] and phages [49]. The work of this thesis will focus on the gut microbiota of healthy adults, and factors shaping it. The adult gut microbiota is influenced by variety of factors, including bacterial infections, antibiotic treatment, surgery, disease, and our diet [48, 50-52]. These factors can perturb gut homeostasis and lead to an altered disease-associated state [47] found in conditions like inflammatory bowel diseases (IBD) (phages [40, 53] and bacteria [54]).

Here, we will highlight the importance of diet and medication consumption as two of the most important factors influencing the diversity and metabolism of the adult microbiota [55]. Both have been explored extensively *in vitro* and in animal models, and are used to modulate both gut bacterial and phage communities.

1.2.2. Diet

Diet is an important factor in the tripartite relationship between host, bacteria, and phages. Researchers have known for decades that diet plays a major role in human

health and disease development (see the extensive list in [56]). Specific bacterial species have been shown to be influenced by high-fat diet vs. a carbohydrate-rich diet (reviewed here [57]). At the community level, bacteria in the gut of healthy individuals, can be shaped over long periods of time, based on protein/animal fat consumption or carbohydrates [58]. Bacterial communities are compositionally stable in the long-term and resilient to short-term dietary interventions [58], but they can nevertheless alter the functional gene profiles, specific bacterial phylotypes, and SCFA concentrations and fermentation processes of the gut bacteria [58, 59].

Viral communities show similar inter-individual variation and stability as bacterial communities in healthy individuals [33-35]. Yet, viral community composition can converge due to dietary interventions in the short-term, in contrast with bacterial community composition [33, 60]. A study of the viromes of 930 healthy individuals found that diet had the second largest effect size on the gut virome composition after geography [52].

1.2.3. Medication

The healthy adult gut is defined by an absence of diagnosed GI diseases, but it is still exposed to oral medications. Medication consumption, by its very nature, is used to treat medical conditions, however the gut is not usually the intended site of action, and consequences in the gut are typically side effects. Medications are increasingly taken orally rather than intravenously to lower the cost of care and ease of treatment [61], and medication consumption is increasing in the United States [62] and globally [63]. We include antibiotics in this section, although it is usually an exclusion criterion for most gut

microbiota studies [61], because healthy individuals are regularly exposed to antibiotics due to over-prescription, and self-medication [64].

We have long known that antibiotics have an impact on the gut bacteria [65]. Our understanding of the long-term consequences of antibiotics to gut commensal bacteria [66, 67] has been improved with the advent of 16S [68]. These studies highlight that the impacts of antibiotics are individual-specific [68], despite some generalizable trends. For example, healthy individuals treated with a fluoroquinolone antibiotic, ciprofloxacin (500mg for five days), showed decreases in the relative abundance of one third of the bacteria, which returned to pre-treatment state 4 weeks later [68]. Similar results were shown with a clarithromycin and metronidazole treatment used against *Helicobacter pylori* [69], as well as in a patient given beta-lactam therapy for an infected pacemaker [70]. Importantly, fluoroquinolone and beta-lactam treatments do not always decrease overall bacterial load, as measured by qPCR, despite altering bacterial diversity and relative abundances [71]. As with short-term diet interventions, the gut bacteria of healthy individuals appear to be resilient to antibiotic exposure.

While antibiotics have a larger impact on the microbiome than other medications [72], non-antibiotics such as proton pump inhibitors and antipsychotic medications can also reduce gut bacterial diversity [73]. In addition, bacteria can directly metabolize drugs in the gut [74] and non-antibiotic medication can inhibit the growth of gut bacteria [75]. As these drugs are not routinely tested against bacteria, we know less about their mechanisms of action, but non-steroidal inflammatory drugs (e.g., carprofen, bromfenac, and vedaprofen) were shown to inhibit *Escherichia coli* DNA polymerase III beta-subunit [76], possibly explaining their impact on the gut microbiome [77].

Less is known about the changes in the phage populations with regards to medication exposure. On a population level, it appears that medication consumption has the third largest effect size for shaping the virome, just after diet [52], but the underlying mechanisms are not known. In pigs, antibiotic exposure was shown to lead to prophage induction [78], which is also supported by bacterial gene-expression profiles in humans [72]. Antimicrobials have also been shown to make bacteria sensitive to phage replication [79] but medication consumption is not likely spreading antimicrobial resistance genes [80, 81]. We still do not know if medication consumption is directly or indirectly shaping the virome.

1.3. Bacteriophage Replication Cycles and Ecological Consequences

1.3.1. Overview

Our understanding of phage replication gives context to the correlations we observe between bacteria and phages in the gut [82, 83]. In some ecosystems, like the ocean, viruses are the source of an estimated $1e^{23}$ phage infections per second [84] which is fuel to an evolutionary arms-race between bacteria and phages [85]. Therefore, it should be unsurprising to those studying phages that we continually discover phages behaving in ways we would never expect (e.g., forming a nucleus [86], using an alternative genetic code [87], communicating with each other [88], or encoding their own CRISPR-cas system [89]). This section will be a modest overview of how phages replicate [90], which has important ecological implications [91] and is entering a new era, as a result of increased metagenomics data [92].

All phages must insert their genomes within their host cell before transcription can occur (Figure 1.1). We will not cover the steps precluding to insertion (attachment), despite its important role in phage-host range and its ecological implications [93]. Phage replication, phage lifecycle, or phage lifestyle are terms used interchangeably, although replication can be used more specifically to reference transcription, and lifecycle more broadly encompasses all steps of generation of new phage virions. Nothing is livelier than a discussion about whether a virus is alive or not, but for this reason, I will avoid using ‘lifestyle’ or ‘lifecycle’. For simplicity and continuity in this thesis, I will define ‘replication’ as the propagation of the phage genome.

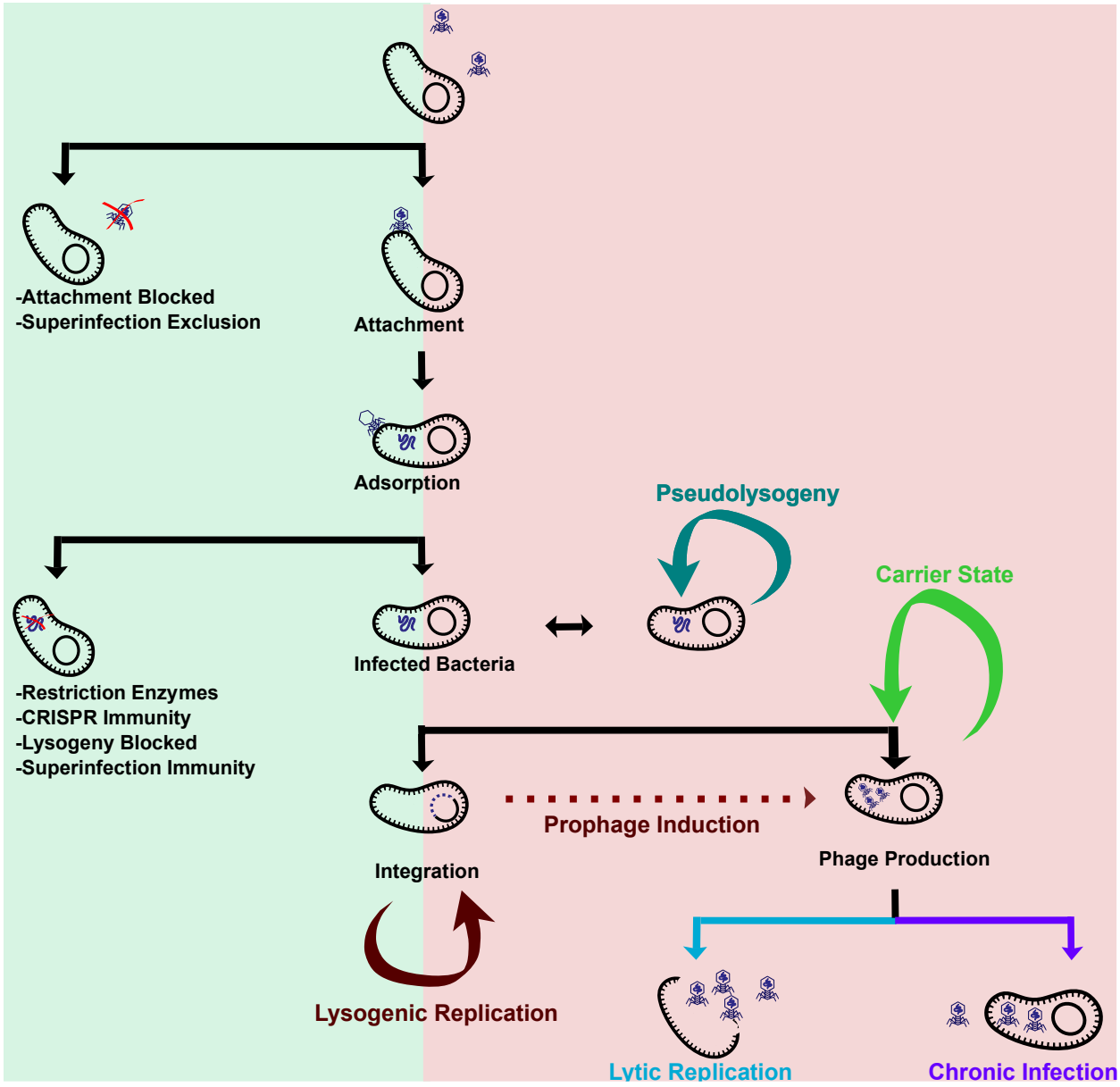

Figure 1.1 Summary of Phage Replication and Bacterial Defenses:

A summary of phage replication (pink) and bacterial defenses (green). Attachment: Free, or extracellular phages, come in contact with bacteria and attach to phage-specific surface receptors. Bacteria and prophages modify receptors to block attachment. Adsorption: The phage inserts its genome into bacterial cytoplasm. Bacteria can block infection through a variety of mechanisms, including degrading the phage genome (restriction enzymes, CRISPR-immunity), block integration (lysogeny), or by prophage mediated defenses (superinfection immunity). Infected bacteria can continue to replicate with phage genome either integrated into the bacterial genome (lysogenic replication) or not integrated (pseudolysogeny). Phage production is initiated after infection or prophage induction. Once fully formed phage particles are produced, bacteria can continue to replicate without releasing phages (carrier state) or release them by lysing the bacteria (lytic replication) or by budding (chronic infection).

1.3.2. Lytic Replication

Lytic replication is the *de facto* phage replication, in that other phage replication strategies are defined by how they differ from lytic replication. Lytic replication occurs after the phage successfully attaches to its host bacterial receptor (attachment) and injects its genome into the host bacteria (adsorption) [94] (Figure 1.1.). Obligate lytic phages, or strictly lytic phages, have no intermediate steps before or after phage production, and release phage progeny by lysing the bacteria [94] (Figure 1.1). Phage production occurs by use of the host's replication and translation machinery before packaging new phage genomes into new phage particles (Figure 1.1). Phages can encode almost everything, except ribosomes, and rely on their hosts' ribosomes to translate their mRNA [95]. Post-translation, the phage genome is tightly packed into empty capsids (procapsids), forming new virions [94]. The number of virions formed is referred to as the burst size, as phages lyse their bacterial host to release the newly formed phage virions [96]. Lytic replication has been used interchangeably with phage production, and in this thesis, we will differentiate it from chronic infection based on how phages are released post-phage production (i.e., lysing bacteria, with or without degradation of bacterial chromosome) (Figure 1.1).

Lytic replication is typically a quick process (25-30 minutes for T4 bacteriophage infecting *E. coli* [97]). The speed with which lytic replication occurs, referred to as latency, appears to be determined by bacterial density, phage species, bacterial species, physiological condition of host, nutrient availability, and temperature [98, 99]. When fully formed phage virions delay cell lysis and allow the bacterial to continue to divide, this is referred to as 'carrier state' (see section 1.3.4: Alternative Replication Cycles).

In the adult gut microbiota, it appears that phages are not under typical 'kill-the-winner' dynamics resulting from lytic phage pressure [6]. CrAss-like-phages, thought to be lytic phages, can be abundant in the gut of healthy individuals in an absence of 'kill-the-winner' dynamics [35]. However, the only isolated CrAss phage, ΦCrAss001, appears to replicate more as a lytic-lysogenic intermediate (see section 1.3.4: Alternative Replication Cycles). Based on longitudinal studies of the gut virome, it does not appear that lytic replication dominates the gut [33-35], although it probably still occurs to some degree [6].

1.3.3. Lysogenic Replication

Temperate phages differ from obligate lytic phages, in that they are capable of lysogenic replication. Lysogenic replication occurs when the phage genome integrates into host bacteria as a prophage or circularizes as a plasmid (Figure 1.1). Temperate phages, post-infection, can either undergo lysogenic replication or go directly to lytic replication: the 'decision' of integration vs lytic replication is one influenced by a number of environmental factors, such as quorum-sensing signals, phage species, phage density, or bacterial physiology and metabolism [88, 100, 101]. Temperate phages that integrate their genomes as prophages, replicate alongside their bacterial hosts, taking advantage of bacterial replication machinery indefinitely or until prophage induction occurs. Prophage induction is an intermediate step between lysogenic replication and lytic replication and will be discussed in detail later (Figure 1.1, see section 1.4.4 Prophage Induction).

Lysogenic replication is passive compared to that of virulent lytic replication. The dual view of temperate phages undergoing either lytic or lysogenic states has led to theoretical [102] and experimental studies [103, 104] offering an evolutionary explanation for why lysogeny would be favoured over lytic replication. Experimental methods in aquatic systems demonstrated that lysogeny is favoured when the bacterial host is in low density or in poor growth conditions, acting as a 'refuge' for temperate phages [105]. Experimental methods for determining the proportions of lysogens in a community vary but usually rely on inducing prophages, and therefore can underestimate the number of lysogens [106]. In contrast to the 'refuge' model for lysogeny, observations that the number of VLPs decrease with increased bacterial density led to the proposal of an alternative model: 'piggy-back-the-winner' [107]. In this model, temperate phages integrate as prophages to benefit from the successful replication of their host [107]. Observations fitting the 'piggy-back-the-winner' were observed in many ecosystems, including animals, and correlate with observations of increased temperate features [107]. In studies of the adult human gut virome, where bacteria are in high density, it has been observed that temperate phages are predominant [33, 34], or at least in some individuals [35].

While bacterial density in the gut is high compared to other environments (terrestrial, marine, and fresh water), it is an environment with relatively low bacteria diversity [108], which increases the occurrence of phage co-infections that push microbial communities towards lysogeny [109]. Lysogeny might also be 'making-the-winner' in the gut, as prophages can provide superinfection exclusion and immunity [109], and influence micro-diversity at the bacterial strain level [110]. The gut microbiota offers an exciting

microbial ecosystem for understanding the persistence of lysogeny (see section in 5.2. ‘Why be Lysogenic in the Gut?’).

1.3.4. Alternative Replication Cycles

Phages are sometimes summarized as being either temperate or lytic, but this is an oversimplification. Phage biologists have described many alternative ways that phages replicate, including, but not limited to: pseudolysogeny, carrier state, and chronic infection (Figure 1.1). These terms have been used inconsistently throughout the literature which makes them difficult to define. The topic has been nicely reviewed here [111]. These categories have in common that phage replication results in an absence of clear plaque formation (lytic) or formation of prophages that can be easily induced (lysogeny).

- ***Chronic infection*****:** The host cell is not lysed post-phage production. Instead, bacteria release phage particles from the cell through continuous release through the membrane [111].
- ***Carrier state*****:** Occurs post-phage production, but before phage release. Phage particles are produced, but bacteria continue to replicate [111].
- ***Pseudolysogeny*****:** After bacterial infection, phage production is interrupted, and phages replicate alongside the host. Differs from lysogenic replication in that the phage genome does not integrate [111].

These alternative forms of replication are also important to the gut virome. For example, the filamentous phages, thought to undergo chronic infection, are commonly found in the gut [112]. Phages of the bacteria *Campylobacter jejuni* benefit from carrier state replication to survive extra-intestinal environments [113]. ΦCrAss001, as mentioned in section 1.3.2, might undergo a carrier state or pseudolysogeny [114] and this alternative replication allow it and its *Bacteroides* host to persist in high abundance in the human gut [115]. An umbrella term has been proposed, ‘carrier state life cycle’ describing

a ‘mixture of phages and bacteria that persist in a more or less equilibrium’ [111]. Phages that replicate using alternative means are difficult to cultivate *in vitro* and identify by sequencing, which limits our understanding of their role in microbial ecosystems like the gut.

1.4. Prophages

1.4.1. Prophages Overview

Prophages can represent a sizable fraction of the bacterial genome and can influence bacterial taxonomic classification at the strain level [110]. For example, the cp32-1 prophage of *Borrelia burgdorferi* represents ~20% of the bacterium’s genes [116]. Prophages can alter bacterial phenotype through lysogenic conversion, where phages can encode virulence factors, such as effector proteins produced by Shiga toxin *E. coli* and *Salmonella enterica* subspecies I and cholera toxin [117]. This makes prophages an important feature of not just phage, but also bacterial biology.

1.4.2. Prophage Integration

Where a prophage integrates can be as, or more, important than the genes the phage carries. Prophages, as other mobile elements, can affect gene function and regulation by either disrupting coding sequences or introducing promoters that upregulate gene expression during integration [118]. This has been referred to as ‘active lysogeny’, where prophages are important switches that regulate bacterial gene expression [119, 120]. Integration varies between phages: some prophages integrate into a specific region of the bacterial genome (such as lambda) or the attachment site [121-123], while others exist as a plasmid [124, 125], or integrate randomly (transposable phages such as Mu)

[126]. In *E. coli* and *Salmonella*, prophage integration sites appear to be non-random and are concentrated away from the bacterial origin of replication [123]. Integration into specific sites, such as the loci of the bacteria's CRISPR anti-viral immune system, increases the chances of the temperate phage to evade the bacterial antiviral immunity [127].

1.4.3. Prophages in the Gut

In the gut, prophages could be spreading genes that encode a number of processes: vitamin B12 transport [128]; anaerobic respiration; amino acid, carbohydrate, nucleotide, lipid, and even xenobiotic metabolism [34]. The role of phages as mobile elements might be overestimated, e.g., antibiotic resistance genes [80, 81, 129]. Prophages also offer an important defence strategy for their bacterial host against phage infection by superinfection immunity [130]. Prophages are found in about half of cultured bacterial isolates [131], and are even more highly abundant in the gut of mammals including humans [132, 133]. It appears that prophages could play an important role allowing bacteria to carve out ecological niches in the gut [134], as has been shown in other environments [135, 136].

1.4.4. Prophage Induction

Prophages can, theoretically, replicate indefinitely alongside their bacterial host through lysogenic replication (Figure 1.1). However, for them to infect other bacteria, they need to switch to the productive replication stage through prophage induction (Figure 1.1). Most of what is known about this process comes from lambda phage, as it is used as a model organism to understand molecular switches [137]. Prophage induction has been

studied *in vitro* with DNA damaging agents (e.g., ultraviolet light or mitomycin C (MC)) with *E. coli* infected by lambda prophage. The DNA damage leads to the RecA protein becoming proteolytic and cleaving the repressor (cI), and the Cro regulatory protein being synthesized, resulting in lytic replication [137]. Similar processes have been shown with closely related phages 434 (*E. coli*) and P22 (*Salmonella typhimurium*) [137]. Less is known outside of these model microorganisms, but a repressor-SOS-response model of prophage induction has been commonly described. Indeed, the temperate phage CTXφ that infects *Vibrio cholerae* is induced by DNA-damage repair mechanism through the SOS-response (RecA-dependent manner), but its repressor (RstR) in the absence of a C-terminal protease domain uses LexA as an additional repressor [138]. In the case of phiMBL3, in the absence of RecA-dependent autoproteolytic activity, the cI-like repressor resembles the *Staphylococcus aureus* pathogenicity islands (SaPIs) StI repressor [139]. The SOS-response can occur in the absence of inducers and lead to prophage induction, referred to as spontaneous prophage induction, as in *Corynebacterium glutamicum* [140].

In the gut, many factors could induce prophages. Immune cells could be triggering prophage induction near the epithelial barrier where antimicrobial molecules are produced, specifically in the small intestine [141]. In patients suffering from Crohn's disease, where the immune response is deregulated, there is an increase in prophage induction [53]. Bacterial-based induction may also be caused in the lumen by bacteria-produced antimicrobials [142]. Substances that are produced outside of the body, such as diet-derived compounds and medications, are also likely sources of prophage induction in the gut. Fructose metabolism and SCFA exposure in the gut were shown to activate the Ack pathway which triggers SOS-response and induces prophages of

Lactobacillus reuteri [143]. Dietary interventions appear to induce prophages of gut bacteria in mice [132] and specific dietary compounds (e.g., Stevia rebaudiana, aspartam, tabasco, and clove) have been shown to induce gut isolates *in vitro* [144]. Medications, and not just antibiotics, are capable of inhibiting gut bacteria [75] and upregulating phage replication genes in the gut [72].

Prophage induction is the other side of the coin for bacteria that would otherwise be coexisting with their prophage. Prophages have been referred to as 'molecular time-bombs' [105], which should be under selective pressure to be made incapable of lytic replication [145]. As bacteria replicate and mutations accumulate, some prophages become cryptic, defective, or as I will use in this thesis, inactive, in that they are no longer capable of being induced. Despite the existence of selective forces acting against active prophages in the gut, the majority of prophages are still capable of undergoing lytic replication [132]. Prophage induction is an important process for phage proliferation, and prophages have recently been shown to utilize arbitrium, phage communication peptides, to limit prophage induction in the presence of other lysogens [146]. Prophage induction could thus be playing an important role in the gut microbiome, its stability, and/or resilience.

1.4.5. Prophage Detection

> "Given the immense variation among phages and our incomplete knowledge of that variation, recognition of prophages can be a rather subjective and delicate art"
> Casjens (2003)

Accurate prophage detection is still an unresolved challenge in the study of phages. Detecting prophages *in vitro* requires cultivating the bacterial host and exposing

the bacteria to its corresponding prophage inducer [147]. The most commonly used prophage inducer is MC, a compound isolated from *Streptomyces caespitosus* with antibiotic and antitumor properties [148], that was shown to induce prophages similar to UV [149], and has become the gold-standard for prophage induction. This method has been extended to community-level studies in aquatic and terrestrial systems using MC [99], but can easily under-estimate the proportion of active prophages [106]. MC can induce prophages at variety of concentrations, depending on the bacteria, which can be easily controlled with isolates, but not so much when considering the diversity of whole microbial communities [106].

The second approach is to detect prophages within the genomes of bacteria. Manual curation of a database of known prophages showed that 'phages appear to have settled on a limited number of transcriptional arrangements', namely: operons longer than the bacterial host's, genes orientated in the direction of replication, and with integrase-like genes adjacent to the attachment site [147]. The first automated process of prophage detection relied on a BLASTX search, combined with a semantic selection of prophage related hits (phage, integrase, tail, capsid, terminase, portal) against negative hits (e.g., macrophage, transposase, transposon, insertion) [110]. The first generation of computational-tools combined phage hidden Markov models (HMM), tRNA and dinucleotide analysis, and attachment site recognition (Phage_Finder (2006) [150], Prophage Finder (2006) [151], Prophinder (2008) [152]). Similar approaches have been used to differentiate plasmid-prophages from bacterial-plasmids [125]. The next-generation improved prophage detection by using a regularly updated prophage database and gene clustering density measurements (PHAST) [153], AT and GC skew,

protein length and transcription strand directionality (PhiSpy) [154], or as a built-in feature of a larger viral identification tool (VirSorter) [155]. We now have a long and growing list of tools for prophage detection. Each tool approaches the problem differently, and their differences mean that they vary in performance, prioritizing either accuracy, precision, recall, or a balanced F1 score [156]. The ‘better’ prediction is dependent on user-defined priorities [156]. As with most computational tools, we have yet to create one prophage detection tool to rule them all.

1.5. Rationale and Hypothesis

The microbiota of healthy adults varies between individuals, but they all share the hallmark of stability (diversity and metabolic) [47]. Medication consumption is a perturbation capable of moving the gut into an altered state of equilibrium, but for which the exact mechanism remains to be determined [47]. In this thesis, we investigate the role of medication consumption as an inducing agent of prophages in the healthy adult gut. We hypothesize that prophage induction is rare in the healthy adult gut, as it would disrupt the stability of the gut microbiome, but that medication consumption can induce prophages with marked effects on the gut microbiota composition and function.

To test this hypothesis, we have developed three aims:

- Determine the ability of medications to induce prophages of human gut bacteria (Sutcliffe SG, Shamash M, Hynes AP, and Maurice CF, 2021, *Viruses*);
- Analyze the contribution of active prophages to the virome of the healthy human gut (Sutcliffe SG, Reyes A, and Maurice CF, 2021, *submitted*);
- Measure the impact of antibiotics on reshaping the gut virome by prophage induction (Sutcliffe and Maurice 2022, *in prep*).

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Chapter 2 Preface

Bacteria in the gut are constantly exposed to medications taken orally. In this chapter, we explore the link between bacterial growth inhibition by medication consumption, and prophage induction. We show that a wide range of medications are capable of inducing prophages of gut lysogens *in vitro*. A few bacteria were highly susceptible to prophage induction by medication exposure, and capable of being induced by medications from a variety classes, including: non-steroidal anti-inflammatory, chemotherapy, mild-analgesic, and cardiac drugs. Virus-like-particle production increased in almost all bacteria (84%) showing even mild inhibition (decrease in the area-under-the-growth-curve (AUC) ≥ 15%).

We show that in addition to antibiotics, non-antibiotic oral medications can trigger prophage induction. The majority of bacteria had an inducible prophage, but there is a strong species-specific response to medication and concentration. This chapter highlights that prophages in the gut might be undergoing triggered prophage induction when we take oral medications, but the response is highly-dependent on the bacteria.

Chapter 2. Common Oral Medications Lead to Prophage Induction in Bacterial Isolates from the Human Gut

Abstract

Many bacteria carry bacteriophages (bacterial viruses) integrated in their genomes in the form of prophages, which replicate passively alongside their bacterial host. Environmental conditions can lead to prophage induction; the switching from prophage replication to lytic replication, that results in new bacteriophage progeny and the lysis of the bacterial host. Despite their abundance in the gut, little is known about what could be inducing these prophages. We show that several medications, at concentrations predicted in the gut, lead to prophage induction of bacterial isolates from the human gut. We tested five medication classes (non-steroidal anti-inflammatory, chemotherapy, mild analgesic, cardiac, and antibiotic) for antimicrobial activity against eight prophage-carrying human gut bacterial isolates *in vitro*. Seven out of eight bacteria showed signs of growth inhibition in response to at least one medication. All medications led to growth inhibition of at least one bacterial isolate. Prophage induction was confirmed in half the treatments showing antimicrobial activity. Unlike antibiotics, host-targeted medications led to a species-specific induction of *Clostridium beijerinckii*, *Bacteroides caccae*, and to a lesser extent *Bacteroides eggerthii*. These results show how common medication consumption can lead to phage-mediated effects, which in turn can alter the human gut microbiome through increased prophage induction.

2.1. Introduction

The human gut is at the intersection of host cells, trillions of microorganisms (bacteria, archaea, eukaryotes, and viruses), and all the different compounds we ingest, termed xenobiotics. The bacterial fraction of this microbial community is responsible for the metabolism of a wide range of xenobiotics, including components of our diet [1]. The increase in medication consumption in the United-States [2] and globally [3] makes medication an important xenobiotic shaping our gut microbiota. Medication of a variety of classes can have major effects on the gut bacteriome [4-12] leading to species-specific bacterial growth inhibition [13] or community-level shifts in bacterial diversity [4-12].

Medication can also alter the gut virome [14], which is highly correlated with the bacterial community [15]. This is because the gut virome is dominated by bacteriophages [16] (phages): viruses that infect and lyse bacteria. The majority of phages in the gut are identified as temperate [17-19], meaning they are capable of replicating lysogenically. Lysogenic replication includes the incorporation of the phage genome into the host bacterial genome as a prophage (or as a plasmid) [20]. Bacterial hosts with prophages are termed lysogens. Prophages are found in about half of bacterial isolates [21] and commonly found in complex communities [22], including the murine [23] and human gut [24]. Prophages are not simply hitchhiking genetic cargo, but play an important ecological role in the gut through super-infection immunity [25], lysogenic conversion or transduction [26], and encode genes involved in a number of processes associated with anaerobic respiration, as well as genes involved in amino acid, carbohydrate, nucleotide, lipid, and even xenobiotic metabolism [18].

Lysogeny is not a static state: prophages contain molecular switches that allow for the return to lytic replication, a process referred to as prophage induction. Prophage induction is likely an important driver of phage-bacteria dynamics in the gut. For example, in Crohn's disease patients, the shifts in gut virome diversity appear to be caused by prophage induction [27]. Determining the role of prophage induction as a driver of phage–bacteria dynamics in the gut requires identifying the conditions that trigger prophage induction first.

Prophage induction is typically triggered through bacterial DNA-damage. Work with bacterial isolates and clinical observations suggest RecA activation by antibiotics leads to prophage induction in situ [28]. Other xenobiotics such as specific dietary compounds [29] dietary fructose, and short-chain-fatty acids [30] have been shown to induce gut lysogens, and whole diet changes have also been shown to alter both murine [23] and human gut virome diversity [14, 19]. Non-antibiotic medications are also likely inducers, as they are capable of inhibiting gut bacterial growth through a variety of mechanisms [13], correlating with gut virome variation [14] and an up-regulation of phage genes in the gut bacterial community [12]. We thus hypothesize that many oral medications, including non-antibiotics, induce prophages of human gut lysogens.

We screened a variety of medications for prophage induction against lysogenic human gut bacterial isolates. We quantified virus-like-particles (VLP) by epifluorescence microscopy and confirmed prophage induction of in silico predicted prophages. Our results confirm that bacterial growth inhibition by medications, including non-antibiotic drugs, leads to an increase in phages through prophage induction, and could be altering the virome and resulting in phage-mediated shifts in the gut microbiome.

2.2. Materials and Methods

2.2.1. Bacterial Isolates

We selected eight human gut bacterial isolates: Bacteroidetes (*Bacteroides caccae*, *Bacteroides ovatus*, *Bacteroides eggerthii*), Firmicutes (*Clostridium beijerinckii*, *Clostridium scindens*, *Enterococcus faecalis*), Proteobacteria (*Escherichia coli*), and Actinobacteria (*Bifidobacterium longum subsp. infantis*). All isolates are associated with the human gut microbiota (Table 2.1) and represent the major phyla of the gut [31]. All of our tested isolates had genomes assembled, at least at a scaffold level, with exception of *E. coli* and *C. scindens* (Table 2.1). *E. coli* and *C. scindens* were shown to be lysogens experimentally, and the rest were determined to be lysogens based on prophage prediction. Prophages were predicted on the bacterial genomes using PHASTER [32] and VirSorter (Supplementary Figure 2.1) [33].

Phylum	Bacteria	Gram	Accession/Assembly	Isolated	Media
Actinobacteria	*Bifidobacterium longum subsp. Infantis ATCC 15697*	+	NC_011593	Infant Intestine	BHI w/ hemin
Firmicutes	*Clostridium beijerinckii ATCC 51743*	+	GCA_000016965.1	Likely Soil	ABB
	Clostridium scindens 32-6-S 4 CNA AN	+	N/A	Human Feces	ABB w/ hemin
	Enterococcus faecalis TUSoD Ef11	+	NZ_ACOX02000011	Human Oral	BHI
Bacteroidetes	*Bacteroides caccae ATCC 43185*	-	AAVM00000000	Human Feces	TSB
	Bacteroides ovatus 3_8_47	-	ACWH00000000	Human Colon biopsy	TSB
	Bacteroides eggerthii 1_2_48	-	ACWG00000000	Human Colon biopsy	BHI w/ hemin
Proteobacteria	*Escherichia coli K12 ATCC 25404*	-	N/A	Human Feces	BHI

Table 2.1 Collection of lysogenic bacterial isolates tested for inducible prophages.

2.2.2. Estimation of Medication Concentrations in the Human Gut

Information on the concentration of medications selected in the human gut is currently unavailable. We first selected medications that are taken orally, as they are likely to interact with the human gut microbiota [34]. The human gut contains bacteria along the entire GI tract but is in highest density and diversity in the large intestine [35]. The colon is the site of most gut microbiota studies, specifically the lumen [36]. Orally administered medications rarely target the colon as the site of action and most of the absorption occurs earlier in the small intestine. The amount absorbed and found in the circulatory system, or bioavailability, is therefore well studied. We estimated the concentration in the colon of our tested medications based on loss of oral dose by bioavailability (Supplementary Table

S1). This model does not take into account medications entering the gut through biliary excretion or in a transformed state but is an estimate for the concentration found in the gut.

2.2.3. Preparation of Medication

Stock solutions were made with powdered medications (ampicillin sodium salt, (A0166) CAS: 69-52-3; ciprofloxacin, (17850) CAS: 85721-33-1; norfloxacin, (N9890) CAS: 70458-96-7; diclofenac sodium salt, (D6899) CAS: 15307-79-6; ibuprofen, (14883) CAS: 15687-27-1; tolmetin, (1670502) CAS: 64490-92-2; digoxin, (D6003-1G) CAS: 20830-75-5; streptonigrin from *Streptomyces flocculus* (S1014) CAS: 3930-19-6; busulfan, (B2635) CAS: 55 98-1; fludarabine phosphate, USP (1272204) CAS: 75607-67-9 Sigma-Aldrich Canada Co., Oakville, ON, Canada; mitomycin C, (BP253110) CAS: 50-07-7 Fisher Scientific, Nepean, ON, Canada) dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg mL^{-1}, except where solubility did not permit, for ciprofloxacin (0.2 mg mL^{-1}), streptonigrin, norfloxacin, and tolmetin (1 mg mL^{-1}), and stored at −20 °C. DMSO was chosen as a solvent due to its ability to dissolve non-antibiotics (fludarabine, ibuprofen, and diclofenac) that have low solubility in water. Medications were serially diluted in DMSO such that 2 μL added to 200 μL wells had final concentrations of 0.01, 0.10, 1.00, 10.00, and 100 μg mL^{-1} (with the exception of previous low solubility medications) in media. This was to reduce DMSO concentration in media, as it can inhibit bacterial growth at high concentrations. In addition, we tested a higher concentration of ciprofloxacin dissolved in slightly acidic water (pH 6.5, final concentration 2 mg mL^{-1}) on a subset of bacterial isolates that did not show induction at the lower tested concentrations.

2.2.4. *In Vitro* Treatments

We grew all bacteria anaerobically (Coy chamber with 5% hydrogen, 20% carbon dioxide, 95% nitrogen, Mandel Scientific Company Inc., Guelph, ON, Canada). Simulating the human gut environment temperature at 37 ◦C in nutrient rich environment with general fastidious growth broth (brain heart infusion broth (BHI) BBL 299070 BD, Mississauga, ON, Canada, anaerobe basal broth (ABB) CM0957, Thermo Scientific, Waltham, MA, USA, tryptic soy broth No. 2 (TSB) 51288 Millipore, Oakville, ON, Canada with or without 0.1% hemin chloride in NaOH (5 mg mL^{-1}) (Table 1). Bacteria were grown in 96-well plates, measuring OD600 nm by spectrophotometry (Epoch 2 microplate spectrophotometer, Biotek Instruments, Winooski, VT, USA) and mixing every five min until early exponential phase (1/4 OD of stationary phase). At the early exponential phase, medications dissolved in DMSO were added (2 µL) to reach their tested concentration (n = 3) along with DMSO control (n = 3). Bacterial growth was then monitored with an $OD600_{nm}$ reading/mixing every 15 min until stationary phase (~24 h). Slow growing bacteria (*B. caccae*, *B. ovatus*) were grown for ~48 h and faster growing bacteria (*E. coli*) ~8 h. Then, 96-well plates were fixed with w.v 2% formaldehyde and stored at −20 ◦C for VLP enumeration. The area under the growth curve (AUC) was calculated after medications were administrated and calculated with Prism (version 7, GraphPad Software, San Diego, CA, USA). AUC for each treatment was calculated compared to the DMSO control for each bacterium on the day of their induction (n = 3). AUC decreases >15% were investigated for VLP production.

2.2.5. VLP Enumeration

Fixed samples were from the 96-well plates were thawed and centrifuged at 2000× *g* for 20 min. The VLP-containing supernatant was collected on 0.02 μm Whatman Anodisc filters (GE Healthcare, Chicago, IL, USA) and stained with 2.5 × SYBR Gold stain (final concentration, ThermoFisher Scientific, Waltham, MA, USA) before enumeration on an Axioskop (Zeiss, Oberkochen, Germany) epifluorescence microscope at 1000X. We counted a minimum of 300 events per slide, or 30 regions to increase statistical power of counts.

2.2.6. Prophage Induction of *C. beijerinckii* for DNA Sequencing and PCR

We performed increased in silico prophage prediction on the strain of *C. beijerinckii* with additional computational tools (VIBRANT [37] and PhiSpy [38]), and it was shown to contain eleven unique putative prophage regions. ORFs of the putative prophage regions were predicted and annotated with HMMER (V.3.2.1) [39] and the pVOG (version May 2016) database [40]. Annotated ORFs of putative prophage regions were grouped based on belonging to five functional modules (lysogeny, genome replication, head morphogenesis, tail morphogenesis, and host lysis).

PCR primers (Supplementary Figure 2.2A) were designed for all the complete and uncertain regions (P1, P2, P3, P4, P5, P7, P10) in addition to a bacteria-specific primer for the *C. beijerinckii dnaA* gene. We tested all primers on bacterial gDNA and confirmed their specificity with Sanger sequencing. To generate larger quantities of unfixed VLPs we repeated our induction protocol for ciprofloxacin 2 μg mL^{-1}, mitomycin 1 μg mL^{-1},

norfloxacin 10 μg mL^{-1}, and ampicillin 0.1 μg mL^{-1} in 42 wells of a PCR plate to increase the volume of sample.

2.2.7. Purification of Viral DNA from VLPs

Phage supernatants were concentrated by centrifugation. The phage pellet was resus- pended in SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7\ H_2O$, 50 mM Tris-Cl (pH 7.5)) and incubated sequentially with lysozyme (50 mg mL^{-1}), TURBO DNase and TURBO DNase buffer (ThermoFisher Scientific, Waltham, MA, USA), and proteinase K (20 mg mL^{-1}). Then, 5 M NaCl and 10% CTAB/0.7 M NaCl solution were added, and samples were transferred to phase lock gel tubes (light PLG tubes, QuantaBio, Beverly, MA, USA) with an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1 *v/v*, pH = 8.0, ThermoFisher Scientific, Waltham, MA, USA) and centrifuged. The top aqueous DNA-containing layer was left to precipitate overnight at −80 °C in 100% ice-cold ethanol and samples were then purified with the Zymo DNA Clean and Concentrator 25 kit (Zymo Research, Irvine, CA, USA). DNA concentrations were quantified with the Qubit dsDNA high-sensitivity (HS) assay kit (ThermoFisher Scientific, Waltham, MA, USA).

2.2.8. Extraction of Genomic DNA from Gut Bacterial Isolates

Bacterial genomic DNA was then extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany) and concentrated with the Zymo DNA Clean and Concentrator 100 kit (Zymo Research, Irvine, CA, USA), as per the manufacturers' instructions. DNA concentrations were quantified with the Qubit dsDNA broad-range (BR) assay kit (ThermoFisher Scientific, Waltham, MA, USA).

2.2.9. Shotgun Sequencing of Purified Viral DNA & Processing of Sequencing Data

Purified vDNA from each experiment was sheared using a Covaris ultrasonicator (Covaris, Woburn, MA, USA) and dual-indexed paired-end Illumina sequencing libraries were prepared using the Accel-NGS 1S Plus kit (Swift Biosciences, Ann Arbor, MI, USA). Pooled libraries were sequenced with 250 bp paired-end sequencing technology on the Illumina HiSeq platform at the Swift Biosciences facility and then trimmed with Trimmomatic (v0.83) [41]. Trimmed quality-filtered reads were aligned to the corresponding reference bacterial chromosome with Bowtie2 (v2.3.4.3) [42]. Manual curation of read coverage along the bacterial chromosome was done in Geneious Prime (v2020.0.4; Biomatters). The mean coverage of a given prophage region was calculated using the "bedcov" command in SAMtools. Mean coverage was normalized to the number of filtered reads in the sample, an approach known as total-sum scaling [43]. The "coverage" command in bedtools (v2.29.0) was used to determine the number of reads mapping to each prophage region within a given sample [44]. Circleator (v1.0.2) was used to generate figures containing bacterial genomes annotated with %GC content and the annotated predicted prophage regions [45].

2.3. Results

2.3.1. *In Vitro* Model to Study Prophage Induction of Human Gut Bacteria

We screened 480 different conditions for bacterial inhibition: 12 medications at five different concentrations for each of our eight bacterial isolates. We selected four categories of medications reported to impact human gut bacteria: non-steroidal anti-inflammatory (NSAID; diclofenac, ibuprofen, tolmetin) [9, 10], chemotherapy (busulfan,

fludarabine) [11], cardiac medications (digoxin) [12], and antibiotics (ampicillin, ciprofloxacin, norfloxacin, streptonigrin, mitomycin) [4-8], along with acetaminophen, the most commonly used analgesic (Table 2) [46]. All of the medications chosen are taken orally, which is more relevant to the human gut microbiota than intravenous medications [34]. Diclofenac and ibuprofen have been previously reported to inhibit growth of bacterial isolates [47-49]. Fludarabine and digoxin have been shown to inhibit growth of human gut bacteria in conditions relevant to the human gut [13]. Fludarabine was also shown to exhibit increased cytotoxicity in the presence of bacteria [50]. Ciprofloxacin, ampicillin, digoxin, and norfloxacin led to differential expression of gut bacterial genes, some of which were related to phage replication [12]. Antibiotics were selected based on their reported ability to induce prophages [51-54].

Type of agent	Drug	Mechanism of Action	Estimated Colon Concentration (µg/mL)	Tested Concentrations (µg/mL)
Antibiotic	Ampicillin	β-lactam: Cell wall synthesis inhibition	44.56 - 3565.06	100, 10, 1, 0.1, 0.01
	Ciprofloxacin	Fluoroquinoline: Bacterial DNA gyrase and topoisomerase	106.95 - 1247.77	2, 0.2, 0.02, 0.002, 0.0002
	Norfloxacin	Fluoroquinoline: Bacterial DNA gyrase and topoisomerase	427.81 - 998.22	10, 1, 0.1, 0.01, 0.001
	Streptonigrin	Aminoquinone: Bacterial DNA and topoisomerase	0.10 - 0.19	10, 1, 0.1, 0.01, 0.001
	Mitomycin C	DNA Cross Linker	-	100, 10, 1, 0.1, 0.01
NSAID	Diclofenac	Analgesic, antipyretic, and anti-inflammatory	44.56 - 66.84	100, 10, 1, 0.1, 0.01
	Ibuprofen	Inhibitor of COX	106.95 - 427.81	100, 10, 1, 0.1, 0.01
	Tolmetin	tNSAID heteroaryl acetic acid derivative	35.65 - 1048.13	10, 1, 0.1, 0.01, 0.001
Chemotherapy	Busulfan	Alkylating agent - Alkyl sulfonate	1069.52	100, 10, 1, 0.1, 0.01
	Fludarabine	Inhibits DNA Synthesis	7 - 7.49	100, 10, 1, 0.1, 0.01
Mild Analgesic	Acetaminophen	Not well known	0.0 312.83	100, 10, 1, 0.1, 0.01
Cardiac	Digoxin	Na+/K+ pumps	0.07 - 0.13	100, 10, 1, 0.1, 0.01

Table 2.2 Medication concentrations and estimated concentrations.

Estimated colon concentrations were calculated based on oral dose, bioavailability, and volume of average colon (Supplementary Table 2.1). Mitomycin estimated colon concentration was not calculated as it is taken intravenously. NSAID: Non-steroid anti-inflammatory drug.

A wide range of concentrations relevant to the gut microbiota were tested as prophage induction can occur between maximum and minimum bacterial inhibition concentrations [55]. In the absence of data on the concentrations of our tested medications in the gut or in faeces, we estimated colon concentrations using the common oral dosage and the bioavailability of each medication, with the exception of mitomycin (Supplementary Table 2.1). Tested medication concentrations (Table 2.2) were determined to be physiologically relevant

to the human gut microbiota: half the medications had at least one tested concentration that fell within the range of estimated colon concentrations, the other half

tested were below the estimated colon concentration (Table 2.2). The median estimated concentration in the colon of our tested medications was 86.90 μg mL^{-1}, below our maximum tested concentration of 100 μg mL^{-1}. We limited our study to the relevant medication concentrations in an effort to approximate *in vitro* conditions to that of the human gut.

2.3.2. Antibacterial Activity of Medications on Human Gut Isolates *In Vitro*

Inhibition of bacterial growth can either be caused by the direct antibacterial effect of the medication, or by cell lysis from prophage induction. Here, we used inhibition of bacterial growth as a preliminary screen of 480 different treatments which may lead to prophage induction.

Antibacterial activity was measured by the difference in the AUC between the control (DMSO) and the treatment (Figure 2.1A). Bacterial growth inhibition was defined here by an antibacterial activity that leads to a decrease in the AUC of 15% or more (AUC15). Of the 480 treatments tested, 64 (13%) led to bacterial growth inhibition (Figure 2.1B). All of our bacterial isolates were inhibited by at least one medication at one concentration tested, except *E. faecalis* (Figure 2.1B). As predicted, antibiotics led to the most treatments with bacterial growth inhibition, specifically ampicillin and mitomycin, inhibiting five and seven bacteria, respectively (Figure 2.1B).

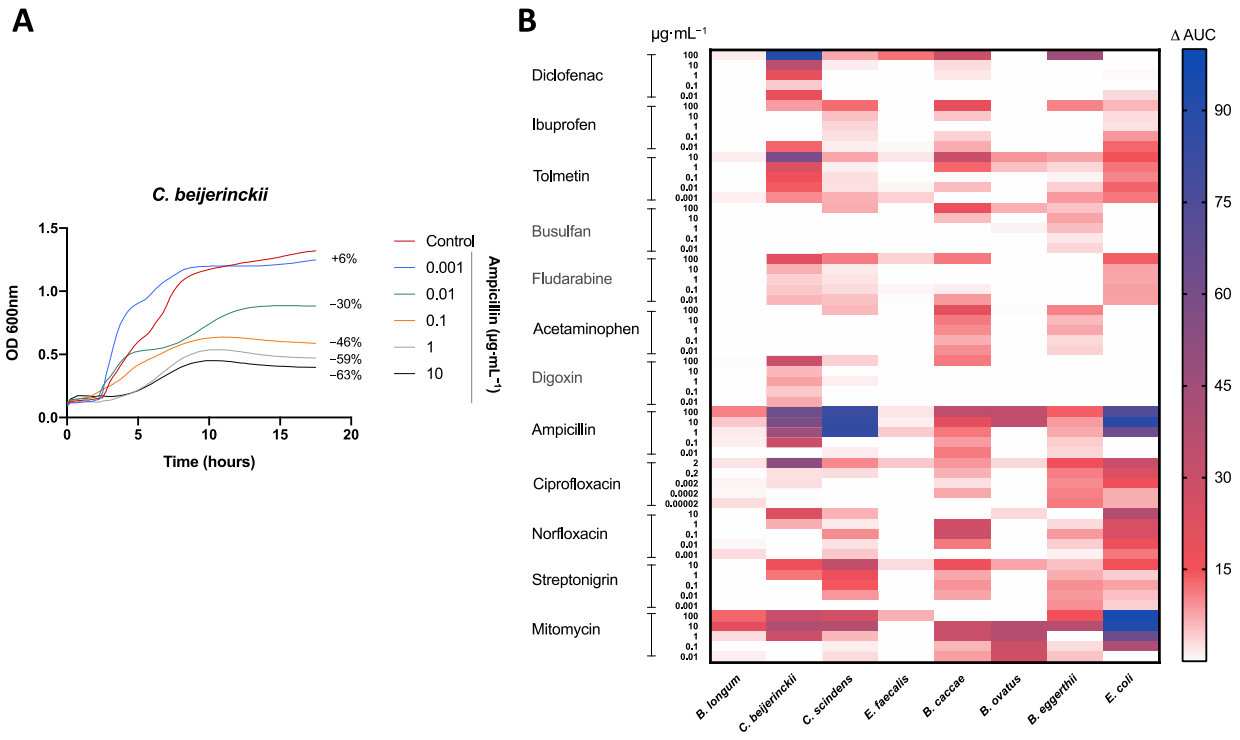


Figure 2.1 Antimicrobial activity of drugs on human gut isolates

(A) Representative growth curve of *C. beijerinckii* (mean $OD_{600\,nm}$ measurements of $n = 3$) with ampicillin treatment and DMSO (control). Percent difference in AUC (treatment to control) labelled for each treatment. (B) Heatmap of the percentage change in the AUC of all five treatments for each drug compared to the control (DMSO) for all tested bacteria. All drugs were dissolved in DMSO. Control consisted of DMSO at a 1% final concentration. Treatments repeated with a $n = 3$.

2.3.3. Medication Caused Prophage Induction of Human Gut Lysogens

We defined prophage induction as the combination of bacterial growth inhibition (Figure 2.1) and a significant increase in VLP compared to control (Figure 2.2A). We thus further studied the 64 treatments leading to the inhibition of bacterial growth, spanning all 12 tested medications, for changes in VLPs (Figure 2.2B), as counted by epifluorescence microscopy (Figure 2.2A).

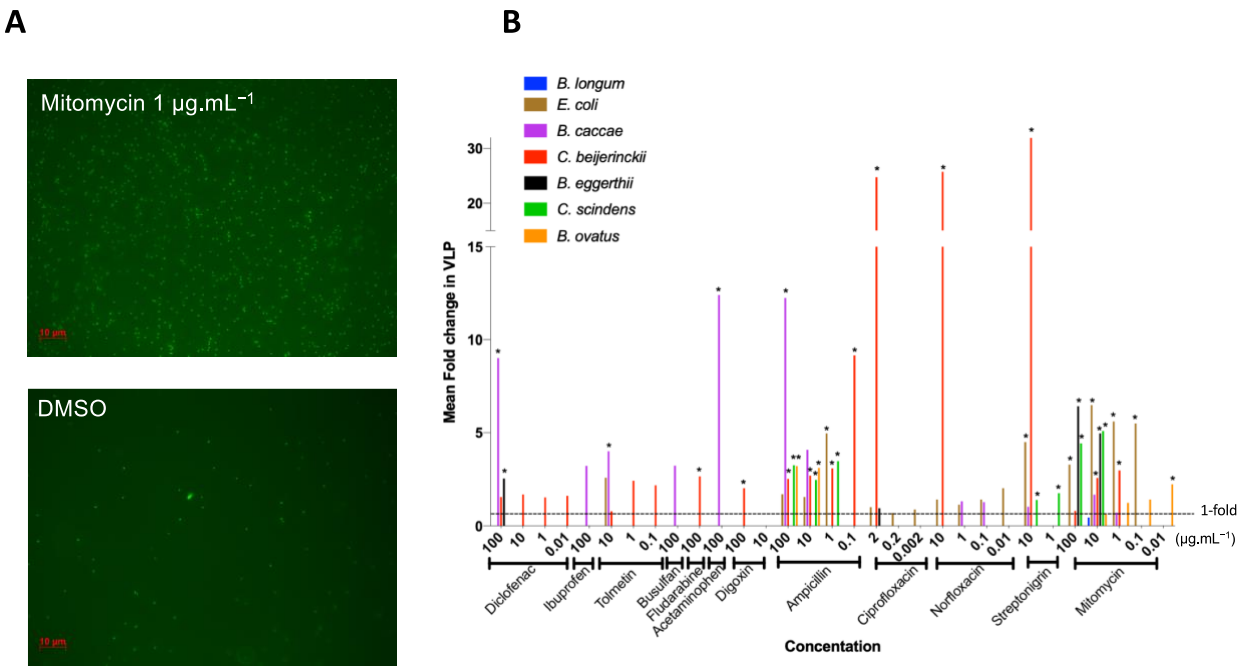


Figure 2.2 Fold increase in virus-like-particles (VLPs) from antimicrobial activity of drugs:

VLPs were counted in drug treatments that resulted in an AUC15. Fold increase in VLPs was obtained by comparing treatment VLP abundance relative to control VLP abundance. (A) Representative images of epifluorescence microscopy of SYBR Gold-stained VLPs at 1000X magnification of *C. beijerinckii*. (B) Fold increase in VLPs resulting from bacterial growth inhibition by all the drugs tested: mean increase in VLPs (n = 3) per treatment compared to DMSO control. * represents $p < 0.05$, Dunnett's multiple comparison test between treatment and control (n = 3).

Most bacterial isolates with growth inhibition had a corresponding increase in VLPs (84%), and over half (55%) increased significantly compared to the controls (Figure 2.2B), indicating prophage induction. Prophage induction is isolate- and medication-specific: no one medication induced all inducible prophages, and on average, bacteria were induced by three different medications (rarely the same ones), with results often concentration specific (Figure 2.2B).

Mitomycin, a commonly used prophage inducer for lysogeny estimates and prophage detection [56, 57] was our most widespread inducer as expected, resulting in the lysis of five of eight strains and representing approximately one third of treatments

where induction occurred (Figure 2.2B). Only *B. caccae* was not inhibited by mitomycin, despite containing an inducible prophage (Figure 2.2B). Ciprofloxacin is also a common antibiotic for prophage induction, yet it did not inhibit many bacteria at the low concentration we tested (Figure 2.1B). We thus increased its concentration to 20 $\mu g\ mL^{-1}$ by dissolving in slightly acidic water (pH 6.5) and tested the non-induced inhibited bacteria with this higher concentration. All bacteria tested with the higher ciprofloxacin concentration were inhibited, but only *C. scindens* was lysed as a result of prophage induction (Supplementary Figure 2.3).

Ten of our twelve tested medications led to prophage induction, including five host-targeted medications, spanning all the medication categories: diclofenac (NSAID), tolmetin (NSAID), fludarabine (chemotherapy), acetaminophen (analgesic), digoxin (cardiac) (Figure 2.2B). Diclofenac was the only non-antibiotic to cause induction in more than one bacterial isolate (*B. caccae* and *B. eggerthii*). *B. caccae* and *C. beijerinckii* make up more than half of the positive results for non-antibiotic prophage induction. This indicates that specific gut isolates are more susceptible to non-antibiotic medications. Only two non-antibiotics did not lead to prophage induction in our isolates: ibuprofen (NSAID) and busulfan (chemotherapy), despite increasing overall VLP counts (3-fold for ibuprofen, adjusted *p*-value: 0.384; 3-fold increase for busulfan, adjusted *p*-value: 0.983; Figure 2.2B).

2.3.4. Confirmation of In Silico Predicted Prophages Induced in *C. beijerinckii*

C. beijerinckii was the most widely induced bacterium tested (Figure 2.2B) and led to the largest increase in VLPs (Figure 2.2B). Several distinct putative prophages were

predicted on the genome of our strain of *C. beijerinckii* by VirSorter and PHASTER (Supplementary Figure 2.1C), more than any of our other bacterial strains (Supplementary Figure 2.1). Due to the abundance of VLPs produced by *C. beijerinckii* induction, we were able to obtain enough viral DNA (vDNA) to perform both PCR and shotgun sequencing. This allowed us to investigate which prophages found in the bacterial genome were being induced in *C. beijerinckii* for each treatment of interest.

We increased in silico prophage prediction on *C. beijerinckii* with VIBRANT [37] and PhiSpy [38] to ensure no potential prophages were missed for primer design (Figure 2.3A). Three prophage regions were scored as complete based on our scoring system: 'complete' genome status was determined with three or more tools predicting the region, a lysogeny module, and at least three other modules; 'uncertain' genome status was determined when at least two prophage prediction tools identified the region, having less than four modules, and one of the following 'head', 'tail' or 'lysis' morphogenesis modules; and 'incomplete' if predicted by just one tool (Figure 2.3B). To determine which prophages were being induced, we designed PCR primers for all the complete (P1, P2, and P3) and uncertain regions (P4, P5, P7, and P10), as well as a bacteria-specific primer for the *C. beijerinckii dnaA* gene (Supplementary Figure 2.S2A). We reran prophage inductions for ciprofloxacin, mitomycin, norfloxacin, and ampicillin at 2, 1, 10, 0.1 $\mu g\ mL^{-1}$, respectively. Primers specific for prophage region P3 amplified DNA in all our treatments, and primers specific for prophage region P1 only amplified in the mitomycin and ampicillin treatments (Figure 2.4A). None of the other predicted regions were amplified (Figure 2.4A). P3 and P1 regions were amplified in controls, due to background spontaneous induction that

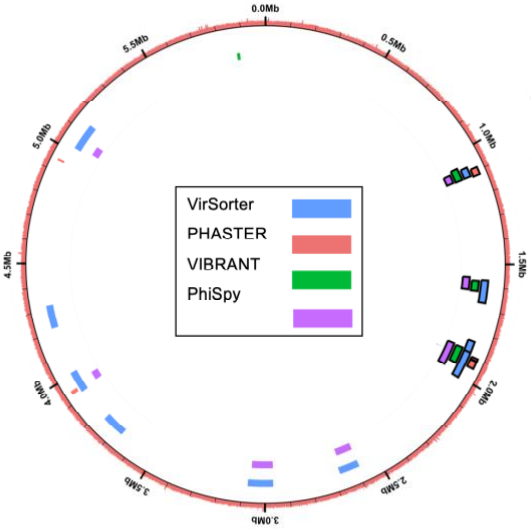
occurs over long growth-curves. We confirmed it is not bacterial contamination, as all vDNA was negative for the bacterial *dnaA* gene (Figure 2.4A).

In addition, we performed shotgun metagenomics on the extracted vDNA used in each PCR reaction. These qualitative data confirm the PCR detected prophages, and that no prophages were missed during primer design or by prophage detection tools. Normalized read coverage increased within induced prophages regions P1 and P3 (>50 fold), relative to the rest of the bacterial genome in all treatments (Figure 2.4B, Supplementary Figure 2.2B). The negative PCR reaction of P1 for the ciprofloxacin and norfloxacin treatments may be due to the limit of detection of our PCR. This is supported by the fact that read coverage of P3 was always higher than in P1 (Supplementary Figure 2.2B), indicating its induction is likely less productive. Our shotgun metagenomics required an amplification step before sequencing and is therefore not quantitative, but for all treatments except ampicillin, read coverage increased in treatment compared to control (Supplementary Figure 2.2C), supporting true prophage induction.

We confirmed our approach using prophage induction of the previously reported inducible prophage found in *B. longum* with 2 mM hydrogen peroxide (Supplementary Figure 2.4) [58]. Whole genome sequencing of vDNA from *B. longum* indicate that our predicted prophages P4 and P6 (Supplementary Figure 2.4C) are being induced (Supplementary Figure 2.4B,D). The P6 prophage corresponds to the previously reported inducible prophage Binf4 [58]. Our P4 prophage corresponds to two prophages predicted by Ventura *at al*. [58] (Binf2 and Binf3). We detected induction of prophage P4, which was not detected by Ventura *at al*. [58] as their primers were designed for complete

circularized phage DNA [58] but Binf2 and Binf3 seem to correspond to one large prophage rather than two smaller complete phages.

A



B

Region	Position	Functional Module: Lysogeny	Genome Replication	Head Morphogenesis	Tail Morphogenesis	Host Lysis	Genome Status	Prediction Tools
P1	1,058,427 - 1,119,559	✓		✓	✓	✓	Complete	PH, VS, PS, VIB
P2	1,561,206 - 1,671,012	✓	✓	✓	✓		Complete	VS, PS, VIB
P3	1,839,801 - 2,005,703	✓	✓	✓	✓	✓	Complete	PH, VS, PS, VIB
P4	2,584,548 - 2,674,268	✓				✓	Uncertain	VS, PS
P5	2,965,678 - 3,076,663	✓	✓		✓		Uncertain	VS, PS
P6	3,669,010 - 3,769,192		✓				Incomplete	VS
P7	3,920,834 - 4,014,153			✓	✓	✓	Uncertain	PH, VS, PS
P8	4,218,606 - 4,318,259	✓				✓	Incomplete	VS
P9	4,943,584 - 4,952,011				✓		Incomplete	PH
P10	5,022,191 - 5,140,277	✓			✓	✓	Uncertain	VS, PS
P11	5,872,941 - 5,886,328	✓				✓	Incomplete	VIB

Figure 2.3 *In silico* computational prophage prediction of *C. beijerinckii*:

Prophages were predicted using PHASTER web-server (PH), VirSorter (VS), PhiSpy (PS), and VIBRANT (VIB) predictive software. (A) Predicted prophage regions located within the bacterial genome, color-coded according to the software predictive tool used. Complete prophages have black outline. (B) Regions with overlap were merged into 11 predicted prophages P1–11. ORFs were aligned to the prokaryotic virus orthologous groups (pVOG) database using HMMER. The following functional modules were used to classify prophage region completeness: lysogeny (integrases, repressors), genome replication (helicases, ssDNA binding proteins, endonucleases), head morphogenesis (terminases, portal proteins, capsid proteins), tail morphogenesis (tail fiber genes, tail tape measure genes), host lysis (holins, lysins). Three prophage regions were scored as complete based on our scoring system: 'complete' genome status was determined with three or more tools predicting the region, a lysogeny module, and at least three other modules; 'uncertain' genome status was determined when at least two prophage prediction tools identified the region, having less than four modules, and one of the following 'head', 'tail' or 'lysis' morphogenesis modules; and 'incomplete' if predicted by just one tool.

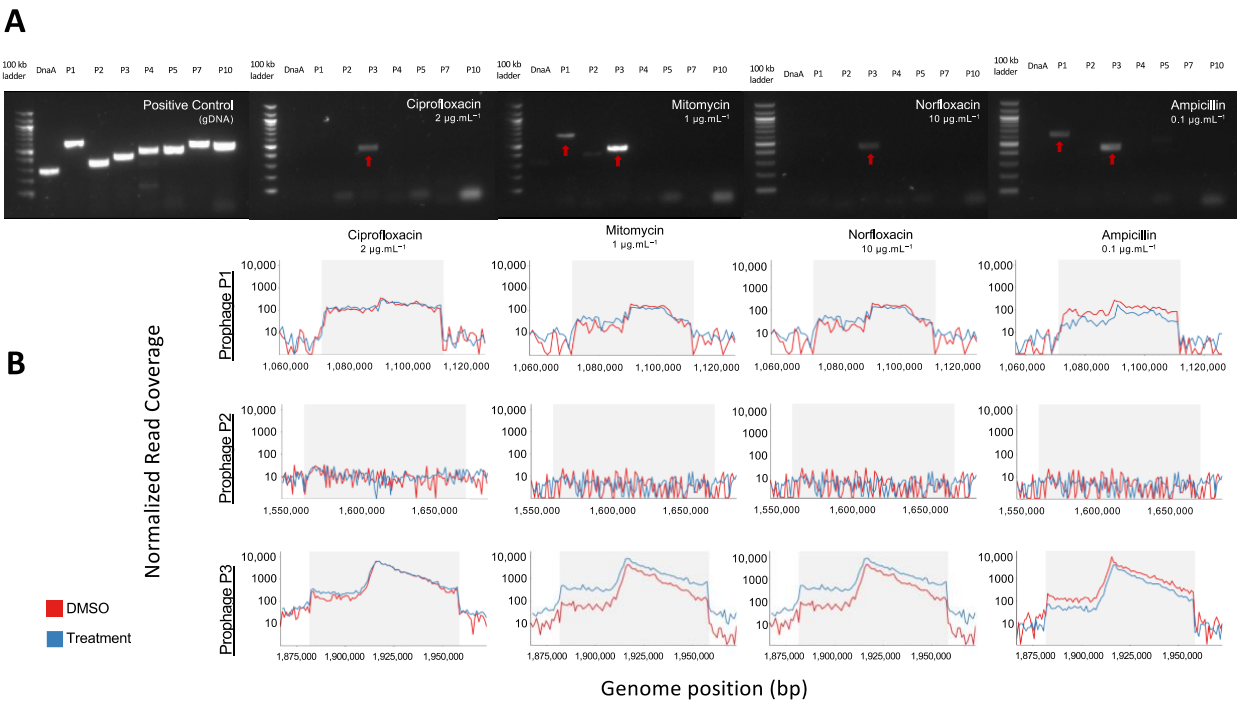


Figure 2.4 PCR and shotgun sequencing of extracted VLPs from *C. beijerinckii*:

(A) Agarose gel electrophoresis of PCR products from bacterial DNA and vDNA after exposure to ciprofloxacin (2 μg mL^{-1}), mitomycin (1 μg mL^{-1}), norfloxacin (10 μg mL^{-1}), and ampicillin (0.1 μg mL^{-1}) (left to right). Each lane corresponds to one predicted prophage region (P1,P2,P3,P4,P5,P7,P10), the conserved bacterial *dnaA* gene, or a 100 kb ladder. Amplification of P1 and P3 regions show their prophage induction with the corresponding treatment. (B) Representative mapping of shotgun sequenced vDNA reads to the genome of *C. beijerinckii* with read coverage increasing within the genome position of predicted complete prophages P1 (top), P2 (middle) and P3 (bottom) for each treatment shown above in the gel electrophoresis. Coverage increased >50× relative to the bacterial genome for prophage regions P1 and P3, but not for P2.

2.4. Discussion

The gut is an environment in which microorganisms are constantly exposed to medications, whose consumption is on the rise [2, 3]. Here, we set out to better understand the role of medications on the gut bacteriophage community, an often-overlooked member of the gut microbiota. Twelve medications from multiple classes were screened to explore their role in prophage induction on eight bacterial lysogens from the human gut. We show that bacterial growth inhibition by these medications leads to prophage induction in at least 55% of cases.

Community-level studies of medications in the gut have shown they are correlated with alterations in bacterial diversity [10-12]. One possible explanation for these differences in bacterial diversity can be explained by the direct antibacterial activity of these compounds. For example, NSAIDs, such as diclofenac, have been shown to have an inhibitory effect on bacteria through DNA replication interference [47] similar to quinolones [48]. This is further illustrated in a recent study identifying that NSAIDs had the largest impact on the gut microbiota in a large cohort of healthy adults exposed to a variety of xenobiotics [10]. Chemotherapy medication, fludarabine [50] showed similar inhibition. Ibuprofen for its part was shown to inhibit *Staphylococcus aureus* in a larger screen of six unrelated bacteria [49]. More recently, Maier *et al.* [13] expanded the study of gut isolates to a large-scale screen of 1000 medications against 40 human gut isolates to understand the direct connection between medications and antibacterial activity. They concluded that 24% of non-antibiotic medications were capable of inhibiting growth of at least one bacterium at concentrations commonly found in the gut. We found a much higher rate of bacterial growth inhibition by non-antibiotics medications, supporting their predictions that increased concentrations would lead to increased antibacterial activity [13] as we often tested concentrations 10-fold higher. Using the same *B. caccae* isolate (*B. caccae* ATCC 43185), we found diclofenac, ibuprofen, tolmetin, busulfan, and acetaminophen to inhibit growth only at concentrations higher than tested by Maier *et al.* [13]. Yet, the concentrations we tested remain biologically relevant according to our estimations of colonic concentrations.

We also conclude that bacterial growth inhibition resulting from these medications is species-specific. In contrast with Maier and colleagues, who found 11 drugs that led to

growth inhibition in all bacteria tested [13], we did not identify “universal” growth inhibitors. Mitomycin, which is often used to detect inducible prophages, was the most effective medication, inhibiting growth in seven isolates. It is important to note that the concentration for mitomycin induction ranged from 0.01–100 μg mL^{-1}, and two of our bacteria with inducible prophages were not induced by mitomycin. This could explain the reported underestimation of lysogeny in communities or isolates [59]. Ciprofloxacin, a common replacement for mitomycin in prophage induction experiments, unexpectedly inhibited only three bacterial isolates when given at 2 μg mL^{-1}, including *E. coli*, which is known to be inhibited by ciprofloxacin at lower concentrations [60]. This low effect of ciprofloxacin could be explained by the low concentrations tested, as seen in previous studies [13, 60, 61]. All bacteria were inhibited at higher concentrations of ciprofloxacin, but we report prophage induction for only one (*C. scindens*) (Supplementary Figure 2.3).

Collectively, our data support the role of drugs inhibiting bacterial growth in a species-specific manner, which can alter the bacterial diversity of the human gut. We further explored if this growth inhibition could lead to prophage induction, thereby compounding unintended consequences of exposure to these drugs on the gut microbiota.

The antimicrobial activity found in our study was strongly linked to prophage induction of lysogens. VLP production increased in 84% of cases where there was bacterial growth inhibition, and 50% of those increases were statistically significant. Importantly, these increases are not resulting only from antibiotics, previously reported to be prophage inducers, but also from non-antibiotic medication, which have not been reported as prophage inducers. Medications tested included common over-the-counter

drugs like acetaminophen and ibuprofen, whose effects on the gut virome have not been reported. Ten of the twelve drugs tested led to prophage induction, and the two drugs for which there was no induction, we nevertheless report an increase in VLPs, suggesting that these compounds can still impact the gut virome.

A limitation to our study was the preliminary screening for bacterial growth inhibition before counting VLPs. First, it is likely that some of our isolates contain prophages inducible by conditions or compounds we have not tested here. For example, we were not able to induce *B. longum*, a strain reported to contain a prophage inducible by hydrogen peroxide [58], with any of our compounds. We thus tested our *B. longum* strain with hydrogen peroxide and saw a significant increase in VLPs without bacterial growth inhibition (2% decrease; Supplementary Figure 2.4AB). In addition, we further quantified VLPs in treatments that were close to our cut-off for bacterial growth inhibition: *C. beijerinckii* (AUC13) exposed to busulfan and *C. scindens* exposed to ibuprofen (AUC7) led to significant increases in VLPs (Supplementary Figure 2.5). Thus, by using bacterial growth inhibition as a preliminary screen, our approach leads to a conservative detection of inducible prophages and we are likely underestimating prophage induction by our drugs.

Lastly, epifluorescence microscopy quantification of VLPs does not allow the direct observation of phages. It is thus possible that our VLPs may not be true phages and correspond to other tightly packaged DNA, or membrane vesicles and gene-transfer-agents [62]. Due to superinfection immunity provided by the prophage to the host, we cannot proceed with plaque assays to confirm they are infectious phages. To partly address this concern, we extracted and sequenced the vDNA from our *C. beijerinckii*

induction experiments and were able to confirm that the VLPs were indeed true phages induced from within the lysogenic bacterial chromosome.

In our study, we show that a wide range of medication can alter the interactions between phages and bacteria in the gut through prophage induction. The species-specific response to these compounds and resulting differential prophage induction patterns suggest distinct mechanisms of induction, which remain to be investigated. Importantly, such prophage-mediated responses to medications could explain the correlations observed between medication and alterations in the gut phage community [12, 14]. Going forward, it will be necessary to tease apart the direct effects of these medications on prophage induction in the gut. Co-culturing bacterial isolates *in vitro* or using gnotobiotic mouse models, as well as simulated gut communities, will be essential to evaluate the role these species-specific responses have on the gut microbial community, and will allow comparisons with other community-level perturbations such as an inflamed gut environment as in Crohn's disease [27]. Investigating the downstream consequences of the increased phage abundance and resulting pressure on gut bacterial communities will also help understand the role of prophages in the gut microbiome and their importance for human health.

Supplementary Materials: The following are available online at https://www.mdpi.com/1999-491 5/13/3/455/s1, Figure 2.1: Location of putative prophages within bacterial chromosome or contig, Figure 2.2: PCR Identified Prophages of C. *beijerinckii*, Figure 2.3: Bacteria not inhibited by low dose of ciprofloxacin grown with higher concentrations (20 μg mL^{-1}) of ciprofloxacin dissolved in water (pH 6.5) as vehicle, Figure 2.4: Induction of *B. longum* prophage by hydrogen peroxide in absence of bacterial

growth inhibition, Figure 2.5: Prophage Induction without antibacterial activity of *C. beijerinckii* and *C. scindens*, Table 2.1: Maximum and minimum oral dose concentrations calculations.

Author Contributions: Conceptualization, S.G.S. and C.F.M.; methodology, data curation, formal analysis, S.G.S. and M.S.; writing—original draft preparation, S.G.S.; writing—review and editing, S.G.S., M.S., A.P.H. and C.F.M.; supervision, C.F.M. and A.P.H.; project administration, funding acqui- sition, resources C.F.M. All authors have read and agreed to the published version of the manuscript.

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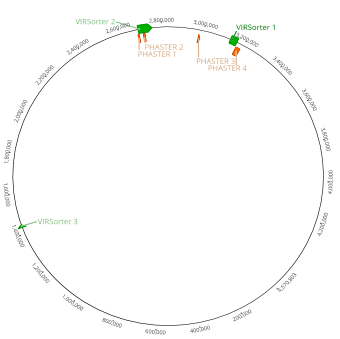
2.6. Supplementary Material

2.6.1. Figures



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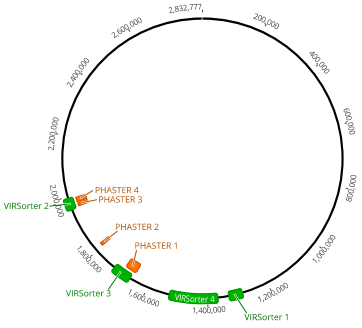

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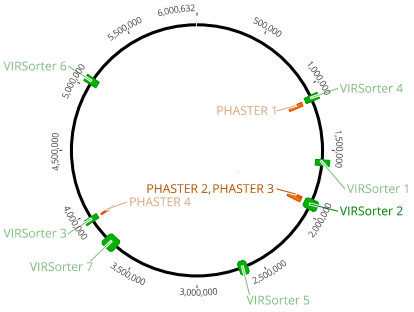

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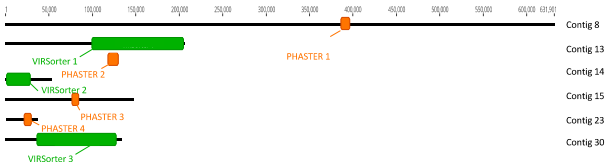

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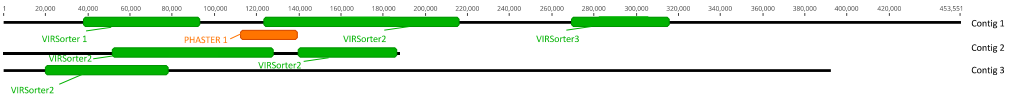

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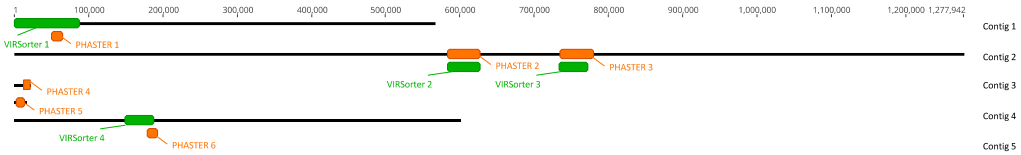


Enterococcus faecalis

Supplementary Figure 2.1 Location of putative prophages within bacterial chromosome or contig:

Prophages were detected by PHASTER Web Server (Default settings) and VirSorter. VirSorter was run with the default options except for the following: use viromes reference database (this database includes sequences from viral RefSeq as well as those obtained from aquatic and human gut, lung, and saliva environments) (--db 2), use DIAMOND for protein alignment (--diamond). For contiguous genomes (*B. eggerthii*, *B. ovatus*, and *E. faecalis*) only contigs containing prophages are shown (Made in Geneious 2020 0.05).

A

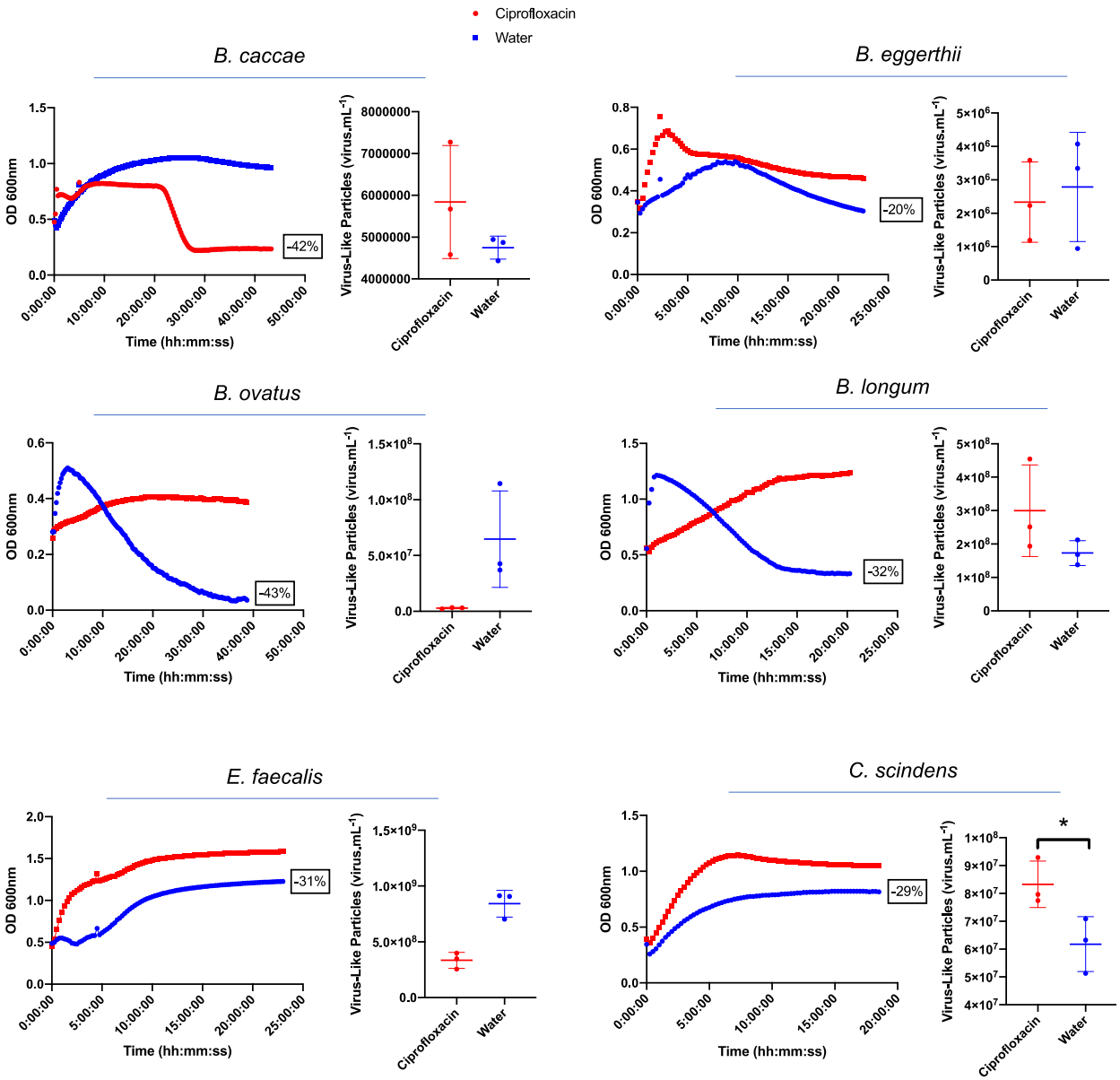
Primer	Sequence (5'➡3')	Description
Clostridium beijerinckii (ATCC 51743)		
C.beij-P1-1F	TTGGCGATACACCACAAGAG	portal protein (CBEI_RS04885)
C.beij-P1-1R	TCTTCACCTCCTCCCAAGTC	
C.beij-P2-1F	ATCCTGTTTTTGCTGACGATGG	portal protein (CBEI_RS07205)
C.beij-P2-1R	AGCACCTTGGAATGGTTGTCC	
C.beij-P3-1F	CGGTTAAAGCTAATTGGGGAC	tail sheath protein (CBEI_RS08600)
C.beij-P3-1R	ATTGTTCCTGTGGCTCCTGA	
C.beij-P4-1F	TTAGATAAATGCCAGGGCTGC	RNA polymerase sigma factor (CBEI_RS11590)
C.beij-P4-1R	TGTTGCCTACGCTACCAATG	
C.beij-P5-1F	TATGGCGATGGTCCTGACAC	hypothetical protein (CBEI_RS13335)
C.beij-P5-1R	ATCTCTCGCATGGGTCTTCC	
C.beij-P7-1F	TGCCTTTCCACTTCCTTCACC	baseplate assembly protein (CBEI_RS17380)
C.beij-P7-1R	GAAGCTCCAGAGGTGCCCAA	
C.beij-P10-1F	AGCCTGTCCCATCTTGTTGAG	putative tail protein/transcrp. regulator (CBEI_RS22455)
C.beij-P10-1R	ACAATGAAACCAAGGGTGCC	
C.beij-DnaA-1F	GCTGCGAACCTCTGTCTATTTC	DnaA gene, chromosomal DNA replication initiation factor
C.beij-DnaA-1R	TGGTGATTCTGCAACTGCCA	

B

			Normalized Coverage		
Xenobiotic	**Dose**	**Prophage**	**Vehicle Control**	**Treatment**	**Fold Change**
Clostridium beijerinckii (ATCC 51743)					
Ampicillin	0.1 μg/mL	P1	111.399	53.556	0.481
		P3	1144.519	649.626	0.568
Mitomycin C	1 μg/mL	P1	77.860	74.031	0.951
		P3	716.779	1691.936	2.360
Norfloxacin	10 μg/mL	P1	157.537	246.761	1.566
		P3	839.069	1544.530	1.841
Ciprofloxacin	2 μg/mL	P1	152.458	162.869	1.068
		P3	1307.908	1436.539	1.098

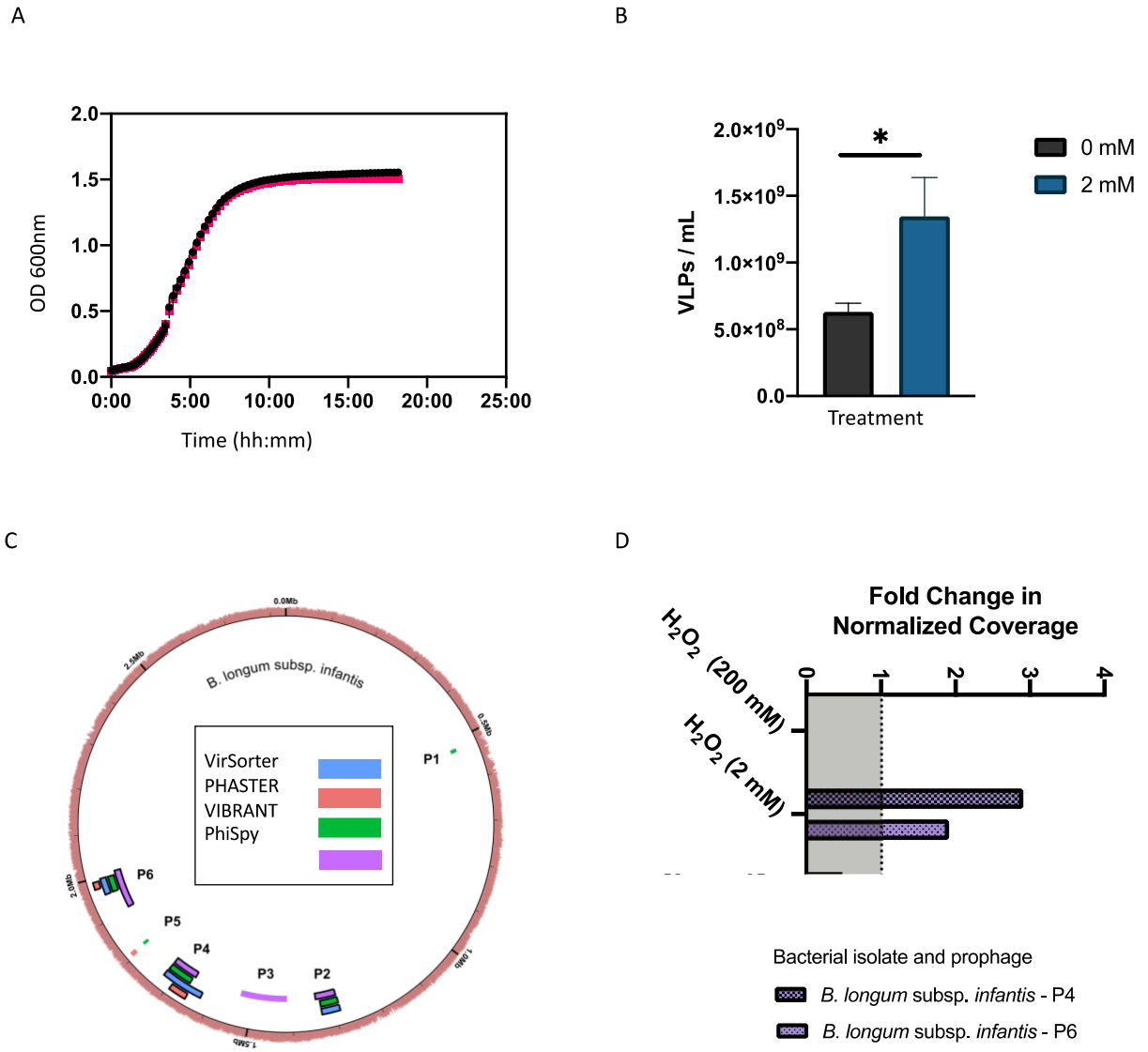
Supplementary Figure 2.2 PCR Identified Prophages of *C. beijerinckii:*

(A) Primer sequence for each prophage region and description of protein associated with sequence. (B) Normalized read coverage by each prophage region after induction treatment of shotgun sequenced vDNA.



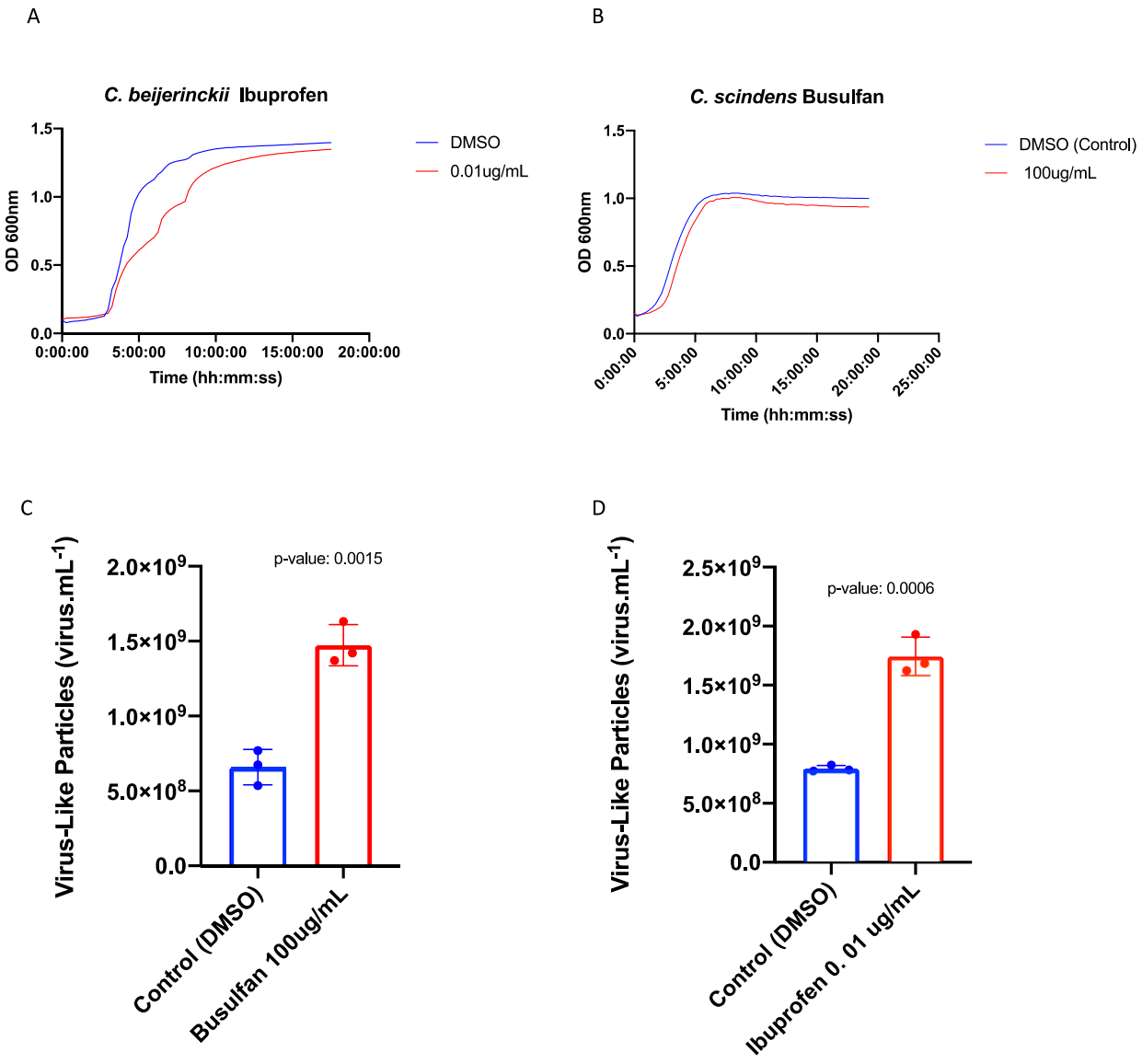
Supplementary Figure 2.3 Bacteria not inhibited by low dose of ciprofloxacin:

Grown with higher concentrations (20µg.mL^{-1}) of ciprofloxacin dissolved in water (pH 6.5) as vehicle. Growth curves are mean OD 600nm values. Virus-like-particle plots show individual values (n=3), with mean (horizontal bar) and standard deviations (vertical bars). * represents $p < 0.05$ (unpaired t-test) between control and treatment.



Supplementary Figure 2.4 Induction of B. longum prophage by hydrogen peroxide in absence of bacterial growth inhibition:

(A) Growth curve of *B. longum* in Control (Black) compared to exposure to 2mM hydrogen peroxide treatment showing no change in growth curve compared to (red) water control. (B) Increase in VLPs after exposure to 2mM hydrogen peroxide compared to control (*, p<0.05) (C) Detection of predicted prophage regions using VirSorter, PHASTER, VIBRANT, and PhiSpy (D) Fold change in read coverage compared to control of vDNA obtained by shotgun sequencing after hydrogen peroxide treatment for prophage regions P4 and P6.



Supplementary Figure 2.5 Prophage Induction without antibacterial activity of *C. beijerinckii* and *C. scindens*:

(A) Growth curve of *C. beijerinckii* with Ibuprofen and the corresponding (B) significant increase in VLPs as well as (C) *C. scindens* with Busulfan and corresponding (D) significant VLP increase. (Significance calculated by unpaired two-tailed t-test)

2.6.2. Tables

Drug	Minimum Range of Dose (mg)	Maximum Range of Dose (mg)	Minimum Range of Bioavailability (%)	Maximum Range of Bioavailability (%)	Minimum Amount in Colon (µg.mL-1)	Maximum Amount in Colon (µg.mL-1)
Ampicillin	250	4000	50	90	44.56	3565.06
Ciprofloxacin	100	1000	30	40	106.95	1247.77
Norfloxacin	400	800	30	40	427.81	998.22
Streptonigrin	0.2	0.2	48	72	0.10	0.19
Mitomycin C*	-	-	-	-	-	-
Diclofenac	50	75	50	50	44.56	66.84
Ibuprofen	300	1200	80	80	106.95	427.81
Tolmetin	200	700	50	90	35.65	624.00
Busulfan	2000	2000	70	70	1069.52	1069.52
Fludarabine	10	10	58	58	7.49	7.49
Acetaminophen	650	650	73	100	0.00	312.83
Digoxin	0.125	0.25	70	70	0.07	0.13

Supplementary Table 2.1 Maximum and minimum oral dose concentrations:

Were calculated using oral doses[1] (Drug@FDA[2], ATC/DDD).[3] Bioavailability data[1] (t[4], q[5], and j[6]) was used as an approximation for how much of the drug is absorbed into the blood before entering the colon. The minimum and maximum estimated concentration were calculated based on the remaining dose in the estimated volume of the colon (561mL).[7]

2.6.3. References

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Preface to Chapter 3

In Chapter 2, we showed that gut lysogens contain inducible prophages, and Chapter 3 follows up by determining if gut lysogens contain prophages that are stably integrated or if they are undergoing regular prophage induction. In Chapter 2 we show that most lysogens only undergo triggered prophage induction when exposed to antibiotics. We explore if a healthy individual undergoes triggered prophage induction in the absence of antibiotic usage, or if it is only a mechanism that occurs during perturbation.

In this chapter we scaled up a bioinformatic method from Chapter 2 that successfully showed which prophages of *C. beijerinckii* underwent triggered prophage induction. This method has been used in other studies [1, 2] and shows promise as tool for tracking prophage induction in healthy individuals bioinformatically. With this method we can identify which prophages are ‘active’ or ‘inactive’, and what their role is within the gut of a healthy individual. We will perform this investigation on the sequence data from the landmark study by Minot *at al* (2013). We selected this dataset as it was published prior to the development of tools to computationally identify active prophages. Importantly, both bacterial and viral communities were sequenced over 2.4 years by shotgun metagenomics.

We show that active prophages regularly contribute to the viral population throughout the 2.4 years the healthy individual was sampled, but they only represent a small fraction of the gut viral population. We propose that spontaneous prophage induction is the means for which prophages maintain their activity and presence in the

gut, as triggered prophage induction was limited to only one sampling time point (of 16). We show that regular spontaneous prophage induction is the source of a stable subset of phages in the viral community, while triggered prophage induction transiently disrupts the viral community composition at the family level.

Chapter 3. Bacteriophages Playing Nice: Lysogenic Bacteriophage Replication Stable in the Human Gut Microbiota

Abstract

The human gut is a dense microbial community, of which bacteria and bacteriophages, viruses of bacteria, exist stably, without major fluctuations in the gut of healthy individuals. This stability appears to be due to an absence of 'kill-the-winner' dynamics, and the existence of 'piggy-back-the-winner' dynamics, where lysogenic replication rather than lytic replication occurs. Revisiting the deep-viral sequencing data of a healthy individual studied over 2.4 years, we were able to improve our understanding of how these dynamics occur in healthy individuals. We assembled prophages from bacterial metagenomic data and show that these prophages were continually switching from lysogenic replication to lytic replication. Prophages were the source of a stable extracellular phage population: continually present in low abundance. In comparison to the lytic-phage population, where taxonomic diversity diverged over 2.4 years. The switch to lytic replication, or prophage induction, appears to occur mostly through spontaneous prophage induction. The observed phage replication dynamics of regular spontaneous induction are ecologically important as they allow prophages to maintain their ability to replicate, avoiding degradation and their loss from the gut microbiota.

Significance Statement

It has been eight years since Minot and colleagues published their landmark longitudinal study of phages in the gut. In the years following, the bioinformatic field

improved in great strides, including the methods of bacterial-genome assembly, phage-identification, and prophage detection. We leveraged the unprecedented deep sequencing of phages in this dataset by adding bacterial assembly and prophage detection. We show clearly for the first time that 'piggy-back-the-winner' dynamics are maintained in the gut through spontaneous prophage induction, not widespread triggered prophage induction. These dynamics play an important ecological role by creating a stable subpopulation of phages, which offers an explanation of how phages are maintained over the 2.4 years timeframe that this individual was studied.

3.1. Introduction

The human gut is home to a diverse and abundant community of microorganisms that are central to human health and development. The most abundant members of this community are bacteria, found in the trillions, and bacteriophages (abbreviated phages), with abundances almost equal to bacteria [3]. Phage and bacterial communities co-evolve over the lifespan of the human host through a variety of replication dynamics [4] shaped by several factors: age, diet, medication consumption, and disease [4-6]. Years of host-bacteria-phage interactions during human growth and development result in an adult gut microbiota that is unique to each individual [7], with strongly correlated viral and bacterial communities [8]. The gut microbiota shows remarkable taxonomic stability where the diversity of bacteria is a source of resilience to perturbation [9]. Understanding host-bacteria-phage interactions is of great importance to maintain, or improve, gut health.

Bacteria-phage relationships are driven by complex and dynamic interactions (see [10] for an overview). These interactions range from strictly parasitic to symbiotic,

depending on the replication cycle undergone by phages (lytic and lysogenic, respectively) [4, 11]. The lysogenic replication cycle differs from lytic replication as the phage genome integrates into the bacterial genome as a prophage [4, 11]. Phages capable of lysogenic replication are referred to as temperate, and their bacterial host as lysogens. Both lysogens [12] and temperate phages [13, 14] have been observed in high abundance in the gut of healthy individuals. A longitudinal study of ten healthy adults also showed that the proportion of temperate phages varies between individuals, yet is relatively stable over time [7]. Understanding how lysogeny persists in the gut of healthy individuals will help contextualize the uniqueness of an individual's gut virome [7], and the resulting viral-bacterial dynamics [8].

Lysogeny in the adult healthy gut has been hypothesized to stem from 'kill-the-winner' dynamics that play out during infancy [4, 15], leading to 'co-adaptation as a means to stabilize the interactions between phages and hosts' [16]. Co-adaptation has also been observed with CrAss-like-phages (e.g., ΦCrAss001) that co-exist with their *Bacteroides* host and persist in high abundance in the human gut [17] through a lytic-lysogeny intermediate [16, 18]. Dense populations of replicating bacteria [19, 20], as found in the gut, show increased rates of lysogeny through 'piggy-backing-the-winner' dynamics [21, 22]. Increased bacterial density increases the rate of lysogeny by phage coinfection [22], or through host-density regulated molecular switches [23]. Once integrated, prophages can provide a fitness advantage to their bacterial host through; protection from further infection, by superinfection exclusion, or immunity [24]; and introduction of new genes encoding for, virulence factors, antibiotic resistance, or novel metabolic functions [25]. In this case, prophages persist by 'making-the-winner' [22].

The benefits of lysogeny for bacteria are counter-balanced by the competitive cost of prophages being a genetic element that can switch to lytic replication through induction. Bacteria limit this switch by accumulating mutations within prophage regions at higher rates, rendering prophages inactive and incapable of lytic replication [26]. Hence, it is important to distinguish active prophages which can still be induced from inactive prophages. Prophage induction is typically triggered by extrinsic stimuli that result in DNA damage [27]. In the mammalian gut, external factors such as diet [2] and antibiotics [28] have been shown to trigger prophage induction. Prophages of gut bacterial isolates have been induced by dietary compounds [29], short-chain-fatty acids [30], antibiotics, and other medications [28]. Human pathologies, such as Crohn's disease, could also lead to an increase in prophage induction [31]. Yet, prophage induction can also occur in the absence of external triggers, in a process referred to as spontaneous prophage induction [32]. In contrast with triggered prophage induction, spontaneous prophage induction leads to a small subset of prophages undergoing lytic replication [33] and is thought to be caused by intrinsic factors like stochastic gene expression or sporadic DNA damage [27].

We sought to determine how prophage induction contributes to the gut virome of healthy individuals. We hypothesize that most prophages in the gut are capable of active replication and replicate by regular spontaneous prophage induction. Spontaneous prophage induction is the most likely cause of prophage induction in the absence of disease, antibiotic use, or drastic dietary changes. Active prophages undergoing regular spontaneous prophage induction would translate to a small but stable fraction of extracellular phage population present in the gut.

To test our hypothesis and better understand the role of lysogenic bacteria and temperate phages in the gut, we revisited a previously published dataset of sequenced bacterial and viral metagenomic gut samples of a healthy individual over the course of 2.4 years [34]. This dataset was selected based on daily longitudinal sampling, which has been previously reported to detect prophage induction [35] and the detection of active prophages [1, 2]. We report that prophages contribute a stable, continuous source of free temperate phages in this healthy individual through spontaneous induction, while triggered prophage induction is rare and by few prophages. Our results suggest evolutionary or adaptive constraints between bacteria and phages in the gut that limit the highly disruptive triggered prophage induction in favour of spontaneous prophage induction.

3.2. Methods

3.2.1. Data Set

We used the previously published data of a healthy male, whose fecal samples were collected at sixteen time points spread over 884 days (~2.4 years) [34]. The healthy twenty-three-year-old did not take antibiotics over the course of the experiment. The viral fraction was separated and sequenced at all sixteen timepoints, and eight timepoints were sequenced twice (Supplementary Figure 3.1A). Bacterial metagenomics were also obtained from fecal samples at three time points (once per week) during the same experimental time frame (Supplementary Figure 3.1A). For more details, see [34].

3.2.2. Viral Assembly

Sequence reads from viral-enriched libraries were trimmed with Trimmomatic V.0.36 [36], minimum quality 35 and minimum length of 70 (SLIDINGWINDOW:4:35 MINLEN:70 HEADCROP:10). As recommended [37], we assembled viral contigs for each sequence run separately with Spades [38] V.3.13.0 using the metaSpades option [39]. Viral assembled contigs were pooled, resulting in 291,758 contigs. Contigs less than 1kb in length were removed, resulting in 24,845 viral contigs. We used CD-HIT-EST V.4.8.1 [40, 41] with 0.95 similarity threshold, 8-word size, 0.9 length cut-off to cluster the contigs from the different samples, resulting in 22,091 non-redundant viral contigs. We then selected for phage contigs, as those fulfilling at least one of the following three criteria: 1) Detected as viral by VirSorter (V.1.0.6) with custom database option additionally using the Gut Virome Database [42]; 2) at least three ORFs (predicted by Prodigal V.2.6.3 with metagenomic mode) with homology (HMMER V.3.1b2 hmmscan minimum e-value 1e-5) to PVOG database (Downloaded on Dec 1, 2020); or 3) BLASTn homology (e-value 1e-10, with 80% coverage of shortest contig) to Gut Virome Database [42]. This resulted in 14,444 phage contigs, of which 6,176 viral contigs were greater than 2.5kb in length.

3.2.3. Bacterial Assembled Genomes

Bacterial metagenomic reads were trimmed with Trimmomatic V.0.36 [36] (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) and decontaminated for human sequences by aligning reads to *Homo sapiens* GRCh38 genome with Bowtie2 [43] V.2.3.5.1. Remaining trimmed and decontaminated reads were pooled and assembled into contigs with megahit [44] V.1.2.7 using the default settings. We generated

bacterial bins with contigs using MetaBat2 [45] V.2.12.1 -m 1500 (41 bins), CONCOCT [46] V.1.1.0 (77 bins) and MaxBin2 [47] V. 2.2.7 (14 bins). Bins were merged using DAS-Tool [48] V. 1.1.2. We used a score threshold of 0.35 to retrieve 27 bins. We then used CheckM [49] V.1.1.3 to confirm that all bins were unique. We selected bins that met the criteria of being either >40% complete and <10% contaminated by CheckM or having a DAS-Tool bin score of >0.4. This resulted in 25 medium-to-high quality bacterial bins. We assigned taxonomy to the bins using GTDB-Tk [50] V.1.4.1 using the reference database [51] version r95. We determined the relative abundance as the percentage of reads that aligned to one of the 25 bins we detected. The total number of aligned reads per bin was normalized by bin size (Figure 3.1A).

3.2.4. Prophage Detection and Identifying Active Prophages

Prophages were detected within bacterial bins by combining various tools: PHASTER [52], VIRSorter (V.1.0.6) [53], VIBRANT (V.1.2.1) [54], PhageBoost (V.0.1.7) [55], and mVIR (V.1.0.0) [56]. We also used a custom alignment method, where we aligned the viral reads to each bacterial bin using Bowtie2 (V.2.3.5.1), then used samtools mpileup to calculate coverage per base (with default perimeters). Using a sliding window of 1,000bp, if the average coverage was >10x, we considered the region as a possible prophage region. In total, we found 2,719 putative prophages. We merged prophage regions detected by multiple tools that overlapped by at least one base-pair resulting in 1,844 putative prophages. Only 17 prophages overlapped less than 100bp. Out of these 1,844 putative prophages, 651 prophages met one of our three phage criteria (see Viral assembled contigs methods). We then ran PropagAtE [57] (V.1.0.0) on these remaining 651 detected prophages with a modified script (available upon request) that replaced

host-coverage with the entire bin coverage when the flanking host region of the prophage was less than 5bp in length. It has been reported that prophages can be incorrectly binned when having multiple bins of closely related bacteria (in particular of the same species/genus) and when the prophage contig presents an absence of host-flanking regions [58]. We included prophages without host-flanking regions as the only bacterial bins assembled that shared genus (*Alistipes obesi*, *Alistipes putredinis*, and *Alistipes onderdonki*) did not contain any active prophages (Figure 3.1B). Finally, we used PropagAtE's criteria (default Cohen's *d* test and prophage:host ratio) to identify 52 predicted active prophages in this dataset [57].

3.2.5. Viral Community

The viral population used in this study consisted of 6,176 assembled phage contigs and 52 active prophages. Out of the 6,176 assembled phage contigs, we removed 338 that had homology to the set of active prophages (BLASTn e-value 1e-5), resulting in a total of 5,890 non-redundant viral contigs. Quality trimmed reads were aligned to viral contigs using Bowtie2 (V.2.3.5.1). Read coverage was normalized by sample using DESEQ2 V.1.30.1 [59], then by viral contig length. Viral contigs were considered 'present' in a sample if their genome was covered in 75% of the length by at least 1x coverage [37]. Family-level taxonomic classification was performed by using a voting-approach after comparing genes on the amino-acid level against the viral subset of TrEMBL by Demovir (github.com/feargalr/Demovir). No CrAss-like-phages were predicted, and their absence was confirmed through additional comparisons of our viral contigs against Guerin CrAss-like phage genomes [17], through BLASTn similarity and shared viral clusters (using VCONTACT2 [60]). Less than 0.05% of viral reads aligned to the Guerin

CrAss-like phage genomes [17], indicating a low-abundance of CrAss-like-phages in this individual.

Before predicting the replication strategy of viral contigs we selected the high-quality viral contigs (i.e., ≥ 40% complete and classified as high-quality by CheckV [61](V.0.7.0.)). These high-quality viral contigs (557) represent a mean of 82% (std. dev. 7.94) of quality controlled viral reads. We used Bacphlip [62] V.0.9.6 on our high-quality viral contigs to predict which were temperate (that is with a >50% chance of being temperate). Bacphlip is designed to be used on complete genomes and will under-report temperate phages when applied to incomplete or fragmented phage genomes.

3.3. Results

In order to study the prophages present within the genomes of bacterial lysogens, we used whole community metagenomic sequences and assembled 25 medium-to-high quality bacterial bins. All bacterial bins were taxonomically identified at the genus level, and 23 at the species-level. The assembled bacterial community consisted mostly of *Firmicutes* and *Bacteroidetes* and one *Proteobacteria*, *Sutterella wadsworthensis*, a commonly found gut bacteria. These bins represent approximately 46%, 56%, 54% of metagenomic aligned reads on days 182, 852, 881, respectively. Bacteria not represented in the bins were likely at too low abundance for assembly and binning of adequate quality bins for prophage detection. Four of the bacteria represented between 67-79% of the mapped reads: *Prevotella sp003447235, Phocaeicola dorei, Bacteroides uniformis,* and *Butyrivibrio_A crossotus* (Figure 3.1A). Our bacterial diversity and bacterial

bin abundances data are in line with what was previously reported in the original work using read-based methods [34].

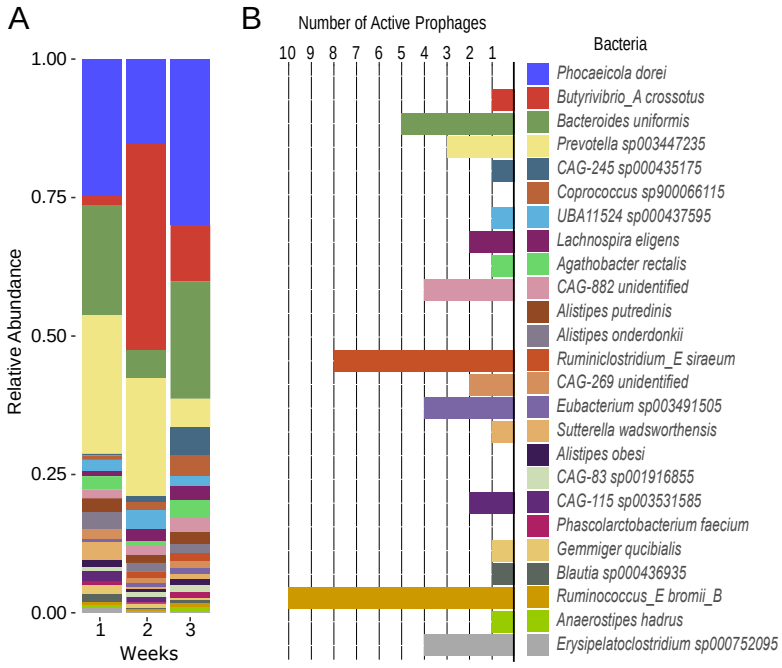

Figure 3.1 Distribution of Bacterial Lysogens in the Gut of One Healthy Individual:

(A) Relative abundance of all (25) medium-to-high quality assembled bacterial bins from metagenomic sequencing for each experimental week over the 2.4 years (B) Number of prophages determined to be active in each bacterial bin.

Each bacterial bin was investigated for prophages using multiple tools (see Methods). Most prophages detected were detected by several tools (Supplementary Figure 3.2A), which led to the detection of 651 non-redundant putative prophages (Supplementary Figure 3.2B). We used PropagAtE [57] to separate prophage-like artifacts, or prophages no longer capable of replicating, from true prophages on the assembled bacterial bins. The prophages that were found to be actively replicating in our samples were deemed as ‘active prophages’. Of these 651 putative prophages, we found 52 putative active prophages (Supplementary Figure 3.2C). We excluded non-active

prophages from this study as differentiating non-active prophages that are still capable of replication (true-positives) from prophage-like artifacts (false-positives) is not possible with our liberal prophage detection approach. We quantified that most bacteria (72%) contain at least one putative active prophage, and 40% of these bacterial lysogens contain multiple active prophages (Figure 3.1B).

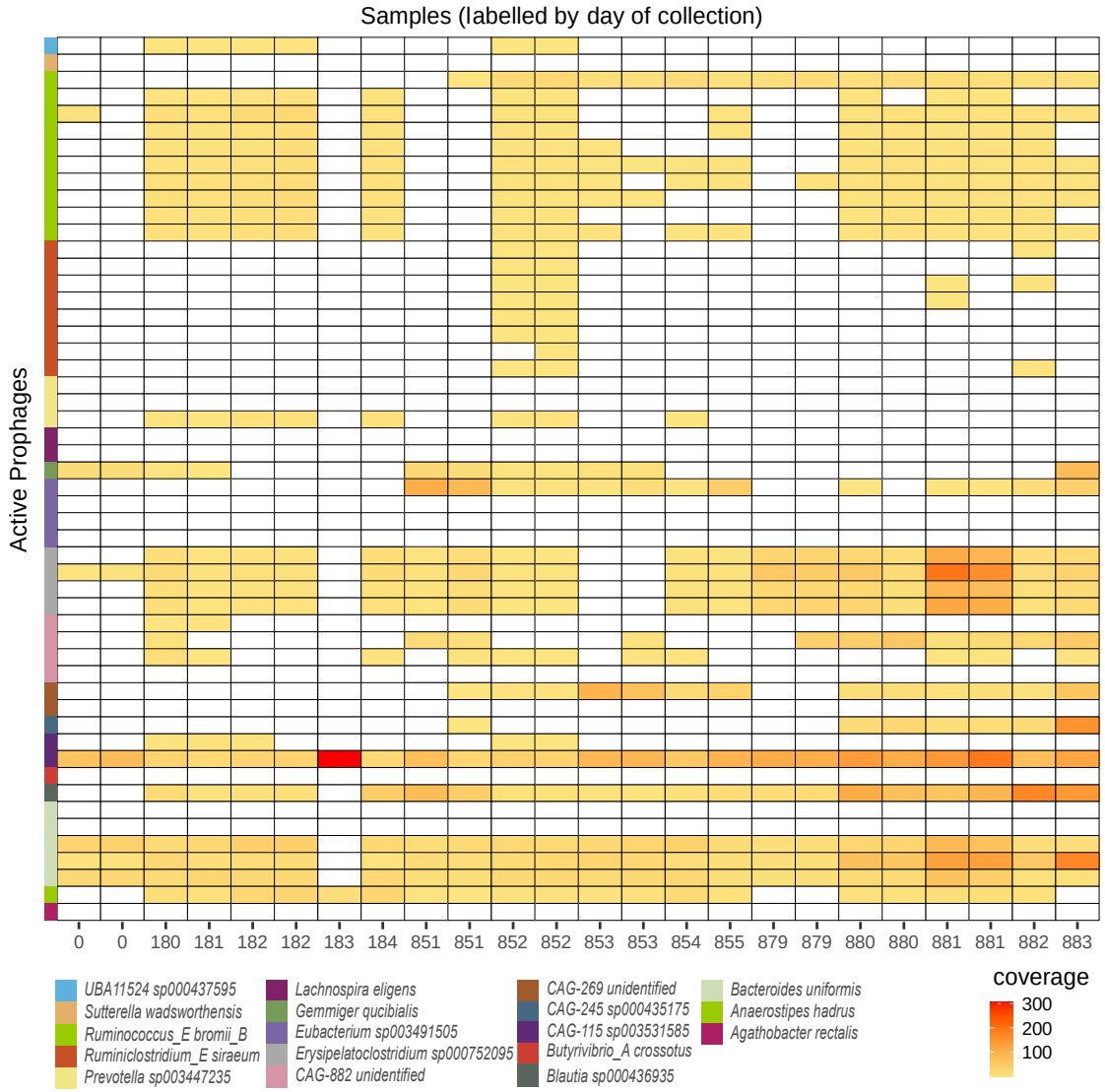

Figure 3.2 Presence of Active Prophages in the Gut of One Individual over 2.4 years:

Heat map showing the relative abundance (coverage) of active phages (rows) in each of the viral-enriched sampled time points. White time points indicate an absence of prophages detected in sample. Presence was defined as viral reads covering prophage regions by at least 1x for more than 75% of prophage length in the sample. A total of 38 prophages were confirmed out of 52 putative active prophages through detection viral sequences. Normalized coverage of active prophages is displayed on timepoints when an active prophage was present in the sample.

We aligned all the viral metagenomic reads to see when each putative active prophage was found over the 2.4 years (Figure 3.2). Thirty-eight (73%) of the putative active prophages were confirmed as ‘active prophages’ by detection in the viral

metagenomes (presence determined as in [37]) (Figure 3.2) (56% of bacteria contain at least one confirmed active prophage found in the viral sequenced fraction). More than half of active prophages were found at 9 separate time points and three different weeks (Figure 3.2). Over the 2.4 years, 19 prophages significantly increased in abundance during at least one time-point compared to the other time points (DESEQ2, *p.adj* <0.05). The significant increase in abundance indicates a possible triggered prophage induction event. Eight prophages within six bacterial lysogens reached DESEQ2 normalized coverage 100x (Figure 3.3A), and of those, five were significantly increased (z-score >1.96 of log-transformed coverage) at one time point. Increased coverage occurred almost entirely during week 3, between days 881-885 (Figure 3.3A). *CAG-115 sp003531585* prophage 1 was the exception, as it rose to significant abundance during week 1 (day 184) as well as week 3 (day 881) (Figure 3.3A). Significantly increased prophages at week 3 were found in five different host-bacteria belonging to both *Firmicutes* and *Bacteroidetes*; meaning the stimuli triggering prophage induction is not phyla-specific and likely an external stimulus. In contrast to week 1 where triggered prophage induction was a species-specific event. Active prophages were found in low abundance, with a cumulative abundance typically less than 0.5% of the community, with the exception of day 881, which rose above 1% (Figure 3.3B). The continuous low-abundance of active prophages (Figures 3.3AB), and continuous presence of active prophages over the 16 sampling times (Figure 3.2) together support a model of regular spontaneous induction. Triggered prophage induction on the other hand is limited to fewer time points (4 of 16 timepoints) and small fraction of lysogens (5 of 18).

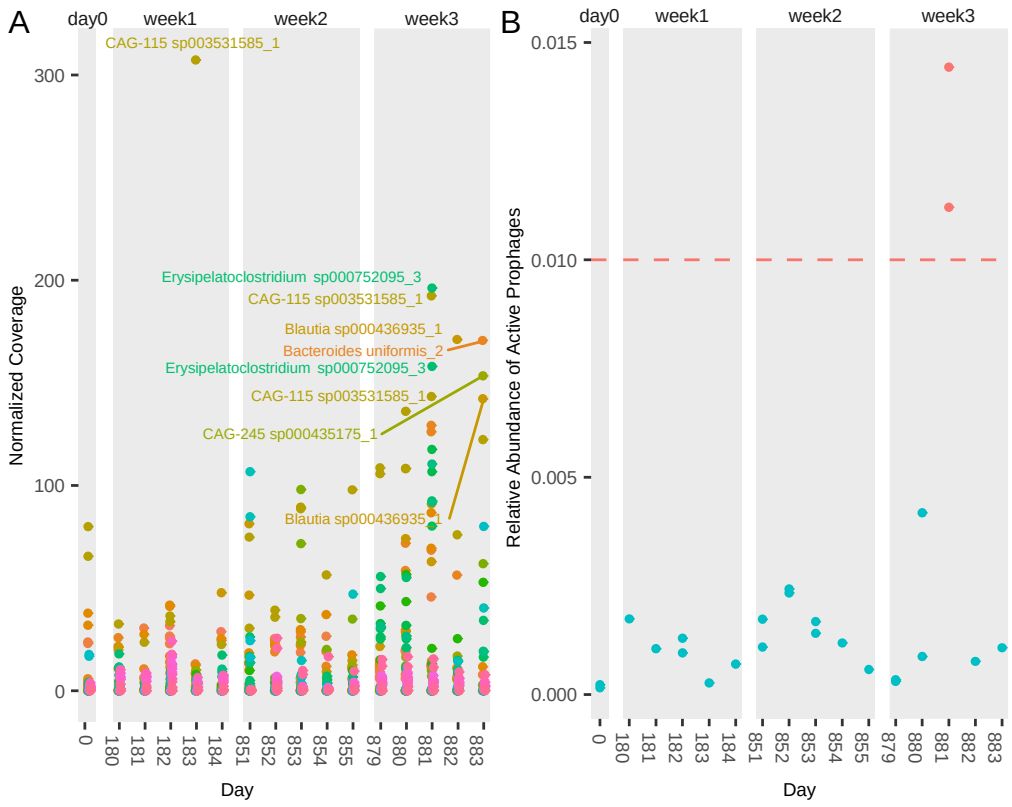

Figure 3.3 Spontaneous Induction of Prophages in a Healthy Individual Dominates in Comparison to Rare, Triggered Prophage Induction Events:

(A) Normalized coverage of each active prophage over the course of the experiment. Active prophages with significantly increased coverage (z-score > 1.96) are labeled by text. (B) Relative abundance of all active prophages in the virome fraction. Red time points are above the 1% relative abundance.

From the viral metagenomic reads we assembled 6,176 phage contigs. We combined the 52 prophages to our analysis and removed 338 viral metagenomic assembled phage contigs that had homology to the set of active prophages: resulting in a total of 5,890 non-redundant viral contigs. We were able to classify 44% of these phage contigs taxonomically at the family-level (Supplementary Figure 3.3). A large percentage of phages at weeks 2 and 3 were unknown (Supplementary Figure 3.3). Non-prokaryotic viruses (*Circoviridae*, *Mimiviridae*, *Phycodnaviria*, *Genomoviridae*, *Marseilleviridae* and *Poxviridae*) were found in negligible abundance. The relative abundance of classified phages at the family-level shows most members belonging to the *Microviridae* family

(Figure 3.4A), which contrasted with the absence of CrAss-like-phages. CrAss-like-phages have also been observed to be at low-abundance in individuals with a high-abundance of *Microviridae* phages [7]. At the family-level, the viral community appears to be stable over the 2.4 years. However, we see an expansion of families belonging to *Caudovirales* (*Siphoviridae* and *Myoviridae*) at day 881 (Figure 3.4A) which corresponds to the increase in active prophages (that belong mostly to the *Siphoviridae* and *Myoviridae* families) from triggered prophage induction (Figure 3.3). In contrast triggered prophage induction at day 183, which only impacted *CAG-115 sp003531585*, did not have an impact on the viral diversity. Triggered prophage induction, unlike spontaneous prophage induction, can significantly and rapidly alter the phage community diversity. Here, the effect of triggered prophage induction is transient, only lasting one day as *Microviridae* phages return to their high relative abundance the following day (day 882).

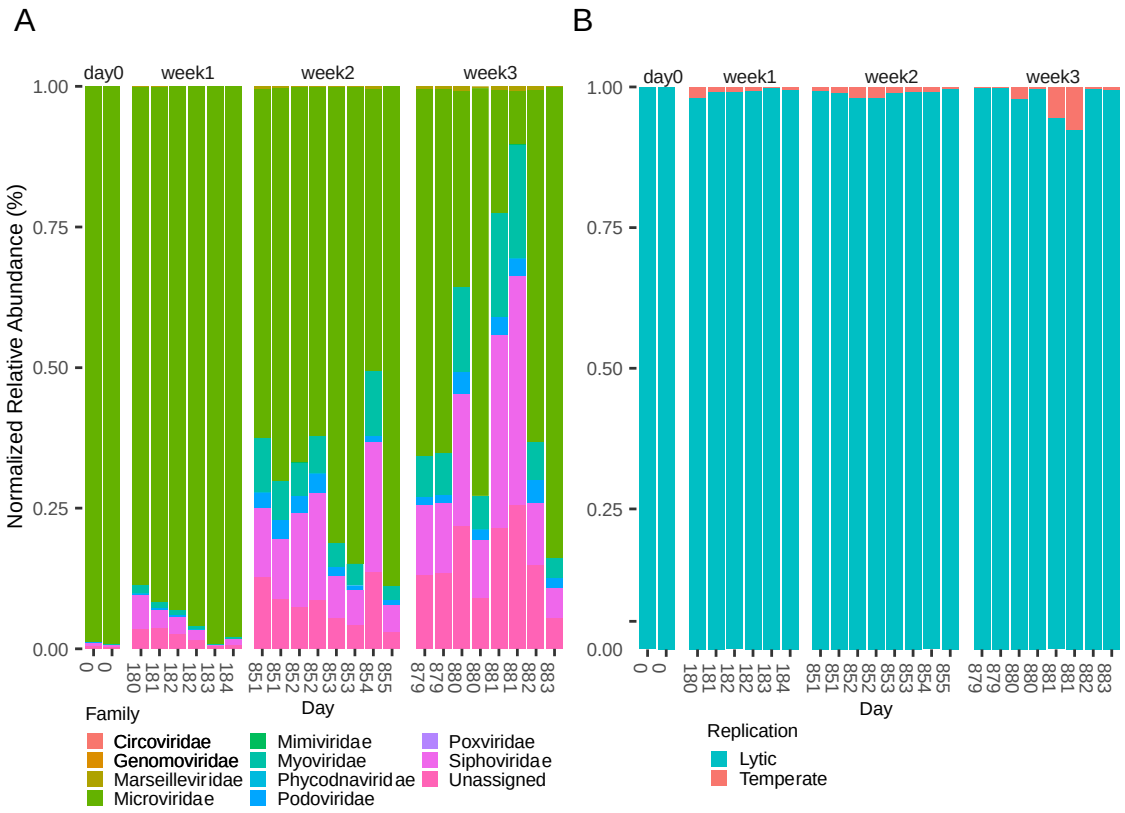

Figure 3.4 Diversity of Phages in the Gut of a Healthy Individual over 2.4 years:

(A) Relative abundance at the phage family-level showing disruption of stability at day 881 by triggered prophage induction (B) Relative abundance of subset of high-quality viral contigs predicted to be either temperate or strictly lytic.

To determine if active prophages influence the proportion of temperate phages in the gut, we determined which of our phage contigs are potentially temperate using Bacphlip [62]. Lysogenic replication cycle prediction by Bacphlip is designed to be run on complete phage genomes, as incomplete genomes underestimate temperate phages due to an absence of genetic hallmarks. For temperate phage analysis, we took a subset of our phage contigs that were high-quality (>90% complete) or complete by CheckV [61]. This resulted in 557 phage contigs that represented a mean 82% (std. dev. 7.94) of viral reads. We found few temperate phages in high relative abundance (Figure 3.4B). However, at week 3, there is an increase of the temperate fraction from an average 1.3%

to 2.1%, and peaks at day 881 (6.5%) (Figure 3.4B). This once again supports the idea that triggered prophage induction occurred at week 3, temporally altering the phage community.

To better understand how frequent spontaneous prophage induction and rare triggered prophage induction events shape the stability of phage community over time, we looked to discriminate between different patterns of community composition change (stochastic variation, directional change, and cyclical dynamics) using the approach of Collins *et al*. [63]. We investigated the change in phage taxonomic composition change with beta-diversity (Bray-Curtis dissimilarity) between time points of the whole phage community (5,890 phage contigs) over time. The regression line was significant (*p-value* = 2.2e-16) with a positive slope (0.025, adj. R-squared 0.9) indicating the community is diverging over time. In comparison, the active prophages are stable as significantly less divergent (*p-value* = 1.36e-05) with almost no positive slope (0.003, adj. R-squared 0.038) indicating the active prophages are more stable than the whole phage community (Figure 3.5AB). To confirm that the results from active prophages are not a sub-sampling artifact, we randomly sub-sampled 52 viral contigs from the whole community (20 iterations), and all were more divergent than active prophages. The rate of divergence over 2.4 years leads to three compositionally distinct viral communities that significantly grouped together by week (PERMANOVA Pr(>F) 0.001: day 0/week 1 (days 0-184), week 2 (days 851-855), and week 3 (days 879-883), with later groups clustering closer due to temporal proximity (Figure 3.5C). The stable active prophage population clusters less by week (PERMANOVA Pr(>F) 0.11) (Figure 3.5D). The slower divergence rate in active prophages leads to less separation by week, despite a higher baseline dissimilarity (0.58

for active prophages compared to 0.18 for the whole community). Active prophages thus appear to be a stable community of phages where composition is maintained over long periods of time, despite prophage induction events.

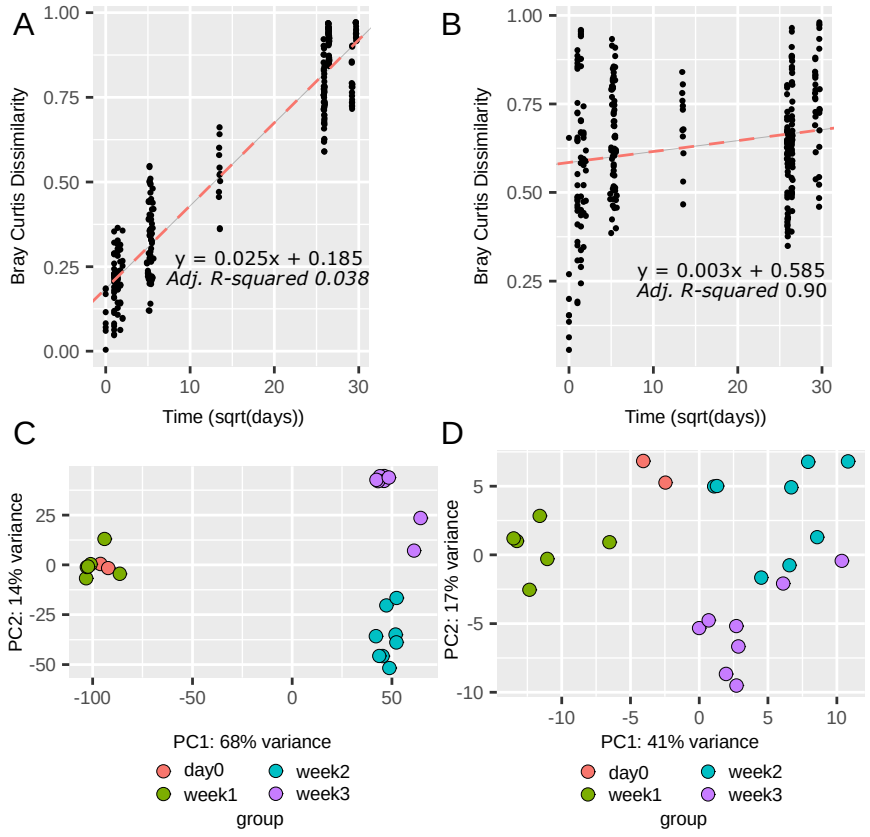

Figure 3.5 Stability of both Viral and Prophage Communities over 2.4 years:

Bray-Curtis Dissimilarity between samples by time elapsed distance to track the degree the rate of community change (slope of dotted line) in both the (A) whole viral community (*p-value* = 2.2e-16) with positive slope (0.0245), and linear (adj. R-squared 0.9) and the (B) prophage community (*p-value* = 1.36e-05) with a positive slope closer to zero (0.003) (C). PCA plot of DESEQ2 relative abundance of samples by time in the whole viral community (D) and in the prophage community.

We confirm that active prophages are commonly found in the genomes of gut bacteria, and that they replicate continually through spontaneous prophage induction. In the 16 time points sampled over 2.4 years, only four time points that showed signs of triggered prophage induction (day 881). Triggered prophage induction at day 881, which

involved multiple lysogens, was able to disrupt viral composition at a family-level and increase the abundance of temperate phages.

3.4. Discussion

By studying both the bacterial and viral communities of a healthy individual over the course of 2.4 years, we were able to link prophages to the extracellular phage community. We found that active prophages represent a taxonomically stable fraction of the phage community (present throughout the 2.4 years at low abundance). Triggered prophage induction involved only a small fraction of lysogens with active prophages (5 of 18). Only one lysogen underwent triggered prophage induction during week 1 (Figure 3.3A). This event did not have an impact on the phage community (Figure 3.4A), as there was no shift in phage taxonomic composition or change in the relative abundance of prophages (Figure 3.3B) or temperate phages (Figure 3.4B). In contrast, during week 3, all five lysogens underwent triggered prophage induction, and led to a disruption of the phage community (Figure 3.4A), and relative abundance of prophages (Figure 3.3B), and temperate phages (Figure 3.4B). For the other 12 time points, prophage abundance support events of spontaneous prophage induction. The ecological consequence of this dynamic was a stable prophage fraction of the community (Figure 3.5CD) when compared to the rest of the community (Figure 3.5AB). Spontaneous prophage induction could be the source of stability in the phage gut community or result from the absence of perturbation.

Prophages are commonly found in the genomes of bacterial isolates [64], as well as bacteria found in the guts of humans [65] and mice [2]. We show that the gut microbiota

of this healthy individual contains numerous active prophages (Figure 3.1B), similar to what was observed in the gut of healthy mice [2]. We leveraged multiple prophage predictors [66] and focused on medium-to-high quality bacterial bins, as well as prophages found on bacterial scaffolds without host-flanking regions [2]. Prophages from scaffolds without host-flanking regions decrease accuracy of prophage assignment to host [58], but was necessary as few prophages were assembled with host flanking regions. In addition, few assembled bacteria belonged to the same genus, thus reducing the likelihood of mis-assigning hosts. When the prophages in the mouse gut [2] were revisited using only prophages with flanking host regions, there were less active prophages observed [67] than in the original study [2]. We confirmed that most bacteria contained active prophages, through their detection in the viral-sequenced fraction. Detection of prophages in the viral sequenced fraction despite their low abundance, is possibly due to the high sequencing depth of the original study. This allowed for the detection of spontaneously induced prophages. Future studies might benefit from hybrid assemblies of short-read and long-read sequences to improve the quality of bacterial metagenomic assembled genomes, and prophage prediction [68] so that all prophages can be assembled with host-flanking regions.

It has been argued that prophages, especially active prophages, represent a fitness cost to bacteria, as prophages represent extra genetic cargo that can act as a ‘molecular time-bomb’ [69], and therefore should be under selective pressure to be rendered inactive [26]. Despite this, we see most bacteria present here have active prophages in their genomes (Figures 3.1B, 3.2). This individual was sequenced well-above saturation [34], which allowed us to track low-abundance phages, and suggest that

active prophages are undergoing spontaneous prophage induction and not triggered prophage induction (Figure 3.3AB, 3.4AB). The differentiation between the two types of prophage induction offers an explanation as to how temperate phages might mitigate the fitness cost of actively replicating (reviewed in [27]). Spontaneous prophage induction would result in less bacterial death compared to triggered prophage induction (Supplementary Figure 3.1C), and prophage release could act as 'bacterial warfare' to closely related bacteria vulnerable to infection [70]. This process ultimately promotes lysogeny in the long-term [70]. These results support the hypothesis that spontaneous prophage induction is a mechanism by which prophages maintain their ability to remain active over long periods of time [27, 71].

The gut virome of a healthy individual is considered stable over time. At the family level, this individual's gut virome was stable (Figure 3.4A), but at the contig level it was undergoing directional change (Figure 3.5AC). The family-level stability was altered temporarily by a triggered prophage induction event at day 881 but not at week 1 (Figure 3.4A). Phage contigs in this data set, which represent more closely phage species or strains, were previously reported to be stable, as 80% of phage contigs were found at day 0 and day 883 (the end of the study) [34]. These findings relied on the Jaccard Index, focusing on the number of shared contigs, whereas we defined stability using Bray-Curtis dissimilarity, including contig relative abundance as well as presence/absence between time points. In doing so, we found that phage community composition is diverging (Figure 3.5AC) [63]. Original findings focused on the most abundant phage contigs through manual curation, and since then, progress has been made to automate the identification of phage contigs using command-line tools (e.g., VirSorter [53]), annotated phage protein,

(e.g., pVOG [72]), and databases of gut viruses [42]. These improvements allowed us to include rare phage contigs, which increase dissimilarity. Active prophages undergo less compositional divergence over time than expected from the whole phage community, even with prophage induction. The decreased divergence is likely in part due to slower mutation rates of temperate phages compared to lytic phages [34], and not just spontaneous prophage induction.

We did not identify any CrAss-like phages in this individual, probably because CrAss-like phages have been shown to be in high abundance when temperate phages and *Microviridae* phages are in low abundance [7], which is not the case here. Lysogenic replication fits into a larger category of passive replication which includes pseudolysogeny and chronic infection [11]; an important characteristic for success in the gut, as this is how CrAss-like phages appear to be replicating [17]. Interestingly, it is a feature specific to adults, as the infant gut undergoes rapid changes where prophage induction appears to play a more important role [15, 73]. Passive-replication in the adult healthy gut might be the consequence of continuous 'kill-the-winner' dynamics that occur during infancy [4, 15]: over time, this pressure drives phages and bacteria to co-adapt, leading to increased stability between bacteria and phages in healthy adult gut [7, 16].

Numerous factors can alter both bacterial and viral compositions in the gut, including age, disease, drugs, diet, etc. [5, 6]. These factors are also capable of triggering prophage induction. It appears that in the absence of disease or antibiotics, this individual had a triggered prophage induction event at week 1 and 3. At week 3, and not week 1, prophage induction impacted multiple bacteria belonging to both *Bacteroidetes* and *Firmicutes* phyla, it is likely that some environmental change is responsible, and not a

species-specific trigger (as we expect for week 1). Unfortunately, no metadata was collected for this study, so we hypothesize that non-antibiotic medication consumption [28] or a switch in diet [2] are likely responsible. We will need to test these hypotheses moving forward to have a better understanding of prophage induction in the gut. Currently, we do not know how species-specific triggers differ from community-level triggers of prophage induction. To test these hypotheses, large-scale studies with comprehensive metadata are needed [74] or implementing gnotobiotic mouse models to explore features causing prophage induction of gut prophages *in vivo* [1].

Longitudinal studies are important when studying the gut microbiota [75], including the virome. With daily sampling of this individual, we could detect triggered prophage induction occurring at day 881, but changes in the phage community were undetectable at days 880 or 882, or the rest of the week (Figure 3.3B, 3.4AB). This highlights how detecting prophage induction at the community level is difficult [76], and therefore using time-series of bacterial and viral metagenomics can aid in our understanding of how active prophages contribute to extracellular intestinal phage communities [2]. Daily sampling of the gut community is necessary to detect triggered prophage induction [77] with sequencing depth above saturation [34]. This scale of daily observations is important to increase our understanding of phage-bacteria dynamics in the gut despite the challenges of increased sampling and cost.

3.5. Conclusion

Our work supports the hypothesis that lysogeny is a stabilizing force between bacterial and phage communities in the gut [16]. Prophages in the gut of this healthy

individual appear to be balancing the benefits of stable integration with the risk of inactivation through regular spontaneous prophage induction. As phages undergo divergent evolution, and we speculate that lysogeny offers a refuge from genetic divergence. Bacteria balance the benefits of accumulating prophages against the costs of having extra genetic cargo that can trigger cell lysis. Regular triggered prophage induction would increase the fitness cost of harbouring prophages and increase selective pressure for prophage inactivation. In conclusion, bacteria and temperate phage balance competing priorities to form a stable equilibrium in the gut and play nice.

3.6. References

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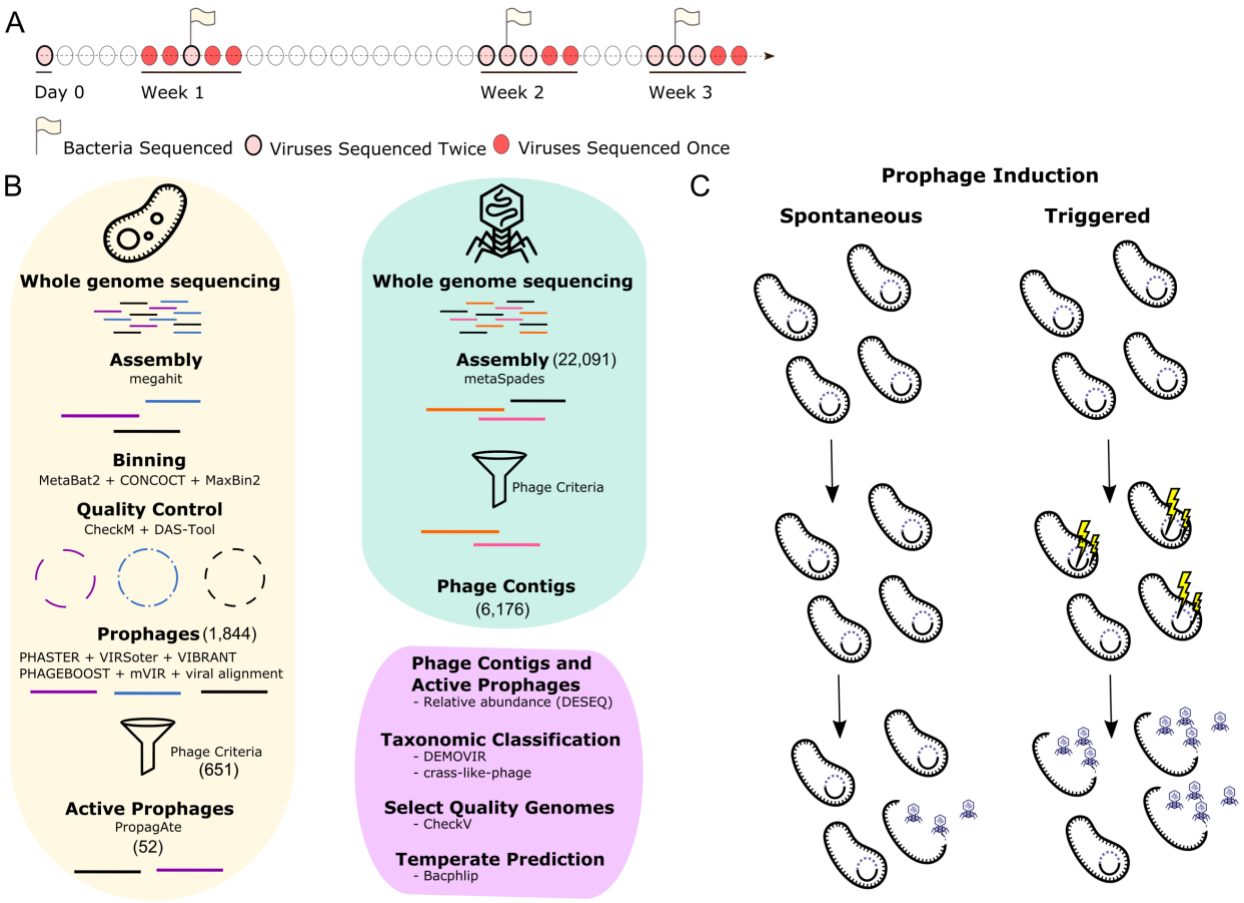
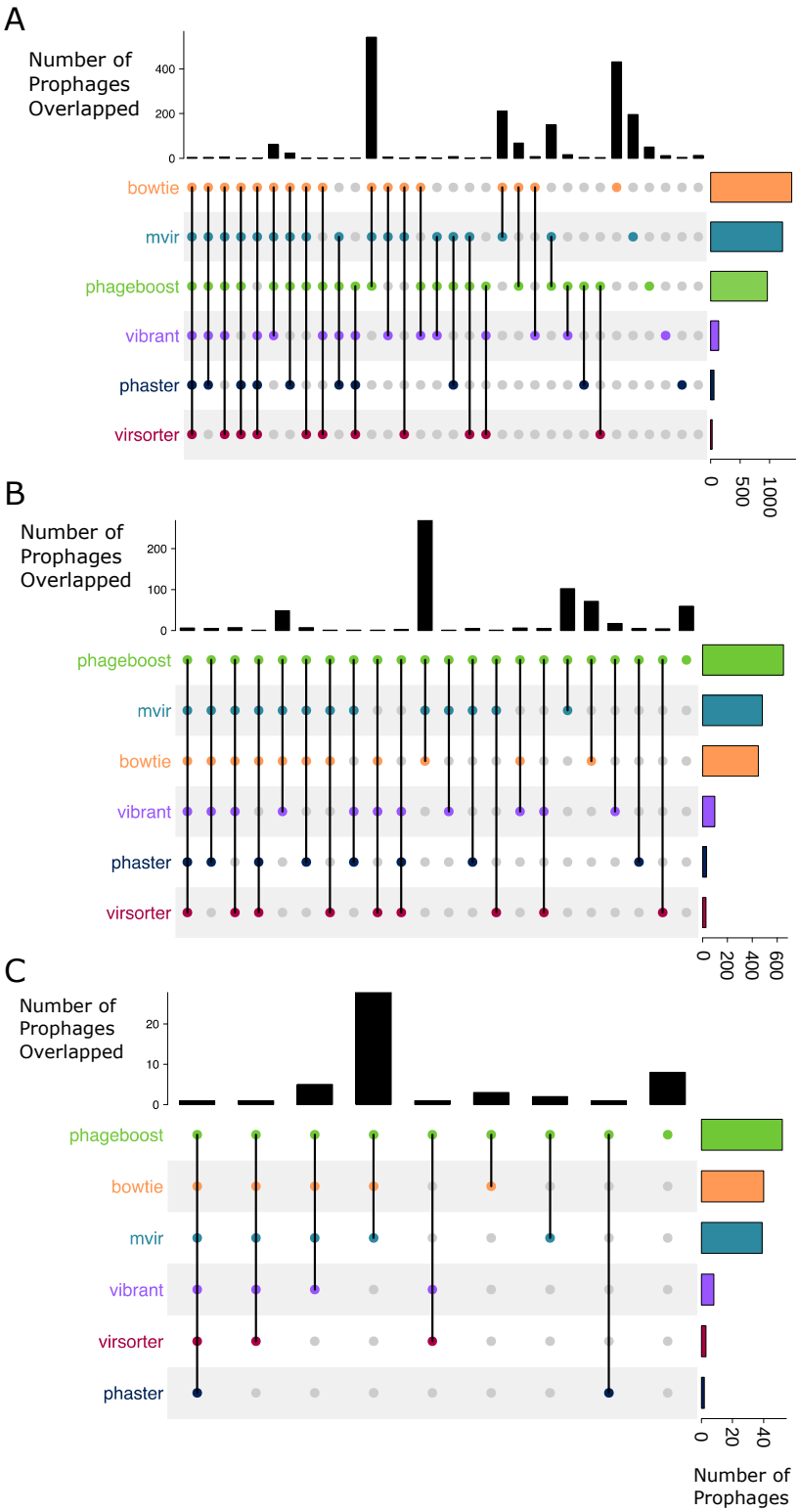
3.7. Supplementary Material



Supplementary Figure 3.1 Study Summary:

(A) Explanation of the sampling for the study (B) Methodology (C) Comparison of spontaneous prophage induction to triggered prophage induction. Lightning bolts represent external prophage induction triggers.


A
Number of Prophages Overlapped
400
200
0
bowtie
mvir
phageboost
vibrant
phaster
virsorter
0
500
1000
B
Number of Prophages Overlapped
200
100
0
phageboost
mvir
bowtie
vibrant
phaster
virsorter
0
200
400
600
C
Number of Prophages Overlapped
20
10
0
phageboost
bowtie
mvir
vibrant
virsorter
phaster
0
20
40
Number of Prophages

Supplementary Figure 3.2 Upset Plot Showing Overlap Between Prophage Predictors:

Summary of prophage predictions by Bowtie, mvir, phageboost, vibrant, phaster, virsorter of (A) All the 2, 719 merged prophage regions (B) All the 651 prophages that meet our phage criteria (C) and the 52 active prophages

Supplementary Figure 3.3 Percentage of Phages with Taxonomic Classification at the Family-Level per Sample-Sequence Run:

Percentage of phages with taxonomic classification at the family-level per sample-sequence run. Individual contigs are separated by grey-lines showing the breakdown of individual contigs.

Preface to Chapter 4

In Chapter 3 we show that triggered prophage induction is rare in healthy individuals in the absence of antibiotics or gastro-intestinal issues. When triggered prophage induction did occur, it resulted in a transient disruption of the viral community. We saw in Chapter 2 that when gut bacteria are exposed to antibiotics, several, if not all, active prophages were induced. We sought to see if consumption of oral antibiotics causes prophage induction in healthy individuals and can be detected using the computational approach developed/optimized in Chapter 3. The goal of this chapter is to provide a possible solution to the limitations of other experimental methods used for community level prophage induction and determine if triggered prophage induction has significant impact on the gut virome.

We show that the resiliency of the healthy individual studied over 2.4 years is not uncommon as all individuals studied in Chapter 3 were resilient to antibiotic perturbation. Bioinformatic methods were able to explore specific lysogens that were being induced by antibiotics, improving on count-based methods. However, widespread triggered prophage induction was absent by using count-based methods and bioinformatics.

Chapter 4. Gut Bacterial Communities of Healthy Individuals Resilient to Prophage Induction by Antibiotics

4.1. Introduction

Our gut microbiota, by adulthood, contains trillions of microorganisms [1], made up of thousands of bacterial 'species-level' phylotypes [2], and their associated viruses, bacteriophages (abbreviated to phages). The two communities correlate with each other [3] without major taxonomic fluctuations in healthy individuals [4] and an absence of 'kill-the-winner' lytic replication dynamics [5]. Alternative to lytic replication dynamics are lysogenic dynamics [6-8], or an intermediate of the two [9, 10]. Phages that undergo lysogenic replication are referred to as temperate phages, which integrate their genomes into the bacterial genome and replicate alongside their bacterial host. Despite high bacterial density [1] and growth [11] in the human gut, temperate phages favour lysogeny and integration over lytic replication, leading to the development of the 'piggyback-the-winner' model [8].

The gut microbiota is shaped by numerous factors including health status [12], diet [13], and medication consumption [14]. Oral medications are increasingly replacing intravenous administration to reduce costs and relieve patient discomfort [15]. Administration of oral antibiotics is also associated with gut microbiota-related conditions, e.g., Crohn's [16], colorectal cancer [17], childhood obesity [18], and increased dissemination of antibiotic resistance genes [19]. To date, the observed consequences of antibiotics on the gut microbiota have focused on the loss of bacterial diversity (both taxonomic and functional), and increased susceptibility to pathogenic bacteria [20] (e.g.,

Clostroides difficile [21]). Consequences of oral antibiotic consumption can occur rapidly, and for some individuals, they can have long-lasting consequences [20]. Yet, despite these disruptive consequences and the global antibiotic resistance crisis, antibiotic consumption is still increasing globally [22-24].

A less understood consequence of antibiotic exposure on the members of the gut microbiota concerns the phage community. As phages are mobile elements, capable of generalized transduction, they have been hypothesized to be highly effective spreaders of antimicrobial resistance genes [25], but recent evidence does not support this [26, 27]. Antibiotics have long been known to trigger prophage induction, sometimes with strong ecological consequences on microbial systems [28-30]. In aquatic systems, prophage induction can take place because of environmental parameters (e.g., sunlight, temperature, and pressure) [31, 32], and pollutants (such as Polynuclear Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PABs)) [32, 33]. Prophage induction occurs mainly through the SOS-response caused by DNA damage [34, 35]. In the gut, mortality by prophage induction has the potential to have significant health consequences, as estimates of active prophages in the mammalian gut can reach 80% of the bacterial community [36].

Given this high abundance of prophages that have maintained their ability to switch to lytic replication, prophage induction triggered by antibiotic exposure could be a particularly disruptive event. Antibiotics are both associated with an increased risk of development of Crohn's disease [16] and its treatment [37, 38], where both bacteria and phage communities are disrupted [39]. Prophage induction could be a contributing factor to this condition, as temperate phages are increased in fecal samples from Crohn's

disease patients [39] and antibiotics used in the treatment of Crohn's disease can trigger prophage induction *in vitro* [28]. In this study, we will investigate if triggered prophage induction is widespread and common amongst gut bacteria during antibiotic treatment.

We hypothesize that when a healthy individual is exposed to antibiotics, there is an increase in triggered prophage induction in the gut. This would result in an increase of temperate phages and a decrease in their corresponding host bacteria (lysogen). We sought to test our hypothesis using 1) the frequency of chemically inducible cells (FCIC) 2) and bioinformatically measuring changes in prophage and lysogen abundance. Our results show that antibiotics do not lead to widespread triggered prophage induction, despite 38% of bacteria harbouring active prophages. Prophage induction triggered by antibiotics was instead specific to a small number of bacteria and antibiotic treatments. Our results highlight how the specific-bacterial response to antibiotics observed in the gut microbiota extends to phage-bacteria dynamics.

4.2. Results

4.2.1. Frequency of Chemically Inducible Cells (FCIC) in the Gut Microbiota

In order to determine the FCIC in the gut, we investigated the bacterial community response to mitomycin C (MC), the experimental standard for measuring prophage induction. Fecal samples from three healthy unrelated individuals were incubated in a gut-specific media for 16h and exposed to MC for 8h and 16h. In general, bacterial abundance decreased relative to the drug vehicle control (Figure 4.1A). The decrease in bacterial abundance was significant for individual 2 at 8h and 16h (*p-value* < 0.05, Kruskal-Wallis). Virus-like-particle (VLP) concentrations, measured by

epifluorescence microscopy counts, increased in most individuals, but only significantly in individual 2 at 8h (control: mean 1.32 x 10^8, std. dev. 2.8 x 10^6, MC: 3.68 x 10^8, std. dev. 3.49 x 10^7, VLP.mL^{-1}, *p-value* < 0.05, Kruskal-Wallis) (Figure 4.1B). The virus-to-bacteria ratio (VBR), remained below one, but significantly increased in individual 2 (*p-value* < 0.05, Kruskal-Wallis, Figure 4.1C). Based on previously published models for quantifying FCIC [40] and an approximate burst-size of 30 [31], individual 2 only showed a FCIC of 0.1% at 8h. These results suggest that only some individuals have inducible lysogens within their gut microbial communities. These levels of FCIC are lower than expected for the gut, where 1.5-80% of bacteria are predicted to maintain an active prophage [36, 41].

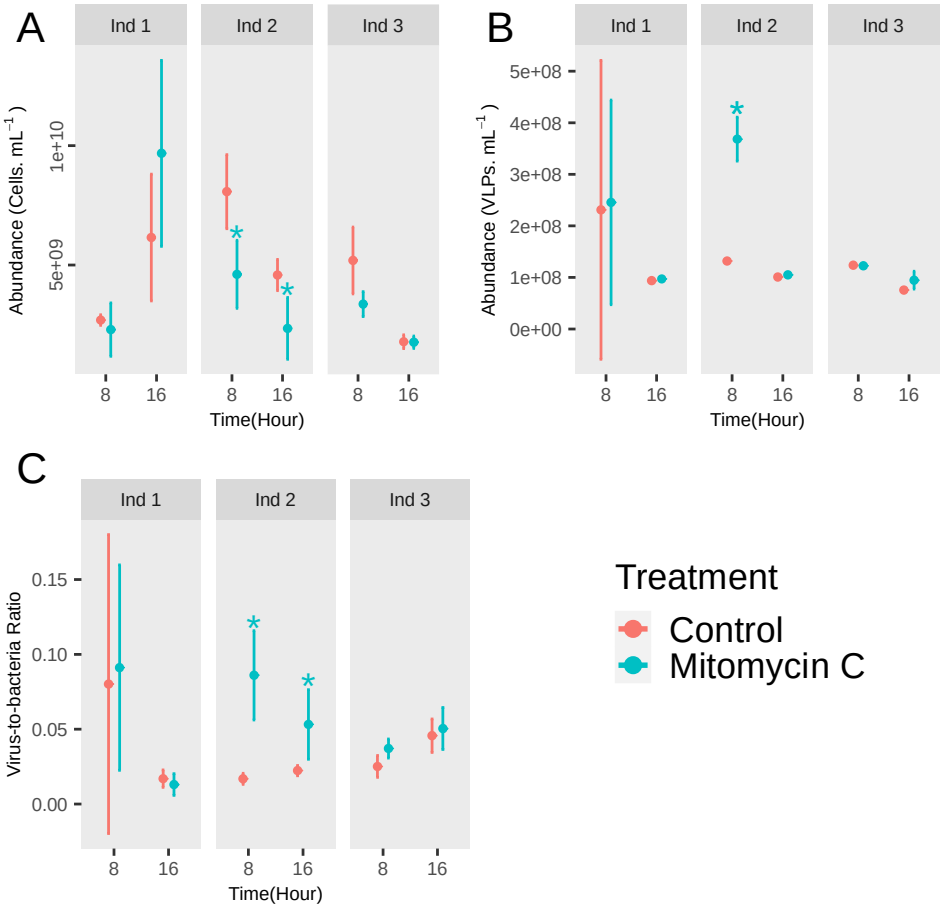


Figure 4.1 *In Vitro* Prophage Induction of Fecal Sample:

Abundance was measured after 8 and 16h after fecal samples were incubated in gut-specific media anaerobically at 37°C with 1 µg.mL^{-1} of either DMSO (control) or Mitomycin C. (A) Bacterial abundance measured by flow cytometry; (B) Phage abundance measured by epifluorescence microscopy counts of virus-like-particles; (C) Virus-to-bacteria ratio obtained from phage and bacterial abundances. Panels (1-3) correspond to individual donors. * Represents *p-value* < 0.05 from Kruskal-Wallis chi-squared test. Error bars represent standard deviation from mean. Counts per time point were repeated in triplicate (n=3).

4.2.2. Prediction of Active Prophages in Healthy Individuals Receiving Antibiotics

We next wanted to see if prophages can be induced in the gut of healthy individuals undergoing antibiotic treatment, using a previously published sequencing dataset of 10 healthy unrelated individuals [27]. Eight volunteers were given one of four antibiotics (ciprofloxacin, azithromycin, doxycycline, cefuroxime), twice daily for five days, and the

remaining two control individuals were given a placebo with the same frequency and timeframe [27]. All individuals donated stool samples at six time points: baseline (T1: 15 days before treatment), during treatment (T2 and T3: days 3 and 5 of treatment, respectively), and post-treatment (T4, T5, and T6: 15, 30, and 90 days after treatment, respectively) [27]. Both the bacterial and viral communities were sequenced by shotgun metagenomics at each time point [27].

We reanalyzed this dataset, and from the bacterial community, we constructed 1,177 metagenome-assembled genomes (MAGs) and predicted 809 prophages within these MAGs (VIBRANT V.1.2.1, integrated virus category [42]). 684 of these prophages were determined to be putative active prophages during at least one time point (PropagAtE V.1.0.0, default Mann-Whitney U Test (*p-value* ≤ 0.05, n= 5) and phage host coverage ratio ≥ 1.75 [41]). This resulted in 42% of lysogenic bacteria (i.e., containing ≥ 1 prophage). Thirty-eight percent of these lysogens have at least one putative active prophage, while the remaining 4% are lysogenic without a putative active prophage (Figure 4.2). We next sought to see how many of the 684 putative active prophages detected in the bacterial MAGs could be confirmed by sequences in the phage community. Defining presence by a ≥ 1x coverage over ≥75% of the prophage genome [43], we found that 49 (7%) of the 684 putative active were also present in the viral community. Due to noted issues of under-sequencing of phage communities (< 1,000,000 reads [43]) and the estimated low abundance of active prophages in the gut (Sutcliffe *et al* 2022, *submitted*), we also applied a less stringent method to investigate the presence of active prophages in the viral community, which is to have at least one viral sequence read aligning (bowtie2 default alignment mode) to the prophage genome [36]. With this

less stringent method, we confirmed the presence of 515 (75%) putative active prophages in at least one sample.

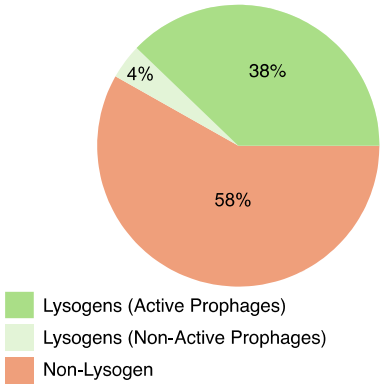


Figure 4.2 Proportion of Bacteria that are Lysogens in the Gut:

In the 1,177 MAGs, from the metagenomic sequencing of ten healthy individuals studied over the course of 90 days with 6 samples, we detected at least one prophage in 492 (42%) of MAGs. Of these 492-lysogens, 445 (38%) of them contained at least one active prophage, while the other 47 (4%) of lysogens appear to be without an active prophage.

4.2.3. Limited Prophage Induction after Treatment with Antibiotics in Healthy Individuals

Within the predicted putative active prophages, we next sought to identify those which underwent triggered prophage induction by antibiotics. We identified triggered prophage induction through a significant decrease in lysogen abundance with a corresponding significant increase in abundance of its active prophage. We found that 52 lysogens significantly decreased during treatment compared to baseline (DESEQ2, Wald test, *p.adj* < 0.05). Nine prophages associated with these lysogens increased in abundance (Figure 4.3A), but only one significantly increased: a phage targeting *Sutterella wadsworthensis* (DESEQ2, Wald test, *p.adj* < 0.05) (Figure 4.3A). These results support the findings from Figure 4.1, in that only a few bacteria in the gut undergo

prophage induction when exposed to prophage inducers. As we have seen previously in healthy individuals that a few induced prophages can disrupt the phage community diversity, we sought to see if this was also occurring here.

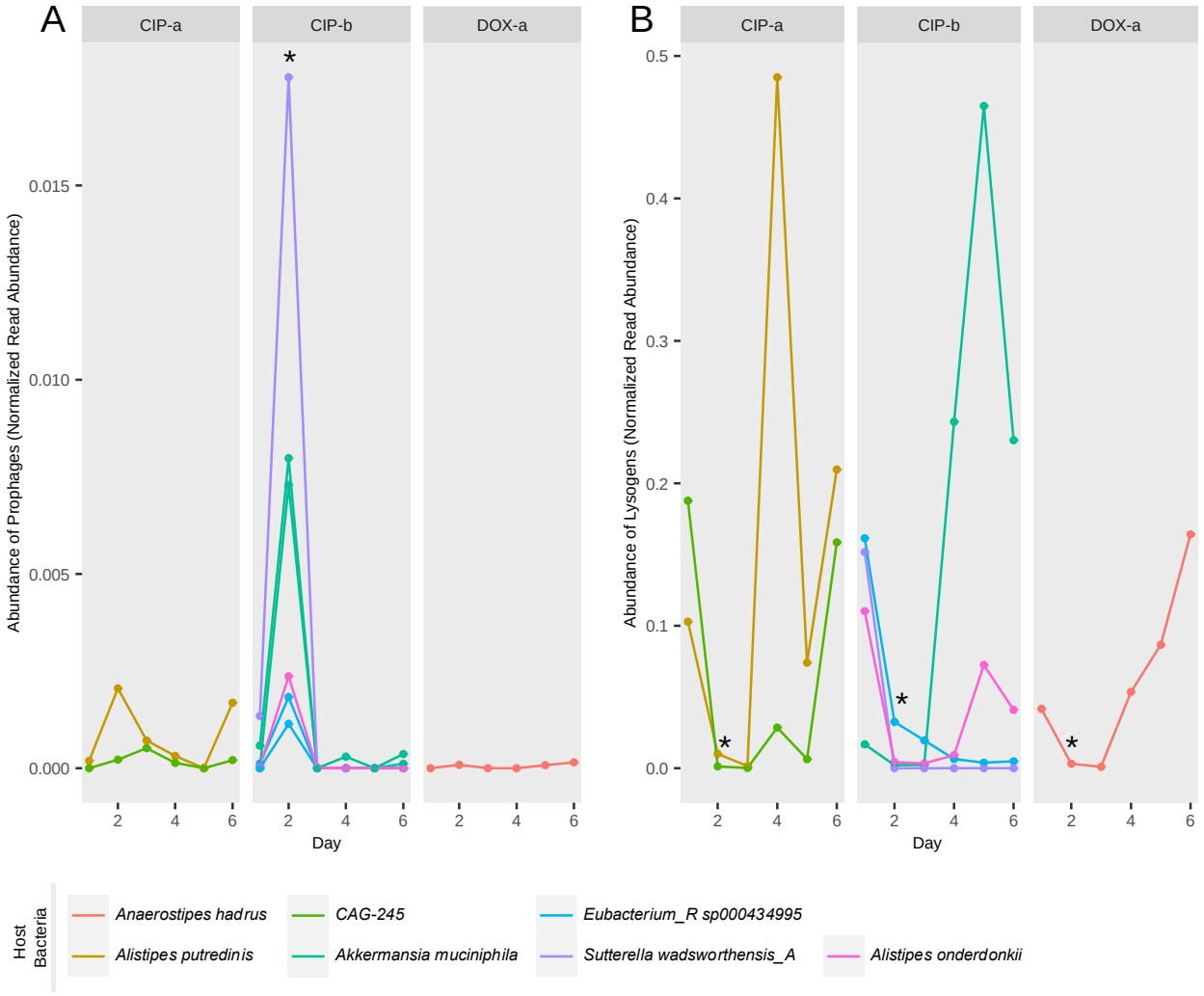


Figure 4.3 Triggered Prophage Induction of Lysogens by Antibiotics:

(A) Normalized abundance of nine prophages that increased in abundance during treatment with a corresponding decrease in abundance of their host. (B) Normalized abundance of the seven lysogens that significantly decreased during antibiotic treatment and had an active prophage that increased in abundance compared to baseline. * indicates significant decrease from baseline, DESEQ2, Wald test, *p.adj* <0.05. AZY-Azithromycin DOX-Doxycyline CFX-Cefuroxime CIP-Ciprofloxacin CTR-Control

As we did not see specific prophages being induced during treatment with antibiotics, we next wanted to see if there were broader trends in the changes in relative abundances of lysogens and prophages. We observed a non-significant trend during treatment, where the proportion of lysogenic bacteria decreased during treatment (from mean 54% at baseline to mean 47% at T2 and T3) and recovered partially post-treatment (mean 52% at T4-T6) (Figure 4.4A). An increase in relative abundance of prophages occurred in some treatments compared to baseline (AZY-b, CIP-a and DOX-a), but not significantly or consistently with other individuals that underwent the same treatments (Figure 4.4B). We expanded our search beyond prophages to include phage contigs classified as temperate (Figure 4.4C). An increase in relative abundance of temperate phages occurred in more treatments compared to baseline (AZY-a, AZY-b, CIP-a, CIP-b), but not significantly (Figure 4.4C). Interestingly, temperate phages increased in both individuals that received either Azithromycin or Ciprofloxacin. Of all the treatments, only individual CIP-a showed a pattern of prophage induction following all three criteria (i.e., decrease in proportion of lysogens, increase in prophages, and increase in temperate phages during treatment). These results support our findings that antibiotics do not appear to trigger widespread prophage induction in healthy individuals.

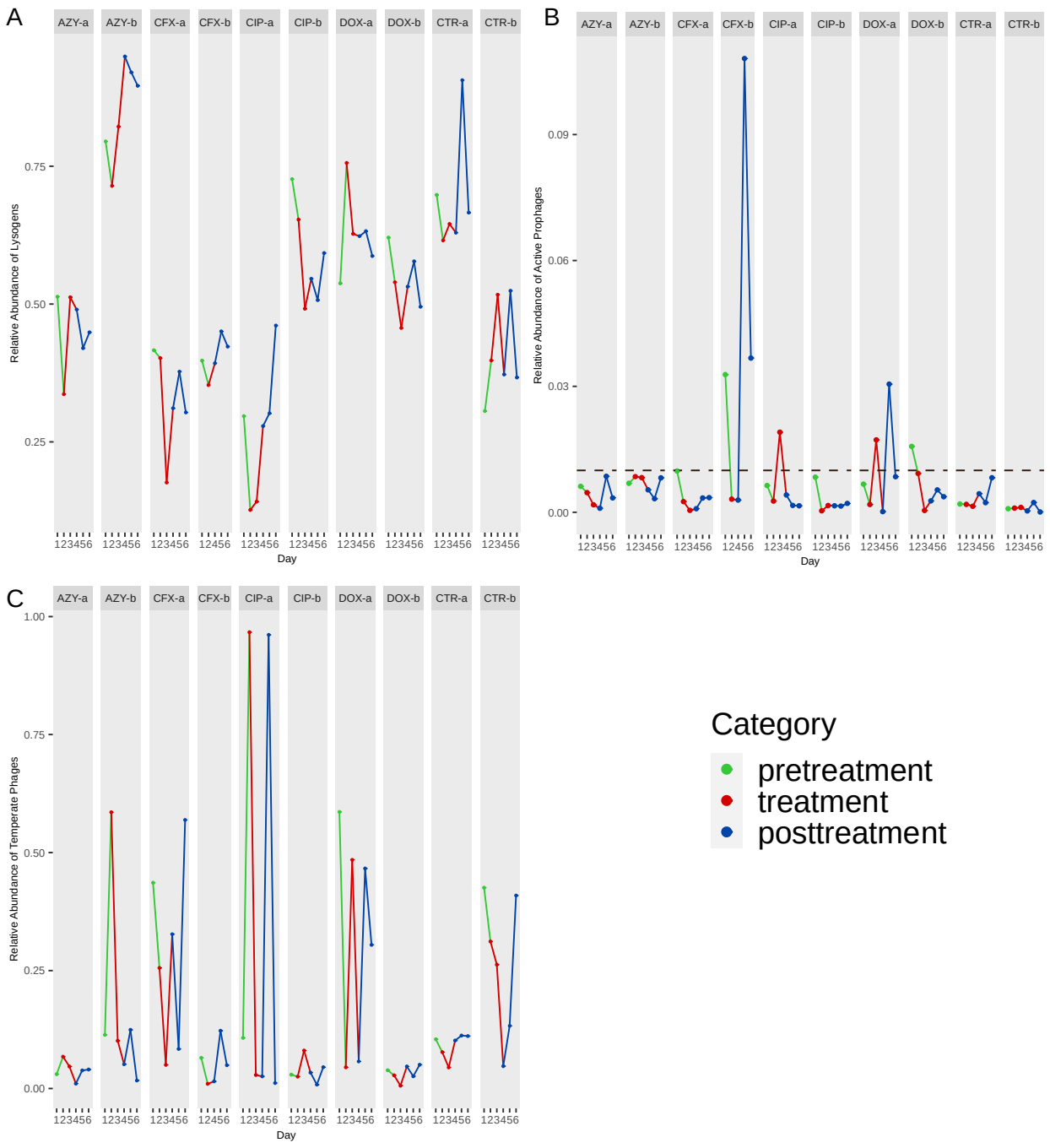


Figure 4.4 Community Shifts in Lysogens and Prophages upon Exposure to Antibiotics:

(A) Relative abundance of lysogens in bacterial population. (B) Relative abundance of prophages relative in phage population. (C) Relative abundance of temperate phages in phage population. AZY-Azithromycin DOX-Doxycyline CFX-Cefuroxime CIP-Ciprofloxacin CTR-Control. Dotted line at 1% relative abundance in panel (B).

4.2.4. Disruption of Phage Diversity in Response to Antibiotics

We next sought to investigate if the whole phage community was altered by antibiotic treatment. Comparing the alpha-diversity (Shannon index) of the phage community of individuals who received antibiotics compared to control individuals, we observed a significant reduction during the early days of treatment (T2) (Dunnett's multiple comparison $p.adj < 0.05$) (Figure 4.5A). Phage diversity recovered by T6, or 90 days after treatment (Dunnett's multiple comparison, $p.adj = 0.0852$) (Figure 4.5A). To investigate each antibiotic separately, we used Hellinger-transformed phage counts per sample by ANOVA-like permutation test for RDA and showed significantly different ($Pr(>F) < 0.05$) clustering of phage abundances during treatment for Azithromycin and Ciprofloxacin only (Figure 4.5B-D), with the treatment explaining over 50% of the variance. This was not observed for the other antibiotics, and treatment period of the controls explained only 35% of variation (Figure 4.5E).

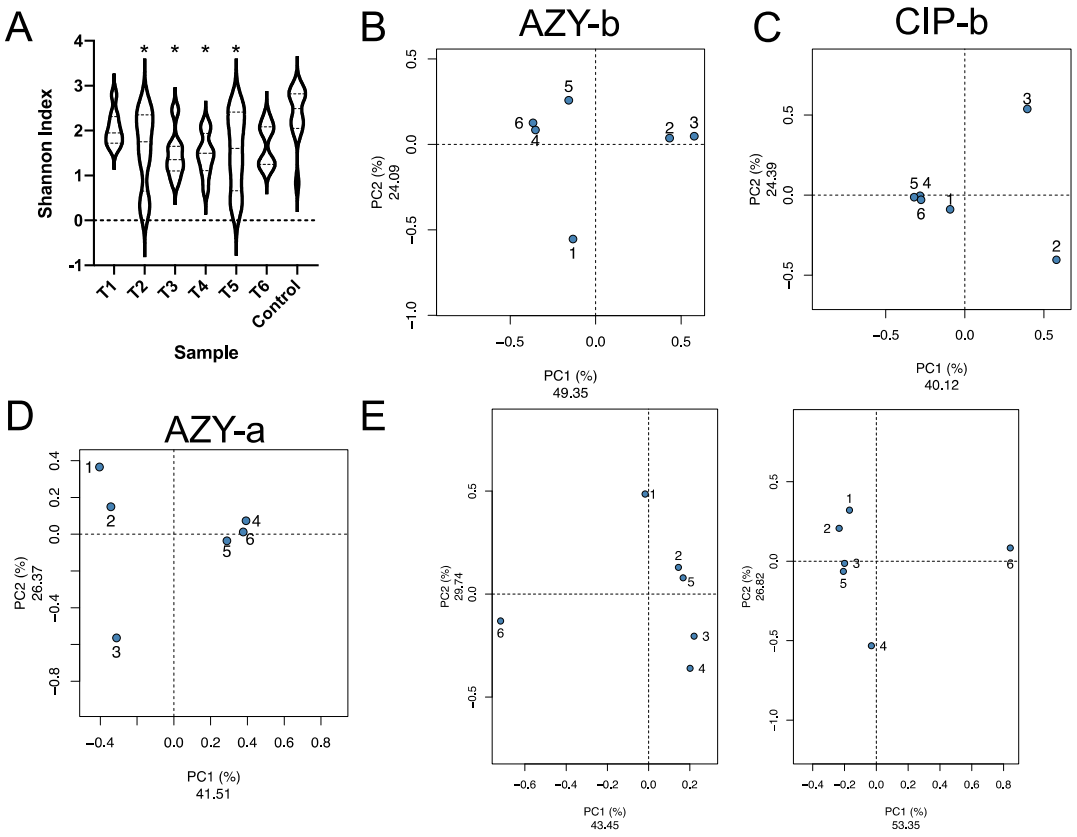


Figure 4.5 Impact of Triggered Induction on Phage Diversity during Antibiotic Treatment:

(A) Violin plot phage alpha-diversity (measured by Shannon Index) per sampling period and pooled for all periods for control individuals. Lines represent median and quartiles. Shannon index significantly decreased during treatment with antibiotics and remained significantly lower until 90 days after treatment began (T6) when compared to controls. (B-D) RDA-plots of species abundances show significant changes in phage communities during antibiotic treatment phage of (B, D) Azithromycin and (C) Ciprofloxacin (Pr(>F) <0.05) (E) Control RDA plots (Pr(>F) >0.05).

4.3. Discussion

Lysogens are common in the human gut [44, 45] and are increasingly recognized for their role in human health [46-51]. As prophages found in the genomes of bacterial lysogens can be cryptic or degraded, and thus incapable of prophage induction, it is important to differentiate between active and inactive prophages. We investigated this differentiation using two methods 1) experimental detection of FCIC and 2) bioinformatically measuring changes in prophage and lysogen abundance. Determining

the FCIC in the fecal samples of three healthy volunteers, we predicted that at most 0.1% of bacteria contain active prophages (Figure 4.1) [40]. FCIC has been shown to under-estimate prediction of active prophages in several ecosystems (freshwater, marine, and sediment) [7]. This FCIC is below the most conservative bioinformatic predictions of active prophages in the gut (1.5%) [41]. We confirmed this discrepancy, as we predicted 38% of bacteria in the gut have an active prophage (Figure 4.2). We further confirmed 75% of putative active prophages were true active prophages bioinformatically. These estimates are lower than what we found using deep-sequencing, as active prophages are typically in low abundance (Sutcliffe *et al* 2022, *submitted*). Variation between studies using different methods is not surprising as no approach is perfect: bioinformatic estimates from the murine gut predicted 80% active prophages [36] but when reanalysed using PropagAtE was only 12.2% [41]. Similar discrepancies have been shown using FCIC methods [31].

Even with the most conservative bioinformatic estimates of active prophages in the gut (e.g., 1.5% of active prophages [41]), widespread triggered prophage induction by MC and antibiotics should still be detected experimentally. Prophage induction might have alluded our detection as it can occur without significant bacterial death [52, 53] or it could have occurred prior to the first sample collection [52]. If prophage induction did not occur, it is likely that the concentration of chemical inducer used was too low [7], as bacterial abundance stayed well above 10^6 cells.mL^{-1} (Figure 4.1A), the theoretical density for which prophage induction should occur [8]. *In vitro* data support this hypothesis, as inducing concentrations of antibiotics responsible for prophage induction varied between

bacterial species [28, 54]. Host-factors (age, lifestyle, and prior bacterial composition) can further influence microbial responses to antibiotics [55].

Of the antibiotics tested, only ciprofloxacin seemed capable of prophage induction. These results are unsurprising as ciprofloxacin is a bactericidal antibiotic previously reported to induce prophages through enhanced *recA* transcription [35] and known to induce gut isolates [28]. Ciprofloxacin was reported to affect the relative abundance of 30% of bacterial taxa in the gut microbiota of individuals receiving treatment [56]. As seen with the phages in this study, ciprofloxacin's impact on gut bacteria varies between individuals [56] and bacterial isolates [28] which could explain why prophage induction was not widespread.

Despite the absence of widespread triggered prophage induction, we observed that *S. wadsworthensis* was induced. *S. wadsworthensis* is a gram-negative proteobacteria, commonly found in the gut of healthy adults, and with limited defined implications for health [57]. Induction of commensal bacteria, such as *S. wadsworthensis*, should have minimal health consequences, but antibiotic-triggered prophage induction could still spread virulence factors of pathogenic bacteria [58].

An absence of widespread prophage induction might be a feature of bacteria and phage coexistence within the gut of healthy adults [3, 46]. Harbouring easily inducible prophages would be a liability for bacteria under frequent exposure to perturbations, such as the gut. Lower induction rates have been predicted for commensal bacteria [59], which dominate in the gut [60]. Bacterial resilience to perturbations, such as antibiotics, has been put forward as a barrier to the development of gut-related illness [61]. Dietary

interventions show similar patterns to those found here with antibiotics [36, 45]. Resilience to perturbations offers an explanation as to why triggered prophage induction is not frequently observed in healthy individuals (Sutcliffe *et al* 2022, *submitted*), and why prophages make poor distributors of antimicrobial resistance genes even under antibiotic exposure [27]. Antibiotics can alter phage replication dynamics through other means than prophage induction (reviewed here [62]), which could explain why they still disrupt the phage community during treatment (Figure 4.5). Destabilization of phage communities by antibiotics in the gut is important to better understand, as an increasing number of diseases and conditions are associated with an altered phage community (e.g., child malnutrition [63, 64], rheumatoid arthritis [65], colorectal cancer [66], IBD [39]) and antibiotic usage [16, 67-69].

Perturbations can vary in frequency and strength, as does the response of the microbiota, which has been beneficial to our understanding of gut microbiota-related conditions [61]. Perturbations capable of significantly reducing bacterial abundance have severe side effects, which include immune-system disruption [70] or increased susceptibility to pathogens [71]. Mouse models would offer valuable insight into phage-bacteria dynamics in the gut [72] and could be used to test if phages are truly stably integrated within their hosts genome upon more variable or stronger perturbations.

Phages have been predominately seen as predatory parasites of bacteria. However, interactions with prophages exist on a spectrum of mutualistic to antagonistic to their host [73]. Mutualistic symbiosis [74] appears to be driving the coexistence of phages and bacteria in the gut microbiota [3]. Bacteria escape top-down control of lytic phages by becoming lysogenic [50], which reduces the number of susceptible hosts [75].

In contrast, lytic replication offers little benefit to the prophage, as after cell lysis, it would find an environment with few susceptible hosts. This ultimately could explain why commensal bacteria undergo lower induction rates [59] with spontaneous prophage induction in lieu of triggered prophage induction.

4.4. Methods

4.4.1. Sample Collection for FCIC

Stool samples were collected from healthy individuals (inclusion criteria: absence of antibiotic usage at least 6 months prior to donation and no diagnosed gastrointestinal (GI) related conditions) and placed immediately in anaerobic conditions (5% hydrogen, 20% carbon dioxide and 95% nitrogen in Coy Anerobic Chamber). From each sample, 2 g of material were mixed and diluted in reduced PBS (rPBS) (1:10). To remove large particles, diluted samples were spun (700g) for 1min, and resuspended in rPBS. 600 µL of sample was diluted in 29.4 mL of complex gut microbiota media [76]. MC was added at a final concentration of 1 $\mu g.mL^{-1}$. Control samples were exposed to DMSO, the drug vehicle. Samples were incubated at 37°C for 16 hours. 1 mL of incubated samples were fixed with 2% formaldehyde for counting bacteria and viruses separately at 8 and 16 hours, respectively separately.

4.4.2. Phage Counts

Bacteria were removed from fixed samples by pelleting bacteria through centrifugation at 3,000g for 10 minutes, and diluting samples 2-fold in 0.02 µm filtered PBS. VLPs were collected on 0.02 µm filters (Whatman Anodisc). VLPs were stained with 2.5x SybrGold dye (30 µL) and rinsed with in 0.02 µm filtered TE following published

procedures [77, 78]. VLPs were counted using an Axioskop (Zeiss) epifluorescence microscope at 1,000X magnification. A minimum of 300 VLP events were counted per slide, or 30 regions.

4.4.3. Bacteria Counts

Fixed bacterial samples were counted by flow cytometry after staining nucleic acid with SYBRGreen I (1x concentration for 15 minutes) on the FACS calibur (BD) as previously reported [53]. Counts were calibrated each experimental day using rainbow fluorescent particles, 3.0-3.4 µm (mid-range FL1 fluorescence BD Biosciences) of a known concentration. Counts were analyzed with FlowJo™ V.10.6.1 software.

4.4.4. Antibiotic Study

Viral and bacterial sequences for the study of antibiotic-triggered prophage induction were originally published by Kang *et al* 2021 [27]. Ten healthy individuals (see criteria for healthy [27]) provided stool samples over the course of four months. Eight individuals received one of four antibiotics: ciprofloxacin (quinolone), cefuroxime (beta-lactam), doxycycline (tetracycline) or azithromycin (macrolide); and two other individuals received a placebo. Stool samples were collected at six time points T1 (15 days before treatment), T2 (third day of treatment), T3 (fifth day of treatment), T4 (15 days after treatment), T5 (30 days after treatment), T6 (90 days after treatment). The viral fraction was separated and sequenced separately from the bacterial community [27]. Both communities were sequenced by short read Illumina approaches, MiSeq PE300 and HiSeq PE125 for virus and bacteria respectively, project ID PRJNA588313 [27]. Metadata were downloaded on, June 2021 from http://sbb.hku.hk/Resistome/.

4.4.5. Viral Assembly

Adaptors were removed from viral sequences and low-quality viral reads trimmed with Trimmomatic V.0.39 [79] (SLIDINGWINDOW:4:20 and MINLEN:75). Quality-controlled viral reads were pooled per individual and assembled into contigs using spades [80] V.3.15.1 and the metaSpades option [81], as recommended for phage assembly [43]. We removed all contigs < 5kb in length. We selected contigs that met one of our three-criterium for being classified as phage by 1) detected using VirSorter (categories 1-2) (Galaxy V.1.0.6) with an additional gut virome database [82]; 2) three or more ORFs (predicted by Prodigal V.2.6.3 -metagenomic mode) with homology (HMMER V.3.2.1 hmmscan minimum e-value 1e-5) to prokaryotic virus orthologous groups (pVOG database [83], downloaded on Dec 1, 2020); or 3) homology to a phage from the gut virome database [82] (BLASTn homology e-value 1e-10, with 80% coverage of shortest contig).

4.4.6. Bacterial Assembly

Adaptors were removed from bacterial sequences and low-quality viral reads trimmed with Trimmomatic V.0.39 [79] (SLIDINGWINDOW:4:20 and MINLEN:75). Human contaminated sequences were removed by aligning trimmed reads to the human genome *Homo sapiens* GRCh38 with Bowtie2 [84] V.2.4.2. Quality-controlled, and decontaminated bacterial reads were pooled per individual and assembled into contigs >1.5 kb with megahit [85] V.1.2.9 default settings. Contigs from each individual were assembled into bacterial genomes using MetaBat2 [86] V.2.14, CONCOCT [87] V.1.1.0, and MaxBin2[88] V. 2.2.7. Bacterial genomes from each individual were merged using

DAS-Tool [89] V. 1.1.2. We selected only medium-to-high quality bacterial bins based on the criteria of either 1) DAS-Tool bin score ≥ 0.5 2) or less than 10% contaminated and greater than 40% complete based on CheckM [90] V.1.1.3. Bacterial genomes were classified using GTDB-Tk [91] V.1.4.1 and their reference database [92] version r95.

4.4.7. Prophage Detection

Prophages were detected within the medium-to-high quality bacterial genomes using VIBRANT (V.1.2.1) [42], and selecting only the integrated prophages. We classified prophages as being putatively active using PropagAtE [41] (V.1.0.0) with bacterial bin coverage profiles based on bacterial read alignments, and those that met their default criteria of MWU and Phage:Host Ratio.

4.4.8. Identification of Temperate Phages

For analysis of temperate phages, we selected phage contigs that were predicted to be greater than 50% complete using CheckV [93] V.0.7.0. We excluded mostly incomplete phage contigs from the temperate analysis, as an absence of protein domains used in identifying temperate phages are increased in incomplete phage contigs based on the incomplete assembly of the genome, and it is not recommended to run this type of analysis on incomplete genomes [94]. We used BACPHLIP V.0.9.6 [94] to predict whether a phage was temperate with over 50% certainty.

4.4.9. Bacterial and Phage Relative Abundances

Bacterial and phage relative abundances were determined by aligning their respective quality-controlled reads to the phages (putative prophages and assembled

phage contigs) and bacterial bins using Bowtie2 [84] V.2.3.5.1, and Samtools V.1.12 coverage. Coverage was normalized by genome size and per sample using DESEQ2 V.1.30.1 [95] size factors.

4.4.10. Statistics

Kruskal-Wallis chi-squared test completed with R stats package V.4.0.3. Dunett's multiple comparison was performed using Prism V.8.4.2 (GraphPad Software). Shannon diversity, helligner distance, and redundancy analysis was calculated using R vegan package V.2.5-7

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Chapter 5. Discussion

5.1. Summary

The gut microbiota is a fascinating microbial ecosystem. By the time we have reached adulthood our microbes have made it through the chaotic first years of life [1], acquired a specialized genetic repertoire [2], all the while avoiding regular washouts [3] and our immune system [4]. Where we live, what we eat, who we live with, and so many other life events further shape our microbial communities, creating a microbiota that is truly our own. Within this ecosystem, phages and bacteria co-exist [5] through complex replication dynamics [6, 7] reaching an equilibrium or stable state [8]. This stable state shows remarkable resilience to perturbations in healthy individuals [9]. In this thesis, we explored how lysogenic replication dynamics between phages and bacteria contribute to this equilibrium and resilience of the gut microbial community.

The dynamics between bacteria and phages are not independent of the surrounding environment. During the early years of life, bacteria and phages undergo lytic replication dynamics [10]. As bacteria in the gut reach carrying capacity (approximately 10^{11} bacteria per mL) [11], temperate phages favor lysogeny [12] over lytic replication [13], or in the case of the CrAss-phages, a lytic-lysogenic intermediate [14]. We show in this thesis that in the absence of antibiotics, phages maintain lysogenic replication through continuous spontaneous prophage induction (Chapter 3). Bacteria exposed to oral medications had their gut phage-bacteria dynamics disrupted by triggered prophage induction (Chapter 2). However, in the gut of healthy individuals triggered prophage induction was rare (Chapter 3), and not widespread when healthy individuals received

antibiotics (Chapter 4). We conclude that lysogeny replication dynamics in the healthy adult gut [6, 7] contribute to phage and bacteria co-existence [5] and the overall stability of the healthy adult microbiota [8].

5.2. Challenges of Studying Lysogeny in the Gut

In research, one should be wary of problems that appear ‘simple’. When I began my PhD, the consensus was that phages in the gut were mostly temperate and bacteria were mostly lysogenic [15]. Based on this prediction, experimental approaches estimating bacterial mortality by prophage induction should have confirmed high levels of lysogeny in the gut, as seen across many other microbial ecosystems (coastal and deep seawaters [12, 16], hydrothermal vents [17], lakes [18-21], hot springs [22], lagoons [23], and soil [24, 25]). We applied protocols used in aquatic systems [26] with additional steps taken from soil-systems (e.g., extracting bacteria before induction, and supplementing filtered fecal slurry with additional nutrients) [24, 27]. These protocols, as we see in Chapter 4, led to low FCIC levels, and not what we expected based on the reported high levels of lysogeny in the gut [15]. In the second year of my PhD, a meta-analysis of FCIC across many ecosystems showed that most studies predict 0-5% of lysogenic bacteria [28]. Both under- and over-dosing of MC can be responsible for underestimates of lysogeny (≤ 0% induced lysogens) [28]. To address the dosage problem, we worked with multiple concentrations and bacterial gut isolates instead of a whole complex microbial community (Chapter 2). The advantage of this approach was that bacteria were studied under ideal growth conditions, and it allowed for differentiation between species. Similar studies had proven successful in showing that dietary compounds can induce gut bacteria [29], and

we confirmed that oral medications can trigger prophage induction of gut isolates (Chapter 2).

Working with bacterial isolates has limitations, notably the inclusion of only a few cultivated bacteria, grown outside of the gut, under *in vitro* controlled conditions. Recent developments in bioinformatics pipelines offered a solution to study lysogeny in complex communities, such as the gut microbiota. Bioinformatic analysis of shotgun sequenced phages introduced to a simplified murine gut microbiota made up of 15 fully sequenced bacterial isolates were able to detect prophage induction [6]. Kim and Bae extended this approach to a natural mouse gut microbiota, where bacterial genomes were assembled from shotgun sequencing [30]. In this study, they showed that dietary intervention triggered prophage induction [30]. We successfully applied these bioinformatic analysis to investigate the role of prophage induction in fecal samples of a healthy individual over 2.4 years (Chapter 3). We confirmed that active prophages can be detected with sequencing data [31], even if the human gut has fewer active prophages than a murine gut [32]. Encouraged, we applied this model to individuals who were given antibiotics, including ciprofloxacin, a prophage inducer identified in Chapter 2 (Chapter 4). By using a bioinformatic approach, we support the conclusion that chemically inducing prophages in the gut leads to low levels of active prophage detection. In the healthy adult gut, prophages appear to maintain their activity by regular spontaneous prophage induction and stable integration resilient to perturbations.

5.3. Why be Lysogenic in the Gut?

Ignoring the problem of viewing phage replication as a dichotomy of 'lytic or lysogenic' [33], the gut is seemingly an ideal environment for lytic replication: an abundance of metabolically active bacteria with high nutrient access and continuous mixing that increases the frequency of interactions. In other ecosystems, lytic replication can lead to top-down control of bacterial populations by phages [34] with 20-30% bacteria being infected at any given time [35]. Surprisingly, phages in the adult gut are found at lower VBRs (between 0.001-1 [36]) than systems with lytic replication (VBRs between 5->85) [37], and both phage and bacterial communities are quite stable [8, 31]. The conclusion was that lysogeny, and not lytic replication, dominates in the gut [38, 39] and 'why be lysogenic?' is an exciting question to ask in this context.

In 1984, Frank Stewart and Bruce Levin put forward two important hypotheses for why temperate phages persist in microbial communities, despite the reduced phage growth rate of lysogenic replication compared to lytic replication. Lysogeny persists:

> 1) "As a consequence of the allelopathic effects of diffusing phage, in physically structured habitats, lysogenic colonies are able to sequester resources and, in that way, have an advantage when competing with sensitive non-lysogens"
>
> 2) "Lysogeny is an adaptation for phage to maintain their populations in 'hard times', when the host bacterial density oscillates below that necessary for phage to be maintained by lytic infection alone" [40].

The 'hard times' has since been referred to as the 'refuge model' [41]. These hypotheses have been highly influential in shaping our understanding of 'why be lysogenic?' but must be modified to explain what we see in the gut.

The first hypothesis of Stewart & Levin, that lysogeny is the product of competition, is applicable to the gut, as closely related bacteria often compete for specific nutrients and space [42, 43]; in particular within bacterial species [44]. Lytic phage proliferation requires an abundance of susceptible hosts [45], while bacteria try to escape lytic replication through a variety of defence mechanisms: receptor modification, CRISPR-immunity, restriction enzymes, amongst others [46]. Prophages offer additional defenses: superinfection immunity and exclusion [46]. Superinfection exclusion can provide an escape from top-down control of lytic phages [47]. This is further supported by the observation that lysogens accumulate 30% less CRISPR-spacers [46]. Superinfection exclusion offers a defence against lytic replication that can occur with minimal fitness cost to lysogens in natural environments [48]. Cycles of 'kill-the-winner' dynamics between lytic phages and bacteria provide an evolutionarily context for which it is beneficial to be lysogenic, as the costs of lysogeny are balanced by benefits of superinfection exclusion [47].

Lysogeny might offer more than just an opportunity to escape lytic replication, it could be used as a weapon against competing bacteria. Lysogens can use the integrated prophage against other bacteria by releasing phages that proceed to lyse closely related sensitive strains [49]. *Bacteroides cellulosilyticus* WH2 strains with active prophages outcompeted closely related strains with inactive prophages, suggesting that releasing phages gave B. cellulosilyticus WH2 a competitive advantage [6]. Essentially, "*amicus meus, inimicus inimici mei*" or the enemy of my enemy is my friend.

Prophages do not just offer offensive and defensive advantages in lytic replication but can also provide the host with additional genes that confer a fitness

advantage. Fast growing bacteria, which are characteristic of the gut [50], are more likely to be lysogens [46] with intact prophages [51]. Prophages have been observed to encode a variety of genes, including increased antibiotic resistance, additional metabolic potential, and resistance to our immune system [52]. In the gut, prophages are a source of carbohydrate metabolism genes [38]. These results align with Stewart and Levin's first hypothesis for 'why be lysogenic?' and support the idea that lysogens in the gut could be 'making-the-winner' [13].

The second hypothesis of Stewart & Levin, whereby lysogeny is favoured when bacteria fluctuate into lower abundance, is at-odds with observations of the healthy adult gut. Low bacterial density has been seen as a source of lysogeny in isolates (e.g., lambda phage [53]), and in aquatic systems [16], but the incidence of lysogeny has been shown to occur at both extreme ends of bacterial density (high and low) (Figure 5.1) [54]. In systems with high bacterial density, as the gut, lysogeny is explained by 'piggy-backing-the-winner' rather than by a 'refuge model' [54]. Of course, some bacteria are found in low abundance in the gut, but they would not represent the highly abundant lysogens observed. Bacterial density is an important factor in shaping the 'lysogeny decision' of temperate phages [55]. Coinfection increases with high bacterial density and lysogeny favoured over lytic replication, in particular in the gut [50]. In the gut, coinfection is frequent even when the VBR is below one, due to high bacterial/phage densities, phage adsorption rates, and longer *in vivo* lysogenic commitment time than *in vitro* [50].

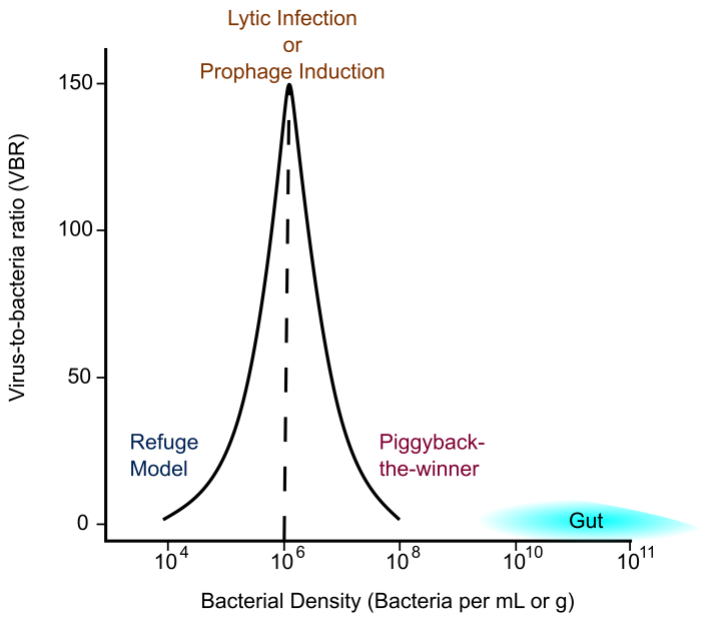

Figure 5.1 Models for Lysogeny at Varying Bacterial Density:

At low bacterial density, bacteria are typically in states of low energy status, with long latency, that increases coinfection, and allows temperate phages to integrate successfully. Low bacterial density decreases the availability of susceptible hosts for lytic replication. As bacterial density increases, and bacterial replication increases, prophages undergo prophage induction, and the bacterial density allows for phages to come in contact with susceptible hosts. As bacterial density increases beyond 10^6 bacteria per mL or gram, coinfection increases, and superinfection exclusion increases, allowing bacteria to escape 'top-down control' of lytic replication. The high levels of lysogeny, at high levels of bacterial density, as seen in the gut [11], are described as 'piggy-back-the-winner'. This figure is adapted from Silveira, Luque and Rohwer, article published in Environmental Microbiology, (2021) 23 (8), 4098-4111, by Society for Applied Microbiology.

The 'refuge model' is based on the idea that low bacterial density is associated with reduced metabolic activity and starvation, which typically result in lysogeny [13]. In the gut, both abundant and rare bacteria are found to be metabolically inactive [56] which could increase lysogeny as well [13]. 'Piggy-back-the-winner' explains this phenomenon as bacteria at high growth rates and densities use less efficient energy metabolism which increases lysogeny, in contrast to the starvation of the 'refuge model' [13]. 'Piggy-back-the-winner' thus offers a more plausible explanation than the 'refuge model' of why bacteria are lysogenic in the gut. As lysogeny becomes established in a system, the system is stable against becoming lytic-dominated [45]. In 'piggy-back-the-winner', as

lysogeny increases, the proportion of sensitive bacteria decreases, boxing out lytic phages. Lytic phages in the gut are thus under increased selective pressure compared to temperate phages [31] as they compete for the few remaining sensitive bacteria. Prophages in contrast, can persist over long periods of time without much divergence (Chapter 3).

Stewart & Levin's original hypothesis helped frame the question 'why be lysogenic?' almost thirty years ago. The 'piggy-back-the-winner' model has improved our understanding by accounting for high levels of lysogeny seen at high bacterial densities. 'Why be lysogenic in the gut?' in adults might be the consequence of early life bacterial competition at densities closer to 10^6 bacteria per gram, which drives diversification [57], colonization resistance [58], lower coinfections, and lytic replication dynamics [10]. Once lysogeny is established, it becomes stable and resistant to perturbations such as antibiotics.

5.4. Spontaneous Induction in the Gut

Spontaneous prophage induction (reviewed by Nanda and colleagues [59]) plays an important role in shaping bacteria-phage interactions. Most of the work to date has been done with bacterial isolates, specifically in pathogenic strains that spread virulence factors [59]. Work done with *Salmonella*, in which lysogens were co-cultured with sensitive strains, showed that spontaneous induction can lead to lysogenic conversion [49]. Spontaneous induction could thus be a mechanism for the spread or maintenance of lysogeny within a bacterial population. Our data showing continuous spontaneous prophage induction (Chapter 3) further support this idea. If a prophage remains inactive

over many bacterial generations, rapid mutation rates that occur in core genes of prophages could render the prophage permanently inactive [60], and result in an evolutionary dead end for the phage. Prophage induction that leads to most lysogens in a population being induced would put prophages under stronger selective pressure to be rendered inactive [60]. Spontaneous prophage induction might thus balance evolutionary costs and benefits for both bacteria and prophage.

5.5. Future Directions

5.5.1. Mechanism of Prophage Induction in Gut Isolates

A number of medications can trigger prophage induction of gut isolates (Chapter 2), yet we still do not know the specific mechanisms underlying these events. Most of what we know about mechanisms of prophage induction comes from *E. coli* and lambda phage [61]. With the diversity of bacteria and phages that exist globally, it is important to understand if these prophages are following typical RecA dependent prophage induction or if other mechanisms are at play. We just have to look at the CRISPR anti-viral system [62] to see the potential diversity that could exist for prophage induction systems. Studying these mechanisms on gut isolates is a challenge, as molecular tools have not yet been adapted for most non-model microorganisms [63]. Regardless, it is an exciting question, that I hope will be explored in the future.

5.5.2. Expanding the Scope of Sampling

A reoccurring issue with gut microbiota studies is the balance between the cost of increased sample collection and benefit of increased biological insight. The strong inter-individual diversity of the gut microbiota is a longstanding issue in the field [64].

Chapter 3 would benefit from increasing the number of individuals enrolled, but as we see in Chapter 4, increasing the number of individuals can come at the cost of reduced sampling per individual (from 24 samples to 6). With the Minot *et al* dataset of Chapter 3, there was an unprecedented level of phage sequencing depth: 16 samples from one individual, for which eight samples had technical replicates, and a read depth well beyond saturation [31]. Phage sequencing depth per sample is important for complex communities like the gut, and a sequencing depth of over a million reads per sample has been recommended [65]. Achieving this level of sequencing depth per sample is a challenge when using fecal samples, where only low levels of viral DNA are often extracted from purified VLPs and an amplification step to increase the viral concentration, at the expense of biasing results towards ssDNA viruses [66, 67]. We saw in Chapter 3 that the increased sequencing depth was important in tracking low abundant prophages over the course of the study.

Sample frequency is an additional important characteristic of longitudinal studies. In Chapter 3, bacteria were not sequenced as frequently as the viruses, and this reduced our confidence in determining prophage induction in comparison to Chapter 4. Even in simplified bacterial communities within SPF-mice, phage-bacteria dynamics fluctuate over small timescales [6]. These fluctuations are important to our understanding of phage-bacteria dynamics: for example, we saw the relative abundance of temperate phages fluctuate between almost 0% to 100% over just a few days in some individuals (Chapter 4). Daily sampling, as completed in Chapter 3, showed that prophage induction is transient. The sampling frequency during treatment in Chapter 4 might have missed prophage induction but was adequate to show that the viral community recovered by day

90 post-treatment. The cost and challenges of sample collection and sequencing must be balanced with the benefits. The work in this thesis highlights both the strengths and weaknesses of both and will hopefully inform future experimental designs.

5.5.3. Improving Prophage Prediction

Prophage detection, even with complete genomes of isolates, is a challenge [68], and problems only increase when using metagenomic assembled genomes [69]. One solution to this problem is to improve the metagenomic assembled genomes by performing long-read sequencing in tandem with short-read sequencing. Hybrid assembly improves on short-read assembly by overcoming intergenomic repeat regions that break short-read assembly and render more complete genomes [70]. When bacterial assemblies improve, so does the prediction of active prophages [30]. This approach increases sequencing costs and computational resources (implementing hybrid assembly pipelines), but most importantly, attaining adequately long DNA fragments from fecal samples is still a major hurdle for gut microbiota studies [71]. Another experimental solution to improve prophage detection is the use of a simplified microbial community of gut isolates with fully sequenced and assembled genomes, where prophages can be confirmed *in silico*. Simplified communities may be simple in composition, but not in terms of maintenance: for example, the Schaedler flora contains some extremely oxygen-sensitive bacteria, that are challenging to grow *in vitro* [72]. They also come at the cost of decreased translatability to humans [73]. The major advantage of complete genomes, as we saw in Chapter 2, is that when viral reads of phages are aligned to a complete bacterial genome, integration sites can be more accurately identified. Identifying integration sites is a particular challenge in prophage prediction tools [74]. Put into practise, the improved

resolution of using complete genomes can help differentiating between lytic replication and prophage induction [6].

5.5.4. Confirming Absence of Inducible Prophages

Negative results are important to research but difficult to present confidently. Antibiotics, when administrated to healthy individuals as in Chapter 4, led to only minor prophage induction. Even MC, the current experimental standard for inducing prophages, led to 0.1% of bacteria undergoing prophage induction, at best (Chapter 4). These results are surprising, as we saw that almost all gut isolates with prophages undergo prophage induction with antibiotics in Chapter 2. We further compared different concentrations of antibiotics, including MC, and we saw that the concentration used can determine whether or not an isolate was induced. Using a single concentration in whole-community studies appears to limit measuring the FCIC [28]. In addition to MC, bacteria show strain-level variations in their response to antibiotics [75] and intensity and frequency of antibiotic perturbations play an important role in how the gut microbiota responds [9]. If prophages can be chemically induced in the gut microbiota at other concentrations, mouse models will need to be used to allow for more intense and frequent perturbations than can be done ethically in humans [76]. Efforts have been made using IL-10 knockout mice (an IBD-murine-model) to design a reproducible perturbation model with antibiotics [76]. This level of perturbation, where there is a significant decrease in bacterial load, has consequences beyond the simple disruption of phage-bacteria dynamics: it can also lead to increased risk of *Clostridoides difficile* colitis [77] and the disruption of the mouse immune system [78]. Increasing the concentrations of antibiotics will determine the limit to which prophages remain stably integrated but not at doses relevant to human health.

5.5.5. Incorporating Biogeography

It is important to acknowledge, as mentioned in the introduction, that our definition of the gut focuses on the lumen of the colon, as that is what is best represented by fecal samples. This limits our understanding of other gut-phage interactions that might be occurring. Indeed, phage replication-dynamics in the lumen differ from those of the mucosal layer [79-81]. The increased rate of interactions observed between bacteria and phages in the mucosal layer was shown to increase phage replication of *E. coli* 1024 strain by T4 phage [80], whereas mucosal *E. coli* Mt1B1 appears to be escaping phage-replication when compared to luminal *E. coli* Mt1B1 [79]. To complicate things further, spatial structuring of the mucus can lead to differential rates of lytic replication vs lysogeny [81] and phage communities in the lumen respond differently to perturbations than in the mucosa (e.g., in diet [82], and disease [83]). Phage-bacteria interactions continue as bacteria move beyond the epithelial layer, to the lamina propria, where bacteria are engulfed by macrophages and eliminated [84], a process which has been shown to induce prophages [85]. Phages are also found well beyond the walls of the epithelial layer [86]. Currently the invasiveness of collecting mucosal samples requires animal models with different biogeography [73]. *In vitro* systems that replicate the gut biogeography, i.e., 'gut-on-a-chip' designed for drug development [87] show promise in being translated to phage applications (e.g., phage therapy [88]). Incorporating gut biogeography was beyond the scope of this thesis but will influence lytic-lysogeny decision [81] and is important to consider in future studies.

5.6. Conclusion and Perspective

We depend on our gut microbiota for nutrient acquisition, immune system training, and protection against pathogens. A system as vital as this to our health is thankfully quite resilient to perturbations, especially given the number of perturbations it must face over our lifetime. This resilience extends to the integration of gut prophages. Prophages in the gut maintain their ability to replicate while minimizing bacterial mortality, a bacteria-phage relationship not antagonistic, but rather mutualistically symbiotic. Mutualistically symbiotic relationships are more common in the viral world than we might intuitively predict. Human cells contain numerous integrated eukaryotic viruses (approximately 8% of the human cellular genome originates from retroviruses [89]). Mutualistically symbiotic viral replication have been observed in viruses infecting yeasts, insects, and plants, as well as bacteria [90]. The fact that phages in the gut coexist with bacteria, should be not just unsurprising, but welcomed. By the time we reach adulthood, bacteria have had plenty of time to establish boundaries, and develop tolerance to their phages. As we all regularly, and increasingly, take medications throughout our lifetime, it is reassuring that these perturbations are not capable of altering the balance of this relationship.

5.7. References

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