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Lactobacillus Metabolite-Mediated Induction of Bacteriophage

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LACTOBACILLUS METABOLITE-MEDIATED INDUCTION OF BACTERIOPHAGE

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ABSTRACT

Once considered sterile in the absence of an infection, the female urinary tract is home to a diverse community (microbiota) of bacterial species and bacteriophages (phages), viruses that infect bacteria. A dominant member of the female urinary microbiota is the bacterial genus *Lactobacillus*. Several *Lactobacillus* species are associated with urinary health. Phages infectious of bacteria in the urinary tract tend to replicate through one of two life cycles: the lytic and lysogenic life cycle. Temperate phages can switch from the lysogenic to the lytic life cycle in the presence of an environmental cue in a process called induction. Within the urinary tract, *Lactobacillus* species naturally produce several inducing agents used in the lab; they release lactic acid, reducing the pH of their environment, and hydrogen peroxide as part of the glycolytic pathway. This observation motivated the investigation into how *Lactobacillus* metabolites induce phages in other urinary bacteria species.

Eight communities of bacterial strains isolated from urine samples from eight different women were examined here. Each community contains either a *Lactobacillus jensenii* or *Lactobacillus mulieris* strain and at least two other bacterial taxa. The genomes of all community members were sequenced and screened for the presence of phages. The pH and hydrogen peroxide outputs were measured for each of the *L. jensenii* and *L. mulieris* isolates. Other taxa in the community were grown in media adjusted to *Lactobacillus*-relevant pH levels based up on the *Lactobacillus* strain in their community. These cultures were screened for the presence of induced phages. Finally, community members were grown with varying volumes of their

community's *Lactobacillus* strain's spent media. These cultures were sequenced and screened for the presence of induced phages. 105 individual phages were induced during this process. These results suggest that *Lactobacillus* is inducing phages in other community members in the urinary tract. Thus, the proliferation and often dominance of Lactobacilli in the female urinary tract may be a result of induction and lysis of other bacterial taxa.

CHAPTER ONE

INTRODUCTION

The Urinary Tract Environment

Advances in microbial techniques have played a key role in increasing our understanding of the human microbiota (the community of bacteria, viruses, and fungi that inhabit the human body) and its relation to human health (Integrative HMP Research Network Consortium, 2019). The microbiome refers to the genetic material of microbes within a certain niche. The Human Microbiome Project played a pivotal role in cataloging the bacterial species of the nasal cavity, oral cavity, skin, gastrointestinal tract and vagina (Turnbaugh *et al.* 2007). Thought to be sterile in the absence of infection, the urinary tract was not included in this original investigation. However, we now know that the urinary tract is home to a diverse microbiota (Wolfe *et al.* 2012; Siddiqui *et al.* 2011; Dong *et al.* 2011). In fact, 90% of urine samples, from women, contain viable bacteria (Hilt *et al.* 2014). We have learned that the urinary tract of healthy individuals is host to a wealth of bacteria (Aragón *et al.* 2018), fungi (Akerman and Underhill, 2017), eukaryotic viruses and prokaryotic viruses (bacteriophage) (Garretto *et al.* 2019).

The urinary tract includes the kidneys, ureters, bladder, and the urethra. The environmental conditions of the urinary tract limit what microbial species grow in this environment as well as the biomass of the niche. The pH of collected urine samples lies between 5 and 8 for most healthy individuals (Simerville *et al.* 2005). However, differing pH levels have been noted for certain health conditions, such as a more acidic urine in diabetic patients (Maalouf

et al. 2007). The urinary environment has temperatures ranging from 36.9-39.9°C (Neugent *et al.* 2020). Oxygen tension within the urinary system is the lowest in the body, although it can cover a wide range from 0.47 to 51.5 mm Hg (Landes *et al.* 1964; Leonhardt and Landes 1963; Shannon *et al.*, 2019). Oxygen levels might be associated with the microbial composition of the urinary microbiome, with individuals with greater amounts of *Escherichia coli*, *Lactobacillus* species, and *Aerococcus* species having lower oxygen tension (Shannon *et al.* 2019).

The production, movement, and voiding of urine allows for nutrients, such as amino acids, electrolytes, and carbohydrates (Forsyth *et al.* 2018), to be continually replenished within the urinary environment. Additionally, the urothelium is protected by a glycosaminoglycan layer, which may be metabolized into smaller sugars and used as a nutrient source by *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* (For a review, see Neugent *et al.* 2020).

The Urinary Microbiome

While the urinary tract is not sterile, there is a significantly lower biomass in comparison to the microbiota at other body sites. For example, the gut contains about 10^{12} colony forming units (CFU) per gram of feces, while the urinary tract contains only 10^{2}-10^{5} CFU per mL of urine (Rowe and Juthani-Mehta 2013). The use of advanced culturing methods, 16S rRNA gene sequencing, and metagenomic studies have allowed us to determine the bacterial species that inhabit the urinary tracts of both sexes with and without urinary symptoms (For a review, see Wolfe and Brubaker 2019). Previous culturing methods heavily selected for common uropathogens, such as *E. coli* (Kass 1962). In addition to its documented association with urinary tract infections (UTIs), *E. coli* can be easily grown under standard laboratory conditions. The diversity of other bacteria within the urinary tract has been captured using new techniques such as the enhanced quantitative urine culture (EQUC) (Hilt *et al.* 2014). Furthermore, high-

throughput sequencing technologies have provided a means of investigating microbes that cannot be grown under laboratory conditions (Mueller *et al.* 2017). In women, the most common bacterial genera isolated by EQUC are *Lactobacillus, Streptococcus, Actinomyces*, and *Staphylococcus* (Pearce *et al.* 2014). Other notable genera include *Aerococcus, Prevotella, Gardnerella, Bifidobacterium*, and *Actinobaculum* (Pearce *et al.* 2014). In contrast, investigation of the male urinary microbiota found *Corynebacterium* species to be common (Fouts *et al.* 2012).

Due to proximity, many bacterial taxa are members of both the urinary tract and vaginal microbiota. Several studies have suggested that the urinary tract is often colonized by vaginal bacteria, leading to an interconnected urogenital microbiota (Thomas-White *et al*. 2018a; Komesu *et al*. 2020). Bacterial species found within both microbiota include *Klebsiella pneumoniae, E. coli, Pseudomonas aeruginosa, Bifidobacterium bifidum, Gardnerella vaginalis*, and *Lactobacillus* species, among others (Thomas-White *et al.* 2018a). Genome sequencing of bacterial isolates from the urinary and vaginal tracts revealed genetic similarities between *L. crispatus, L. gasseri, L. jensenii*, and *Actinomyces neuii* strains, suggesting a common origin (Thomas-White *et al.* 2018a). While many of the same bacterial species inhabit both the urinary and vaginal tracts, there is strong evidence supporting that they are in fact two distinct microbiota (Thomas-White *et al.* 2018a).

The best characterized inhabitant of the urinary microbiome is *E. coli* as it is the most common cause of UTIs in women (Flores-Mireles *et al.* 2015). UTIs annually affect upwards of 150 million people worldwide. As one of the most common infections, UTIs pose a serious risk to individuals of all ages and biological sexes (Flores-Mireles *et al.* 2015). However, women are more at risk for UTIs and can be placed further at risk based upon sexual activity, prior UTIs,

and vaginal infections (Foxman 2014). Additionally, 30 to 50% of women who experience a UTI will experience a second, recurrent, UTI within the following 12 months (Foxman 2014). In addition to *E. coli*, UTIs can be caused by a slew of Gram-negative and Gram-positive bacteria, including *K. pneumonia, Staphylococcus saprophyticus and S. aureus, Enterococcus faecalis, Proteus mirabilis,* and *P. aeruginosa*, as well as certain fungi, including *Candida* species (Flores-Mireles *et al.* 2015). New culture techniques, such as EQUC, and 16S rRNA gene sequencing studies have led to the identification of emerging uropathogens, including *Actinotigum schaalii, Corynebacterium urealyticum, Aerococcus urinae*, and *Haemophilus influenzae* (Yarbrough 2018).

While the aforementioned species have been documented as the causative agents of UTIs, they have also been observed in asymptomatic women (Mueller *et al.* 2017). In fact, *E. coli* has been well documented in women with and without UTIs or other lower urinary tract symptoms (LUTS) (Thomas-White *et al.* 2018b). In a 2019 study, our lab explored the genotypic differences between 66 *E. coli* strains isolated from women with and without UTIs (Garretto *et al.* 2020). Through this analysis, we could not identify any gene(s) associated with pathogenicity (or the lack thereof). Rather, this study suggests that the likelihood of an *E. coli* bloom and UTI occurrence is associated with the overall composition of the urinary microbiota. For instance, perhaps UTI is the result of the absence of a "protective" community member, the diversity of the microbiota, or the relative proportion of the members of the microbiota. While antidotal evidence suggests that the whole microbiota is contributing to UTI formation, this hypothesis has yet to be explicitly tested.

Low bacterial diversity and high prevalence of *Lactobacillus* species are often correlated with a lack of LUTS in women (Pearce *et al.* 2014). Additionally, female patients with a urinary

microbiota with a high abundance of lactobacilli are less likely to develop UTIs after operations or instrument removal (Pearce *et al.* 2014). *Lactobacillus* species, such as *L. crispatus, L. gasser*i, *L. iners*, and *L. jensenii*, have be observed as highly abundant species within the urinary microbiome (urobiome) of women (Price *et al.* 2020). In fact, *Lactobacillus* species are the most abundant species in the female urogenital tract (Ravel *et al.* 2011; Pearce *et al.* 2014). With the exception of *L. gasseri*, which has been associated with urgency urinary incontinence (Ravel *et al.*, 2011), other *Lactobacillus* species are hypothesized to be protective or beneficial species as it correlates with low instances of infection, both within the urinary tract and the vagina (Stapleton, 2016). In previous studies, *Lactobacillus* species have been shown to reduce the growth of *E. coli* strains as well as other bacterial genera such as *Pseudomonas*, *Proteus*, and *Bacillus* (Price and Lee 1970; Martin and Suárez 2010; Hertzberger *et al.* 2014). This is due to the hydrogen peroxide produced by these strains. In a 2021 study, a *L. jensenii* strain isolated from the female urinary microbiota could inhibit the growth of uropathogenic *E. coli* (UPEC) strains *in vitro* (Mores *et al.* 2021). It is theorized that it has the same effects *in vivo*.

Lactobacillus sp. greatly shape the environments they are found in through the release of several key molecules. Several *Lactobacillus* species are documented as producing small molecules, which can inhibit growth of other microbes (Kanmani *et al.* 2013). Furthermore, lactobacilli release lactic acid, lowering the pH of the environments they inhabit. This release of lactic acid is attributed to maintaining the low pH observed in the vagina and may contribute to the pH of the urinary tract as well. (For a review, see Stapleton 2016). Lactobacilli also produce hydrogen peroxide, with *L. jensenii* and *L. crispatus* being the main hydrogen peroxide producers (Antonio *et al.*, 1999; Eschenbach *et al.* 1989). Hydrogen peroxide inhibits the growth of other microbes. In the context of the vaginal microbiome, *in vitro* studies have shown that

Lactobacillus hydrogen peroxide production can reduce the growth of *E. coli* and *Gardnerella* sp. (Klebanoff *et al.* 1991). Moreover, women who have hydrogen peroxide producing *Lactobacillus* strains dominating their vaginal microbiome are less likely to contract bacterial vaginosis, HIV, *Neisseria gonorrhoeae*, and *E. coli* based UTIs. (For a review, see Stapleton 2016.)

Bacteriophages

In addition to bacteria, the urinary tract is home to many viruses, mainly bacteriophages (phages), which are viruses that infect bacteria. Phages are the most abundant biological entities on earth, far outnumbering the bacteria that they infect. Phages have been found throughout the human body (Barr 2017). In the context of the gut microbiome, phage diversity and abundance has even been linked to health and disease states (Carding *et al.* 2017). The role of phages in other organs of the human body, including the urinary tract, has yet to be explored. Armed with the knowledge that the urinary tract is home to a diverse bacterial population (Thomas-White *et al.* 2018a), we have only recently begun to catalog and characterize the phages of the urinary tract (Miller-Ensminger *et al.* 2018).

Phages typically persist in one of two life cycles: the lytic life cycle or the lysogenic life cycle (Figure 1) (Hobbs &Abedon 2016). In the lytic life cycle, a phage attaches to the bacterial cell surface and injects its DNA into the host. The host's machinery then replicates the phage's genomic information and synthesizes phage proteins. These proteins are assembled forming viable phage progeny, which lyse the host cell wall. (For a review, see Young 2014.) These phages then disperse into the surrounding environment until they find another susceptible host cell and repeat this infection cycle. In the lysogenic life cycle, a phage also attaches and injects its DNA into the bacterial host cell. However, the phage DNA is either integrated into the host

chromosome or persists as an extrachromosomal plasmid (Little 2005). We refer to phages in this state as "prophages." These prophages are replicated with the bacterial genome and thus are vertically inherited by subsequent generations of the bacterium.

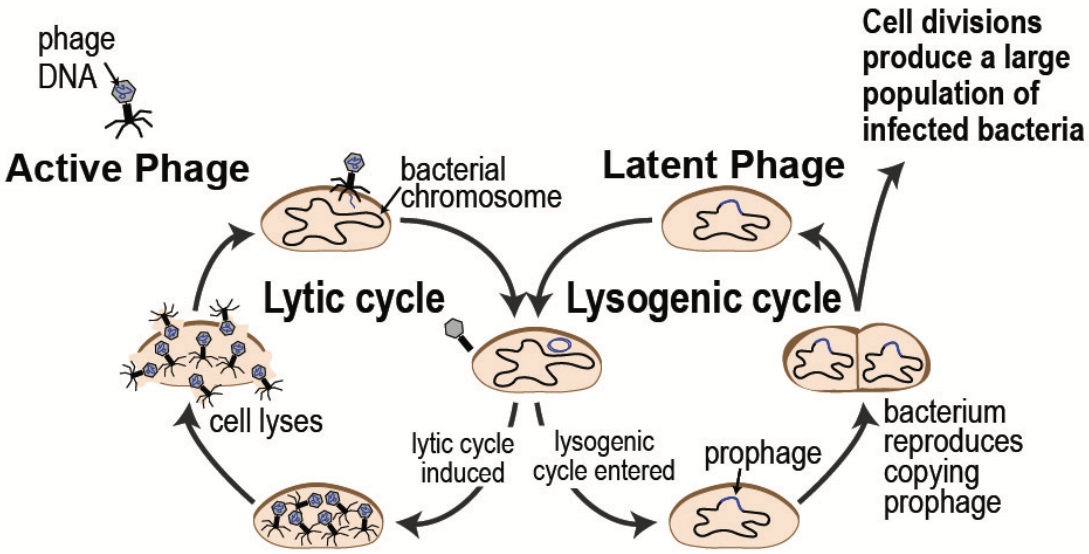


Figure 1. Lytic and Lysogenic Phage Life cycles. Overview of the lytic and lysogenic life cycles as well as illustration of induction for temperate phages.

Phages that only replicate through the lytic cycle are referred to as obligately lytic. However, some phages have the ability to persist in both the lytic and lysogenic life cycles; we refer to this class of phages as temperate phages. A temperate phage can switch between the two life cycles through a process called induction. Induction occurs when an environmental stressor triggers the SOS response in the bacterial host which in turns causes the prophage to transition from the lysogenic life cycle to the lytic life cycle (Figure 1) (Little 2005; Abedon 2008). At this point, the phage genome is replicated, and progeny are assembled and released into the environment to infect new hosts. Environmental stressors that can lead to induction include

antibiotics, shifts in temperature, exposure to UV light, shifts in pH, and the presence of hydrogen peroxide (Kirby *et al.* 1967; Barnhart *et al.* 1976; Meijer *et al.* 1998; Bossi and Bossi 2002; Lunde *et al.* 2004; Williamson and Paul 2006; Martin *et al.* 2009; Los *et al.* 2010; Carlquist *et al.* 2010; Choi *et al.* 2010; Chu *et al.* 2011; Zeaki *et al.* 2015; Zhang *et al.* 2020, Miller-Ensminger *et al*. 2020a). More recently, molecules produced by certain bacteria species have been shown to induce phages. For example, a compound released by *P. aeruginosa*, pyocyanin, is capable of induce a specific phage within a polyprophage-containing *S. aureus* strain (Jancheva and Böttcher 2021). Similarly, colibactin, a secondary metabolite produced by several members of the gut microbiota, induces phages in *E. coli*, *S. aureus*, and *S. enterica Typhimurium* (Silpe *et al.* 2021). Interestingly, bacteria that contain a colibactin resistance gene are immune from colibactin-triggered induction.

Phages have the ability to greatly shape microbial communities. First, phages in the lytic life cycle shape communities through predation, hunting and killing susceptible hosts (Fuhrman 1999; Suttle 2005; Clokie 2011). This reduces the number of susceptible taxa in the system and therefore changes the microbial abundance profiles within a space. Second, phages and their bacterial hosts exist in a constant arms race with bacteria adapting to avoid phage predation and phages adapting to continue to infect hosts (Buckling and Rainey 2002; Rodriguez-Valera 2009; Koskella and Brockhurst 2014; Tellier 2014). This leads to mutations both within the phage and the host, driving genetic diversity within the community (Scanlan *et al.* 2015). Last, phages can also shuttle genes from one host and transfer them to another during infection (Hosseinidoust *et al.* 2013; Wendling *et al.* 2017). This leads to increased genetic diversity across strains within an environment, even potentially increasing the virulence of certain strains.

Bacteriophages of the Urinary Tract

In 1917, the first lytic phage from the urinary tract was isolated by Felix d'Herelle (D'Herelle 1917). The presence of phages in the urinary tract was further reported by Larkum in 1926, with results showing phages present in samples from 25% of patients with UTIs (Larkum 1926). Since then, a variety of phages have been isolated from the urinary tract by our lab and others. This includes *P. aeruginosa*-infecting phages (Brown-Jaque *et al.* 2016; Johnson and Putonti 2019; Johnson *et al.* 2019), *E. coli*-infecting phages (Dallas and Kinsbury 1997; Malki *et al.* 2016; Miller-Ensminger *et al.* 2018), and *S. anginosa*-infecting phages (Brassil *et al.* 2020). Transmission electron microscopy (TEM) and sequence similarity to previously morphologically characterized phages has determined that most of these phages are siphoviruses (Dallas and Kinsbury 1997; Brown-Jaque *et al.* 2016; Malki et al 2016; Miller-Ensminger *et al.* 2018; Johnson and Putonti 2019; Johnson *et al.* 2019; Brassil *et al.* 2020). Two *P. aeruginosa*-infecting phages were found to be tailless phages (Jalil *et al.* 2017). Six coliphages were isolated by our group from the urinary microbiota of women with urge urinary incontinence (UUI) (Malki *et al.* 2016). Two of these phages, phages Greed and Lust, have been tested for their ability to lyse urinary *E. coli* isolates, including UPEC strains (Putonti *et al.* 2017). Both were effective in lysing some, although not all, UPEC strains as well as non-pathogenic strains (Putonti *et al.* 2017). This suggests that phages within the urinary tract may interact with, either infecting or lysing, diverse strains within the community.

To date, two viral metagenomic (viromic) studies have screened the urinary microbiome for viruses. In screening 10 samples from women and 10 samples from men, Santiago-Rodrigues *et al.* (2015) found that over 99% of the viral sequences were phage genes and only 27% of the

sequences showed homology to previously characterized viruses. In a later study, sampling from two healthy patients and four with human cytomegalovirus infections, it was found that there was a large phage community in these samples and the majority of the phages were relatives of *Chlamydia* microviruses, viruses that infect *Chlamydia* (Thannesberger *et al.* 2017). These previous studies suggest that there is a diverse phage community within the urinary tract.

In a 2018 study, our group screened 181 bacterial isolates, representative of the bacterial strains found in the female urinary tract, for the presence of prophage sequences (Miller-Ensminger *et al.* 2018). We found that 86% of isolates harbored at least one prophage, with some isolates harboring up to 10 unique prophages. Additionally, 57% of these prophage sequences exhibited no sequence similarity to previously characterized phages. These findings indicate that a large portion of the phages within the urinary tract are novel, which concurs with the previously described viromic studies (Santiago-Rodrigues *et al.* 2015; Thannesberger *et al.* 2017).

In this same 2018 study, we found evidence of prophages shared across individuals. For example, several similar prophages were found within *Actinomyces* strains isolated from urine collected from different females (Miller-Ensminger *et al.* 2018). Similar observations have been made by our group for *S. anginosus* phages (Brassil *et al.* 2020) and *L. jensenii* phages Lu-1 and Lv-1 (Miller-Ensminger *et al.* 2020b). Furthermore, bacteria screened for the presence of Lv-1 and Lu-1 also contained CRISPR arrays with spacer sequences matching these phages. CRISPR-cas systems provide a level of immunity against phages for bacteria. Evidence of Lv-1 and Lu-1 spaces in CRISPR arrays, as old and new additions, showed that these phages have been highly active and evolving in the urogenital tract for some time. These studies point to evidence of a

core “phageome” within the female urinary microbiome, an observation that has been made within the gut (Manrique *et al.* 2016). However, more studies are needed to support this claim.

While bioinformatic tools can predict prophage sequences, they are unable to determine if they are temperate phages. We have determined that many urinary phages are capable of entering the lytic life cycle and are thus viable temperate phages. In 2018, we were able to induce, isolate, and characterize *E. coli* phage 901. In another study, we aimed to determine if phages could be readily induced using biologically relevant conditions, such as shifts in pH (Miller-Ensminger *et al.* 2020a). Using 66 *E. coli* isolates, we were able to induce over 400 hundred phages (Garretto 2019). We positively identified 13 of these phages. These results show that prophages within urinary isolates are viable and can be induced using methods that are relevant to the urinary tract, most notably changes in pH.

As previously mentioned, changes in pH and increased levels of hydrogen peroxide are two effective methods to induce prophages in the laboratory. Given that lactobacilli produce lactic acid, thus dropping the pH of their environments, as well as hydrogen peroxide, I hypothesized that lactobacilli may trigger the induction of prophages in other bacterial species within their community.

In this thesis, I directly tested this hypothesis through the evaluation of *Lactobacillus*-containing communities of bacteria isolated from urine samples from eight women. Each of these communities contains either a *L. jensenii* or *L. mulieris* strain. The genomes for every bacterial isolate in these communities were sequenced and screened for the presence of prophages. First, I present an evaluation of the pH shifts produced by *L. jensenii* and *L. mulieris* strains as well as the hydrogen peroxide produced by each strain. Next, I determined if *L. jensenii* or *L. mulieris* supernatant can induce prophages in the other bacterial isolates isolated from their same

community. In an effort to ascertain the triggering effect of observed induction, I also grew community members in the pH produced by their corresponding *Lactobacillus*. A more comprehensive evaluation was conducted for *E. coli* members in which both lactobacilli pH and hydrogen peroxide levels were examined. Last, conclusions and future directions are presented.

CHAPTER TWO

METHODS

Specimen Collection and Bacterial Isolation

Urine was collected from patients with or without lower urinary tract symptoms via transurethral catheterization as part of prior IRB-approved studies, see Appendix A for a list of IRB study numbers and associated publications. Urine was spread on plates using the EQUC method as described in Hilt et al (2013). Plates were screened for the presence of bacterial colonies. Individual colonies were selected based upon morphology and regrown for purification. Individual colonies were selected after purification and taxonomic classification was performed via Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) as described in Hilt et al (2013). Isolates were then stored at -80°C.

Isolate Purification and Identify Confirmation

Isolates from freezer stocks were steaked on CNA Blood agar plates by the Wolfe Lab at Loyola University Chicago and delivered to the Putonti lab. Single colonies were picked based upon morphology and streaked on CNA Blood agar plates. Plates were incubated at 35°C in 5% CO_2 for 18 to 36 hours, until colonies appeared. This process was repeated for further isolate purification. Next, individual colonies were selected and grown for 18-36 hours in 3 mL of their respective medium (Table 1). 1 mL of this culture was mixed with 1 mL of 50% glycerol and frozen at -80°C as a freezer stock. Individual colonies were used for 16S rRNA gene sequence PCR and sequencing. PCR reactions included a single colony suspended in 8 ul of nuclease free

water, 10ul of Promega goTaq, 0.5 ul (5 mM) of forward 63F primer (PMID: 9464425), and 0.5 ul (5 mM) of reverse 1387R primer (PMCID: PMC106123). After amplification, the PCR product was run through a 1.2% agarose gel. The remaining product was cleaned for sequencing using the E.Z.N.A Cycle Pure Kit. Sequencing of the 16S amplicon was performed by GENEWIZ at a coverage of 2x using the 63F and 1387R primers individually. Sequencing reads were manually trimmed and assembled in GeneiousPrime. The results were queried against the 16S rRNA sequence database using megablast. Isolates with differing 16S and MALDI-TOF MS identities were reclassified using the 16S sequence identity. For a complete list of bacterial isolates, taxonomy, and growth conditions, see Table 1.

Bacterial Genome Sequencing

Bacteria from freezer stocks were streaked onto CNA Blood agar plates and incubated at 35°C and 5% CO_2 for 18-36 hours. A single colony from each plate was grown in 1 mL of the bacterium's respective medium for 18-36 hours at 35°C and 5% CO_2. The bacteria were extracted using a modified version of the Qiagen Blood and Tissue Kit Protocol. Briefly, Pre-1 lysis buffer was made by combining 120 ul Triton x-100, 100 ul Tris-Cl at pH 8.0, 40 ul of EDTA, and 9.74 mL of nuclease free water. Lysis buffer was made by combining 150 ul of Pre-1 lysis buffer and 50 ul of 120 mg/mL Lysozyme. Samples were centrifuged at 5000 xg for 10 minutes. The supernatant was removed and discarded. The pellet was resuspended in 180 ul of lysis buffer and incubated at 35°C for 30 minutes. After incubation, 25 ul of proteinase K and 200 ul of buffer AL were added to the sample. The sample was heated at 56°C for 10 minutes. 200 ul of 100% ethanol was added to the sample. Steps 4-6 of the Blood and Tissue kit were performed following the manufacturer's protocol. A modified version of step 7 from the manufacturer's protocol was performed using 50 ul of buffer AE instead of 200 ul of buffer AE.

DNA concentrations were quantified using a Qubit fluorometer following the manufacturer's protocol. Samples were then shipped to MIGS (Pittsburgh, PA). There sequencing libraries were prepared using the Illumina Nextera Kit and samples were sequenced using the Illumina NextSeq 550 platform.

Bacterial Genome Assembly

Raw reads from the Illumina sequencing were trimmed via Sickle (https://github.com/najoshi/sickle) using the paired ends, sanger, and length threshold equals 100 parameters. The trimmed reads files from the Sickle output were assembled using SPAdes v.3.14.1 (Bankevich *et al.* 2012). SPAdes was run using the only-assembler option, with specified k values of 55, 77, 99, and 127. Genome coverage was calculated using BBmap (https://sourceforge.net/projects/bbmap/) with a kfilter of 22, subfilter of 15, and maxindel of 88.

Raw sequencing reads and assembled genomes have been deposited in NCBI's SRA and Assembly databases, respectively (Appendix B). Genomes were annotated upon submission to the Assembly database. Several of these genome assemblies have been described in the literature (Putonti *et al.* 2019; Garretto *et al.* 2020; Truckenbrod *et al.* 2020; Scott *et al.* 2020; Khan *et al.* 2020; Markovic *et al.* 2020; Schwartz *et al.* 2020; Kalska *et al.* 2020; Eskander *et al.* 2020; Miller *et al.* 2020; Tsibere *et al.* 2020; Gallian *et al.* 2020; Salgado *et al.* 2020; Kemper *et al.* 2020; Purta *et al.* 2020; Gondi *et al.* 2020; Bhimalli *et al.* 2020; West-Pelak *et al.* 2020; Khan *et al.* 2020; Miller-Ensminger *et al.* 2020c; Allen *et al.* 2020).

Phage Prediction and Taxonomic Identification

Prophage sequences were predicted using VirSorter v.1 (Roux *et al.* 2015) and Phaster (Arndt et al 2016). Bacterial genomes were run through both programs using default parameters. VirSorter was run locally and Phaster was run via the webserver. VirSorter predicts phage sequences,

classifying them into one of 6 categories. Per VirSorter documentation, categories 1 and 4 are the most confident predictions, 2 and 5 are less confident ("likely" predictions), and 3 and 6 are the least confident ("possible" predictions). We separated the VirSorter predictions into two groups: categories 1 and 4 and categories 2 and 5. Category 3 and 6 predictions were not examined further as they unlikely represent complete, intact prophages. Phaster predicts phage sequences as Intact, Questionable, and Incomplete. Phage predictions were separated into these groups for downstream analysis.

VirSorter categories 1, 2, 4, and 5 predictions and Phaster Intact and Questionable predictions were used in BLAST analysis. Predicted phage sequences were queried against the database of all phage genome sequences using blastn. Blastn was run locally. The database of all publicly available phage genome sequences was created by retrieving all publicly available nucleotide sequences with the Division "PHG", to represent phage sequences, and Organism "Virus" from NCBI. An initial threshold of at least 70% query coverage and 70% identity match was used to filter the BLAST results. A less stringent threshold of 70% query coverage and 30-70% identity match or 30-70% query coverage and 70% identity match was applied to the remaining results, allowing for homology results of potentially novel phages. The NCBI phage record with the greatest sequence similarity to each prediction was used for taxonomic analysis. Predicted phage sequences that did not pass the aforementioned thresholds were listed as "Unknown" taxa.

Primer Design for Phage Detection

All Results from VirSorter and Phaster were compared to each other to identify regions predicted as phage by both tools. Predictions that were identified by VirSorter as category 1, 2, 4, or 5 and identified by Phaster as Intact were automatically selected for primer design. Predictions

identified as 1, 2, 4, or 5 by VirSorter and Questionable or Incomplete by Phaster were further evaluated based on the number of genes, length of the predicted phage sequence, and the functionality of the predicted genes. In the case of the latter, we evaluated the predicted phage sequences for essential coding regions, including structural, e.g., capsid or tail fiber proteins, and essential functionality, e.g., integrases, lysin genes, transposases. If the predicted sequence contained these essential coding regions, they were selected for primer design. Predictions categorized as 3 or 6 by VirSorter were only selected for primer design if the sequence was also predicted as Intact or Questionable by Phaster.

Primers were designed for the selected phage sequences using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). Self-dimerization was checked using the ThermoFisher Multiple Primer Analyzer tool (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Primers were synthesized by Eurofins MWG. Primer sequences are listed in Appendix C.

Lactobacillus Bacteriocin Predictions

L. jensenii and *L. mulieris* genomes were screened for the presence of secondary metabolites and bacteriocins using antiSMASH (Medema *et al.* 2011) and BAGEL4 (van Heel *et al.* 2018). Both programs were run with default parameters. Results for each *Lactobacillus* strain were recorded.

Lactobacillus Hydrogen Peroxide Measurements

Lactobacillus strains were grown from freezer stocks in 10 mL of MRS + 1% tween 80 for ~20 hours at 35°C in an incubator with 5% CO_2. Samples were grown and analyzed in triplicate. Cultures were briefly vortexed. 1 mL of the sample was removed and placed in a microcentrifuge tube. The sample was centrifuged at 10,000 xg for 2 minutes. The supernatant was transferred to

a clean microcentrifuge. The pH of each sample was adjusted to fall between pH 7 and 8. This step is required by the Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit used to measure the H_2O_2 of each culture. 200 ul of the sample was transferred into a PCR tube and a tenfold dilution was performed. The reaction was carried out using the diluted and undiluted sample following the manufacturer's directions. Fluorescence was measured using excitation at 520 nm and emission at 590 nm. Nuclease free water served a control. Control and standard curve groups were run in triplicate (Figure 2 and Appendix D). Outlying data points attributed to pipetting error were removed from consideration. Hydrogen peroxide concentrations for each sample were calculated and the average for the replicates was determined. Hydrogen peroxide concentrations calculated from the diluted samples were used in further experiments. This decision was based upon hydrogen peroxide values previous recorded for *Lactobacillus* strains (Ocaña *et al.* 1999; O'Hanlon *et al.* 2010).

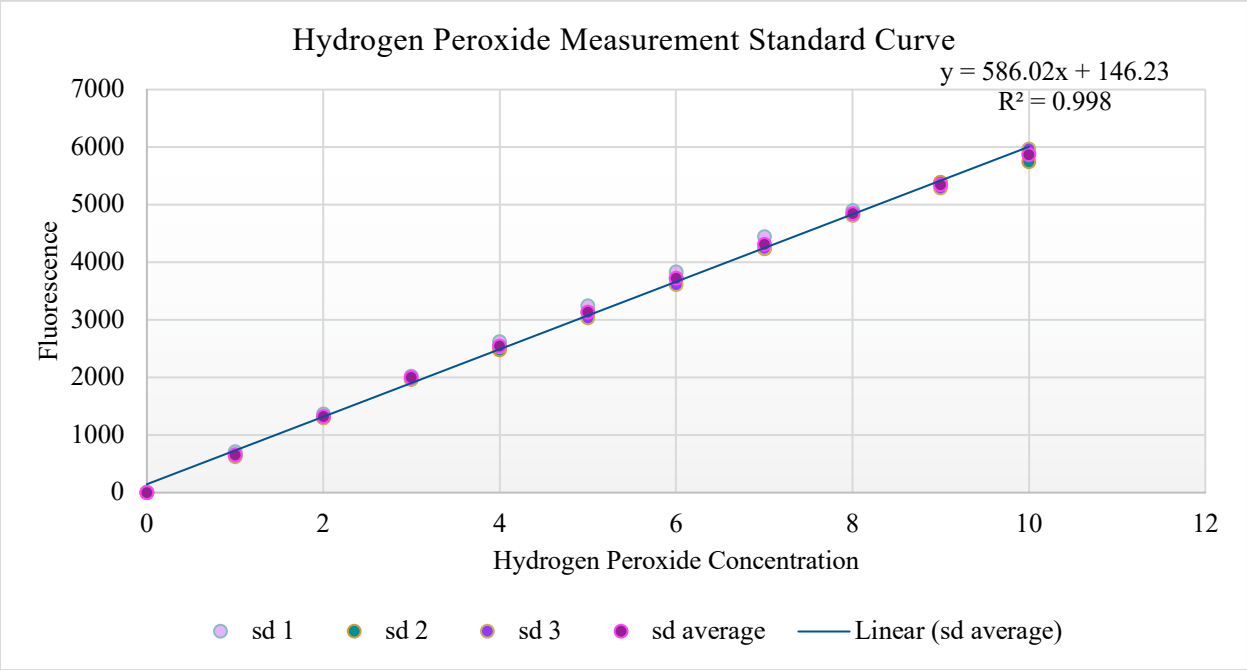


Figure 2. Hydrogen Peroxide Measurement Standard Curve.

Lactobacillus pH Measurements

Lactobacillus strains were grown from freezer stocks in 15 mL of MRS + 1% tween 80 for ~20 hours at 35°C in an incubator with 5% CO_2. Strains were grown in triplicate. A pH meter was calibrated using buffers at pH 4 and 7. Measurements for each replicate were recorded. The average for each strain was calculated and was used in future experiments.

Growth of Other Community Members in Corresponding *Lactobacillus* pH

Bacterial isolates from each community, excluding *L. jensenii* and *L. mulieris* strains, were grown in 13 mL of their respective media for ~18 hours, except for *Corynebacterium* strains that were grown for ~36 hours. All cultures were grown at 35°C and 5% CO_2. Fresh media was adjusted to match the pH of the spent media of the *L. jensenii* or *L. mulieris* strain from each community. These adjustments were made using HCl, NaOH, and a pH meter. The adjusted media was separated into culture tubes in 3 mL aliquots. 1 mL of overnight growth was added to

each tube. All samples were grown in adjusted pH media from their community's corresponding *Lactobacillus* strain in triplicate. 1 mL of overnight culture for each community member was added to 3 mL of "unadjusted" media, i.e., the same media the isolate was originally grown. These cultures serve as a control group, and they were conducted in triplicate. Cultures for the three experimental groups as well as the control group were grown overnight, ~18 hours, at 35°C and in 5% CO_2.

Screening for Phage Induction in Community Members

1 mL of the culture from each replicate and each group in the previous step was transferred to a microcentrifuge tube. The sample was spun at 10,000 xg for 2 minutes and the supernatant, which will contain phages if they were induced from the bacterium, was transferred to a new tube. The sample was DNased following the OPTIZYME DNase I Fisher Bioreagent's protocol. The sample was then heated at 95°C for 10 minutes to burst the phage capsid. Using the primer pairs designed to specifically amplify predicted prophages (Appendix C), PCR was performed to detect induced phages in the supernatant. Briefly, 25 ul reactions were set up using 12.5 ul of goTaq, 0.5 ul of forward primer (5 mM), 0.5 ul of reverse primer (5 mM), 11.5 ul lysate. For a positive control, the freezer stock for each sample was streaked on a CNA blood agar plate and allowed to grow for ~18-36 hours at 35°C in 5% CO_2. A single colony was picked and placed in 200 ul of nuclease free water. The mixture was heated for 10 minutes at 95°C. The positive control was set up by combining 12.5 ul of goTaq, 0.5 ul of the same forward primer (5 mM), 0.5 ul of the same reverse primer (5 mM), 6.5 ul of nuclease free water, and 5 ul of the heated colony mixture. Amplification of the bacterial colony will be the result of amplification of the integrated prophage sequence. PCR products were run through a 1.2% agarose gel.

Coliphage Isolation and Identification

Phages infecting *E. coli* strains, also known as coliphage, were further examined. Laboratory *E. coli* strains, *E. coli* C (obtained from C. Burch, University of North Carolina), *E. coli* B (Ward's, VWR), and *E. coli* K-12 (ATCC 25404), were grown from freezer stocks in 15 mL of LB media for ~18 hours. Bacterial lawns were prepared by combining 3 mL of LB soft agar (0.7% agar) with 500 ul of bacteria and spread on a 1.7% LB agar plate. Plates were left to dry for ~5 minutes. 10 ul spots of potential induced prophages from isolates 1162, 1284, 1346, and 1354 collected from the "Growth of Other Community Members in Corresponding pH and Hydrogen Peroxide Values" section. Spots were left to dry for ~10 minutes before moving the plates into the incubator at 37°C for ~18 hours. Plates were checked for plaques, indicative of phage infection and lysis of the lawned bacteria. Plaques were harvested into 1 mL of LB media and disrupted using a cell disruptor for two minutes. Harvests were then spun at 11,000 xg for two minutes. The supernatant was removed and filtered using a 0.22 um cellulose acetate syringe filter. The phage filtrate was stored at 4°C for future use.

The filtrate was used in PCR to try to identify induced phages. Each filtrate was DNased following the OPTIZYME DNase I Fisher Bioreagent's protocol. The filtrate was then heated for 10 minutes at 95°C for 10 minutes to burst the phage capsid. PCR reactions were set up by combining 12.5 ul of goTaq, 0.5 ul of forward primer, 0.5 ul of reverse primer, 11.5 ul lysate. PCR products were run through a 1.2% agarose gel.

To gain a higher titer of phage for DNA extraction and sequencing, pour plates of phage filtrate and bacterial host were created. Briefly, 100 ul of phage filtrate was mixed with 3 mL LB soft agar (0.7% agar) and 500 ul of the laboratory *E. coli* strain on which it plaqued. This mixture was spread atop a LB agar plate (1.7% agar). Plates were left to dry for ~5 minutes than placed in

an incubator at 37°C overnight, ~18 hours. Pour plates were harvested in 1 mL of LB media. Harvests were disrupted for two minutes then spun at 11,000 xg for two minutes. The supernatant was removed and filtered using a 0.22 um cellulose acetate syringe filter. The filtrate was stored at 4°C for DNA extraction. (Pour plates yielding no plaques were noted and discarded.) Prior to DNA extraction, the phage filtrate was grown in liquid culture with its susceptible host (the *E. coli* strain it plaqued on). Briefly, 3 mL of fresh media was mixed with 1 mL of the laboratory host *E. coli* from an overnight culture (as described above) and 100 ul of the filtrate and placed in a shaking incubator at 120 rpm and 37°C for ~18 hours. 1 mL was removed from the culture and transferred to a microcentrifuge tube. The culture was spun down, then the supernatant was removed and filtered through a 0.22 um cellulose acetate syringe filter. These samples were stored at 4°C for DNA extraction.

The lysate was extracted using the Qiagen UltraClean Microbial DNA Extraction kit with one modification. In the first step, 280 ul of phage lysate, 300 ul of PowerBead Solution, and 50 ul of Solution SL were aliquoted into a PowerBead tube and heated at 70º C for 10 minutes. The protocol was continued from step 4 onward. DNA was quantified using a Qubit Fluorometer. 20 ul PCR reactions were performed by combining 1 ul of this DNA, 0.5 ul of the forward primer (5 mM), 0.5 ul of the reverse primer (5 mM), 10.5 ul of goTaq and 7.5 ul of nuclease free water. After thermal cycling, PCR products were run through a 1.2% agarose gel.

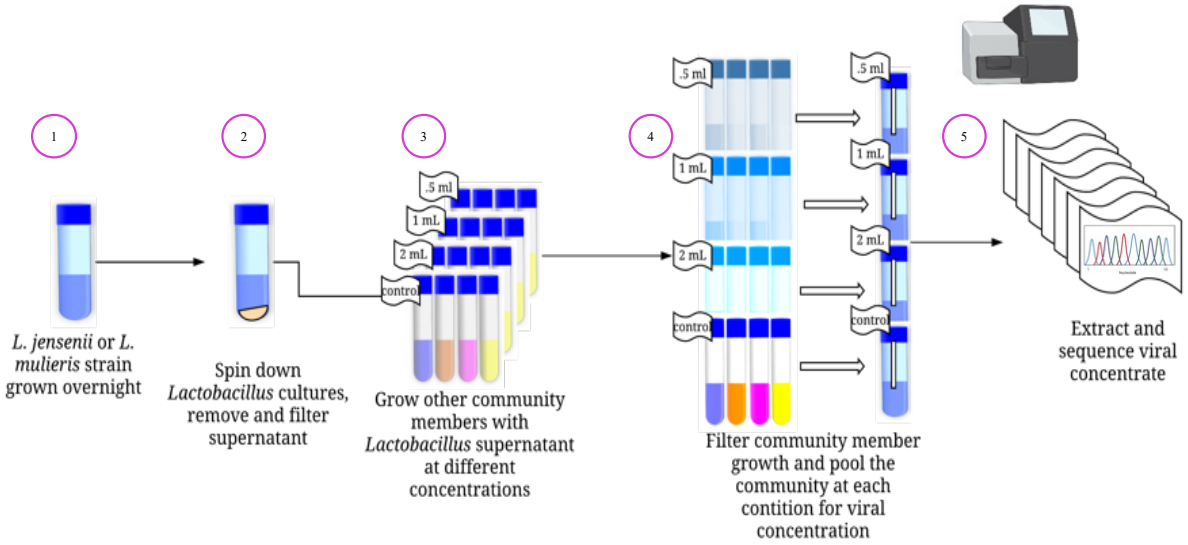


Figure 3. Process For Growing and Sequencing Phages Induced by *Lactobacillus* Spent Media. Overview of process done in "Growth of Other Community Members in Spent Lactobacillus Media", "Concentrating Viral Particles", and "Phage DNA Extraction and Amplification."

Growth of Other Community Members in Spent *Lactobacillus* Media

L. jensenii and *L. mulieris* strains from each community were grown in four 15 mL tubes of MRS + 1% tween 80 media for ~20 hours in 5.0% CO_2 at 35°C (Figure 2, step 1). *Lactobacillus* cultures were spun at 10,000 x g for ~5 minutes and the supernatants for cultures of the same strain were pooled and filtered through a 0.22 um cellulose acetate filter (Figure2, step 2). This filtrate represents the spent media from the *Lactobacillus* cultures. Other community members were grown in 15 mL of their respective media for ~18 hours in 5.0% CO_2 at 35°C with the exception of *Corynebacterium* strains, which were grown for ~36 hours.

Community members were grown under four experimental conditions of different volumes of *Lactobacillus* spent media (Figure 2, step 3). The experimental groups were set up in the following manner:

A. 0.5 mL of *Lactobacillus* spent media: 1 mL bacterial culture (from community member) + 0.5 mL *Lactobacillus* spent media + 1 mL media + 1.5 mL nuclease free water
B. 1 mL of *Lactobacill*us spent media: 1 mL bacterial culture (from community member) +1 mL *Lactobacillus* spent media + 1 mL media + 1 mL nuclease free water
C. 2 mL of *Lactobacillus* spent media: 1 mL bacterial culture (from community member) +2 mL *Lactobacillus* spent media + 1 mL nuclease free media
D. Control: 1 mL bacterial culture (from community member) + 1 mL media + 2 mL nuclease free water

Cultures were grown for ~18 hours in 5.0% CO_2 at 35°C.

Concentrating Phage Particles

Cultures from bacteria belonging to the same community were pooled by experimental group and filtered using a 0.22 um cellulose acetate filter (Figure 2, step 4). Macrosep tubes (Pall; Omega Membrane 100K) tubes were used to concentrate the filtrate using the following protocol:

Macrosep tube preparation-

1. Add 10 mL of 70% EtOH to each tube and spin at 4000 xg for 10 to 25 minutes (until most of the liquid has passed through the membrane).
2. Discard flow through and carefully remove any remaining EtOH without scratching the filter.
3. Add 10 mL of nuclease free water to each tube and spin for 10 minutes at 4000 xg.
4. Discard flow through and carefully remove remaining water without scratching the filter.
5. Add 10 mL of fresh 5% tween 20 to each tube. Incubate tubes for an hour at room temperature.
6. Discard the tween 20 from the tube by pipetting it out without scratching the filter.

7. Add 300 ul of nuclease free water to the tube. Mark the water level on the exterior of the tube.
8. Add 6 mL of nuclease free water and wash the filter by pipetting the water up and down.
9. Discard the water by pipetting it out of the tube without scratching the filter.
10. All 10 mL of nuclease free water. For immediate use, spin for 10 minutes at 4000 xg, discard the flow through, and remove any remaining water. Otherwise, add 10 mL of nuclease free water to each tube and store at 20°C for up to one week.

Processing samples using the Macrosep tubes (Figure 2, step 4)-

1. Add 10 mL of sample to the Macrosep tube.
2. Spin at 4000 xg until ~300 ul of sample has flowed through the filter, approximately 5 minutes. Discard flow through.
3. Repeat steps 1 and 2 until all of the sample has been loaded into the tube.
4. Continue to spin at 4000 xg until 300 ul remains in the tube.
5. Gently scrape filter with a pipet tip and remove concentrate from the tube. Transfer to a clean microcentrifuge tube for future processing.

Phage DNA Extraction and Amplification

Viral concentrates were DNased following the OPTIZYME DNase I Fisher Bioreagent's protocol. Viral DNA was extracted from the concentrates using the Zymo Research *Quick*-DNA Viral DNA Kit following the manufacturer's protocol. Viral genomic DNA was amplified for sequencing using the Qiagen REPLI-g Single Cell Kit (Communities 1 and 3) or the Sigma GenomePlex Single Cell Whole Genome Amplification kit (Communities 2, 4, 5, 6, 7, and 8). The respective manufacturer's protocols were followed for amplification. Products from the Qiagen REPLI-g Single Cell kit were cleaned for sequencing using the Qiagen Qamp DNA Mini

Kit, following the manufacturer's protocol. Samples from the Sigma GenomePlex Single Cell Whole Genome Amplification Kit were cleaned for sequencing using the Omega Bio-tek E.Z.N.A Cycle Pure Kit, following the manufacturer's protocol. Samples were sequenced using the Illumina NextSeq 550 platform at MIGS as previously described.

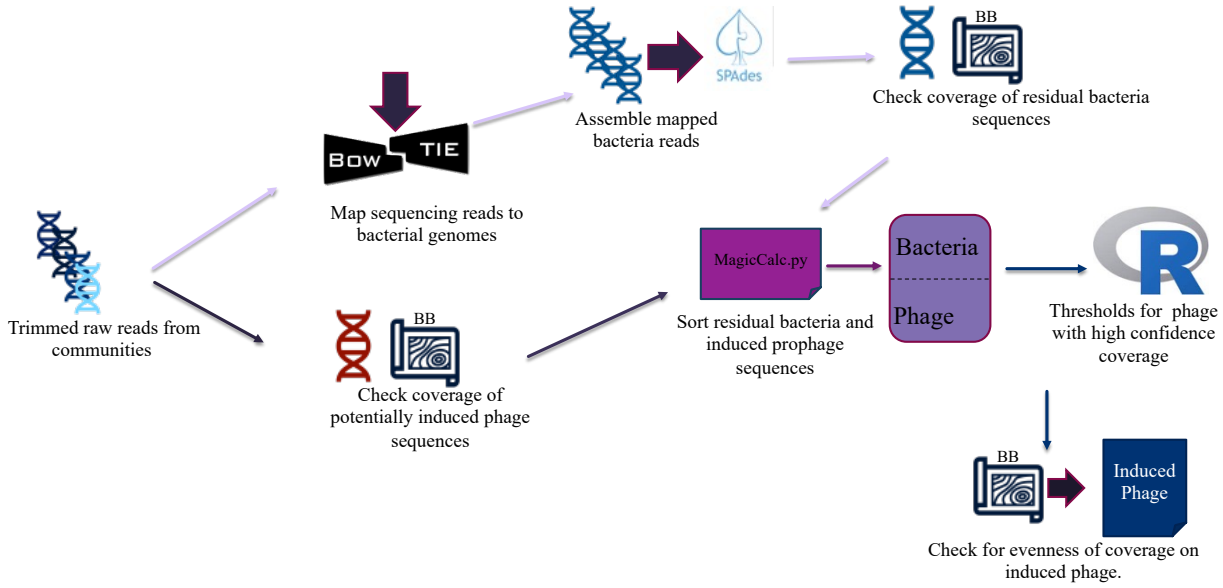


Figure 4. Computational Pipeline to Identify Induced Phages. Overview of process in "Genome Assembly and Phage Identification"

Genome Assembly and Phage Identification

Figure 4 outlines the process of identifying induced phages. First, raw reads from the Illumina sequencing were trimmed via Sickle using the paired ends, sanger and length threshold equals 100 options.

For each bacterial strain in a given community, a Bowtie index was created using the bacterial genome sequence assembly. The trimmed reads from each community's experimental group were mapped to each member of the community's bacterial index using Bowtie2 v 2.2.0

with the local option. In doing this, we could separate the reads representative of each community member present in the pooled sequencing samples. All reads that mapped to a given bacterial index were then assembled using SPAdes using the only-assembler option with specified k values of 55, 77, 99, 127. To determine the coverage of each of the produced contigs, the trimmed reads were then mapped to the assembled contigs using BBmap (https://jgi.doe.gov/data-and-tools/bbtools/) with a kfilter of 22, subfilter of 15, and maxindel of 88. For each bacterial strain in a given community, all of the Phaster and VirSorter phage predictions were concatenated into a single file. The trimmed reads for the community were then mapped to this file to compute the coverage using BBmap (https://jgi.doe.gov/data-and-tools/bbtools/) with the same parameters as above.

These results were run through a series of custom python scripts to categorize each contig as representative of a prophage region or representative of another sequence in the bacterial genome. This script produced a file listing the contig name and coverage value (from the prior BBmap analyses) for both prophage and bacterial contigs. Next, an R script was written to determine which sequences that mapped to a prophage sequence were representative of an induced prophage. Briefly, the output file produced by the python scripts was read into R. For each isolate at each concentration, the values for the bacteria coverages were used to create a bootstrap sample of size 10,000, allowing for a more robust analysis. A threshold at the 99% mark of this data set was determined. Prophage contigs with abundances at this threshold or higher, i.e., sequenced at a depth greater than or equal to sequencing of non-prophage regions of the bacterial genome, were selected and used in further analysis.

These prophage contigs were then screened further to reduce false positive results. We excluded prophage contigs without evenness of coverage, i.e., prophage contigs with a high

average coverage due to a single gene/ region. Phage contigs with an average coverage less than or equal to 1 were removed. Next, the percentage of a phage contigs represented by reads in the assembly was calculated using BBmap. BBmap was run using the file of concatenated predicted prophage sequences for each bacterium as the reference with the basecov option. Prophage contigs with 90% or more of the bases covered were classified as induced phage, while those below 90% were discarded. Finally, the bacterial and phage coverage values for each isolate, at each condition, were combined to make a distribution. Using this distribution, an empirical probability for each phage identified as induced from the previous analysis was determined. Phage contigs with a p-value of 0.01 or less were identified as an induced phage with confidence. This analysis was completed in R.

CHAPTER THREE

RESULTS

Community Structure and Community Members

Eight communities of bacteria were isolated from the urine samples of eight individual female patients. Each community contains either a *L. jensenii* or *L. mulieris* strain. The additional bacteria within these samples vary by community, and they represent the diversity of bacteria found within the female urinary microbiome. Community member taxa were determined via 16S PCR and verified through whole genome sequencing. Table 1 shows the ID number and taxonomic classification for each community member as well as the medium used to grow each member. Community members with an ID number that contains a decimal point, and a letter were isolated through further purification upon arrival at the Putonti lab.

Out of the eight total communities, the smallest contains three members (community 3) and the largest contains seven members (community 4). The average size of the communities is 4.75. The *Lactobacilli* of interest in communities 1, 2, 3, 4, 6, and 8 are *L. jensenii* strains, while communities 5 and 7 contain *L. mulieris* strains. These two Lactobacillus species are closely related, with *L. mulieris* being reclassified as its own species in May 2020 (Rocha *et al.* 2020), after the inception of this project.

Community	***L. jensenii* or *L. mulieris* Strain Number**	**Community Member Number**	**Community Member Taxa**	**Growth Medium**
1	37	37	*Lactobacillus jensenii*	MRS + tween
		38	*Proteus mirabilis*	LB

		38.2I	*Micrococcus luteus*	TSB
		39.1M	*Staphylococcus epidermidis*	TSB
2	847	835	*Staphylococcus epidermidis*	TSB
		839	*Streptococcus anginosus*	BHI
		843	*Enterococcus faecalis*	BHI
		847	*Lactobacillus jensenii*	MRS + tween
3	1165	1162	*Escherichia coli*	LB
		1163	*Lactobacillus crispatus*	MRS NBCS
		1165	*Lactobacillus jensenii*	MRS + tween
4	1303	1284	*Escherichia coli*	LB
		1295	*Actinomyces neuii*	Actinomyces
		1296.1T	*Staphylococcus hominis*	TSB
		1303	*Lactobacillus jensenii*	MRS + tween
		1309	*Enterococcus faecalis*	BHI
		1310.1E	*Corynebacterium amycolatum*	LB
		1310	*Proteus mirabilis*	LB
5	1355	1346	*Escherichia coli*	LB
		1353.1Y	*Streptococcus anginosus*	BHI
		1354	*Escherichia coli*	LB
		1355	*Lactobacillus mulieris*	LB
6	7766	7765	*Staphylococcus epidermidis*	TSB
		7766	*Lactobacillus jensenii*	MRS + tween
		7768	*Streptococcus anginosus*	BHI
		7769	*Corynebacterium aurimucosum*	LB
7	7784	7779	*Klebsiella pneumoniae*	LB
		7780	*Enterococcus faecalis*	BHI
		7781.2F	*Staphylococcus epidermidis*	TSB
		7782	*Streptococcus agalactiae*	BHI
		7783.2Q	*Aerococcus urinae*	LB
		7784	*Lactobacillus mulieris*	MRS + tween
8	8489	8490	*Corynebacterium coyleae*	LB
		8492.1R	*Corynebacterium aurimucosum*	LB
		8492	*Klebsiella pneumoniae*	LB
		8493	*Staphylococcus epidermidis*	TSB
		8489	*Lactobacillus jensenii*	MRS + tween

Table 1. Community Member Information. Community member isolate numbers, taxonomic classification, and growth medium.

These communities include species commonly found within the female urinary tract: *E. coli* (n=3), *S. epidermidis* (n=5), *S. anginosus* (n=3), and *E. faecalis* (n=3). Additionally, these communities include taxa that are found a lower frequency in the female urinary tract than the previously listed species. These species include *M. luteus* (n=1), *A. neuii* (n=1), *A. urinae* (n=1), *L. crispatus* (n=1), *C. coyleae* (n=1), *C. aurimucosum* (n=1), *S. hominis* (n=1), and *S. agalactiae* (n=1). Community 5 is the only community with two members of the same species, containing two different *E. coli* strains.

Predicted Phage – Phaster and VirSorter Comparison

The genomes of each bacterial isolate, except for *L. jensenii* and *L. mulieris* strains, were analyzed for the presence of prophage sequences using two phage prediction tools: Phaster and VirSorter. Phaster categorizes phage predictions as either Intact, Questionable, or Incomplete. VirSorter assigns predictions in categories 1 and 4 ("most confident" predictions), 2 and 5 ("likely" predictions), and 3 and 6 ("possible" predictions). Given the improbability that predictions in Phaster's Incomplete and VirSorter's category 3 and 6 are representative of viable phages, they were excluded from further consideration. Henceforth, when referring to Phaster and/or VirSorter predictions, we are referring only to Intact, Questionable, and categories 1, 2, 4, and 5, unless otherwise stated. A complete list of predictions from both tools for each genome can be found in Appendix E. Figure 5 shows the number of predicted phages within each community. Prophage regions identified by both tools are counted under "Both Predictions." In total, 119 individual phages were predicted across the communities. Community 4, the largest community (n=6, excluding *L. jensenii* 1303), contains the most phage predictions (n=30), and community 5, containing 3 community members and *L. mulieris* 1355, contains the least phage predictions (n=9). On average, each community contains about 15 predicted phages.

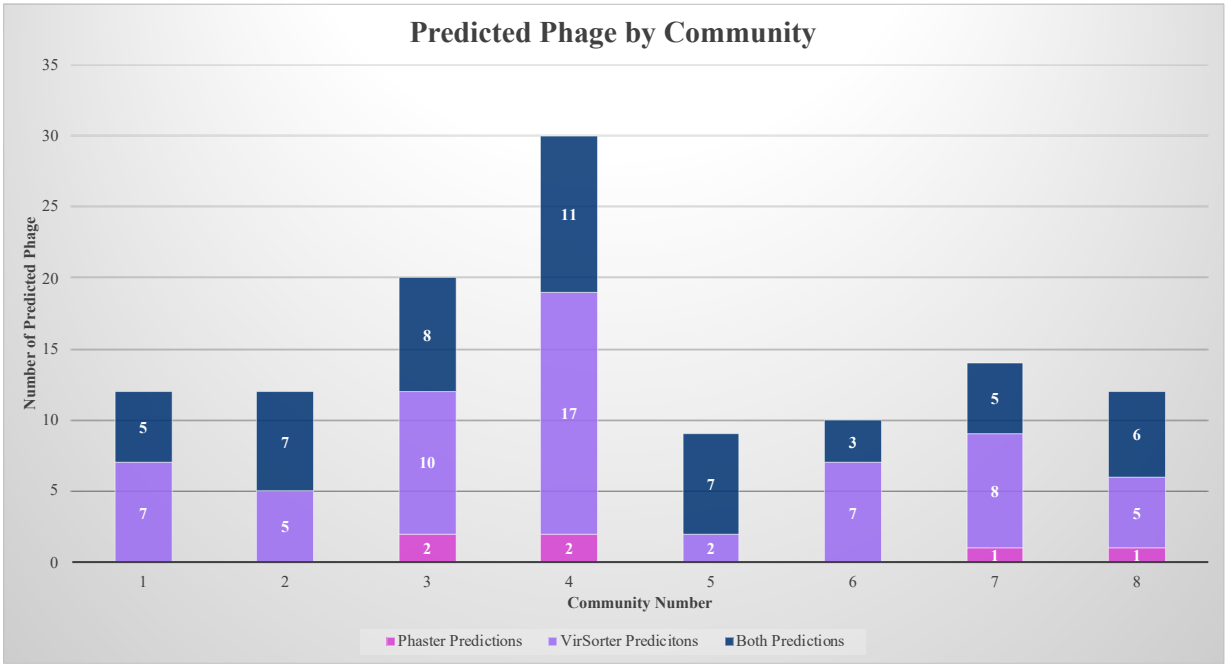


Figure 5. Predicted Phages by Community. Only Phaster predictions labeled as Intact or Questionable and VirSorter predictions in categories 1, 2, 4, and 5 were used. Overlapping predictions from the two tools are counted in "Both Predictions"

Given the variation in the number of prophages identified between communities, we further investigated the number of prophages predicted by community member. In Figure 6, the number of prophage predictions by bacterial strain is shown. The majority of isolates harbor at least one predicted phage with the exceptions being *S. agalactiae* 7782 and *C. coylea* 8490 from communities 7 and 8, respectively. Neither of these strains contained prophage predictions above the confidence threshold used here. Isolates with the most predicted phages were *E. coli* 1162 (n=14) and *E. coli* 1284 (n=15), which belong to communities 3 and 4, respectively. In contrast, the other *E. coli* strains included within the experiment (both in community 5) were only predicted to contain 3 prophages each. Four bacterial strains were only predicted to contain a single prophage. VirSorter alone predicted one prophage in strains *C. amycolatum* 1310.1E

(community 4), *C. aurimucosum* 7769 (community 6), and *A. urinae* 7783.2Q (community 7). Both Phaster and Virsorter predicted the same single prophage in *S. epidermidis* 8493 (community 8).

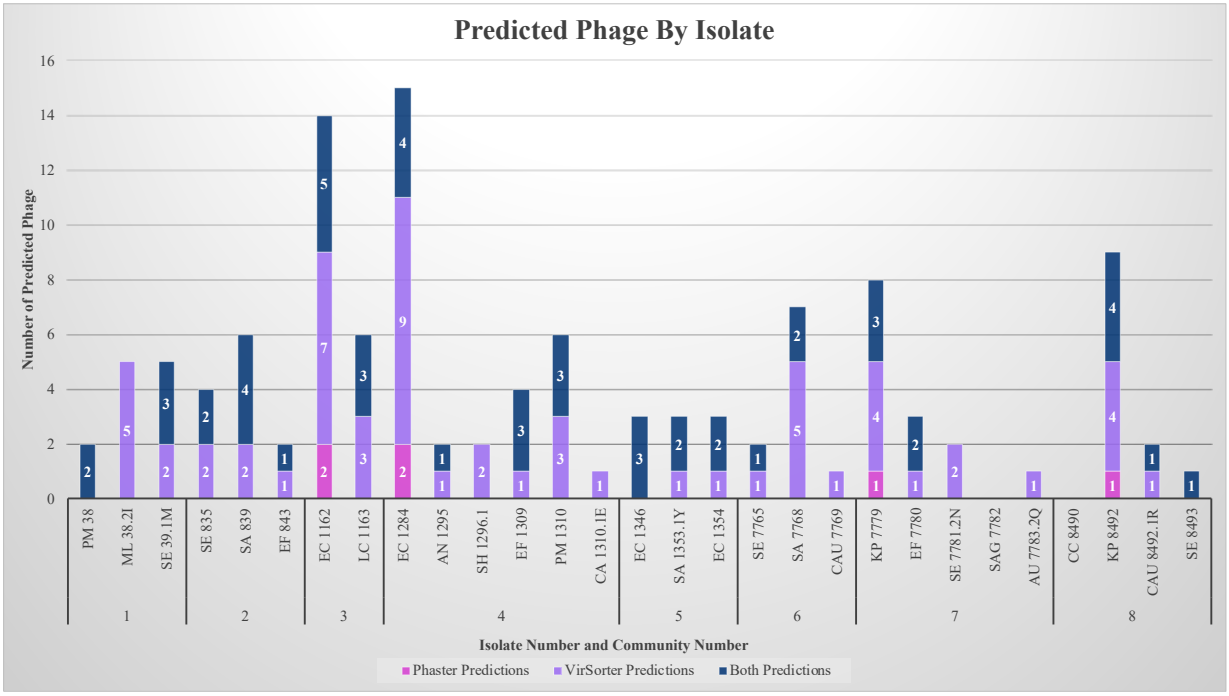


Figure 6. Predicted Phages by Isolate. Only Phaster predictions labeled as Intact or Questionable and VirSorter predictions in categories 1, 2, 4, and 5 were used. Overlapping predictions from the two tools are counted in "Both Predictions." Bacterial taxa codes: PM – *P. mirabilis*; *ML* – *M. luteus*; SE – *S. epidermidis*; SA – *S. anginosus*; EF – *E. faecalis*; EC – *E. coli*; LC – *L. crispatus*; AN – *A. neuii*; SH – *S. hominis*; CA – *C. amycolatum*; CAU – *C. aurimucosum*; SAG – *S. agalactiae*; KP – *K. pneumoniae*; AU – *A. urinae*; CC – *C. coyleae*

In Figures 5 and 6, we see that the majority (87.9%) of phages predicted by Phaster as Intact or Questionable were also predicted by VirSorter (categories 1, 2, 4, 5). There are only six phages predicted by Phaster that were not predicted or not predicted as a high confidence category by VirSorter: 2 in *E. coli* 1162 (1 Intact and 1 Questionable; community 3), 2 in *E. coli*

1284 (2 Questionable; community 4), 1 in *K. pneumoniae* 7779 (1 Questionable; community 7), and 1 in *K. pneumoniae* 8492 (1 Questionable; community 8). However, there are 61 unique prophages predicted by VirSorter into one of its 4 high confidence categories that are not predicted as Intact or Questionable by Phaster. These include: 4 Category 1, 26 Category 2, 0 Category 4, and 31 Category 5 predictions. In this case, 50.8% of these phages (n=31) were predicted as Incomplete by Phaster. Phaster failed to predict the remaining 30 phages.

Predicted Phage – Taxonomic Identification

Phaster and Virsorter predicted prophage sequences were queried against all publicly available phage genomes using blastn. Predicted prophage sequences with at least 70% query coverage and 70% percent identity to an existing phage genome were immediately kept for downstream analysis. They resemble previously characterized phages, and thus it is highly likely that they belong to the same taxonomic lineage as the previously characterized phage. A lower threshold of sequence similarity was then considered for the remaining prophage sequences in order to identify more distant relatives and/or novel phage strains. Thus, two different thresholds were considered: (1) 70% query coverage and 30-70% percent identity and (2) 30-70% query coverage and at least 70% percent identity. Predicted phages meeting one of these thresholds were assigned to the taxonomic group of the most similar characterized phage.

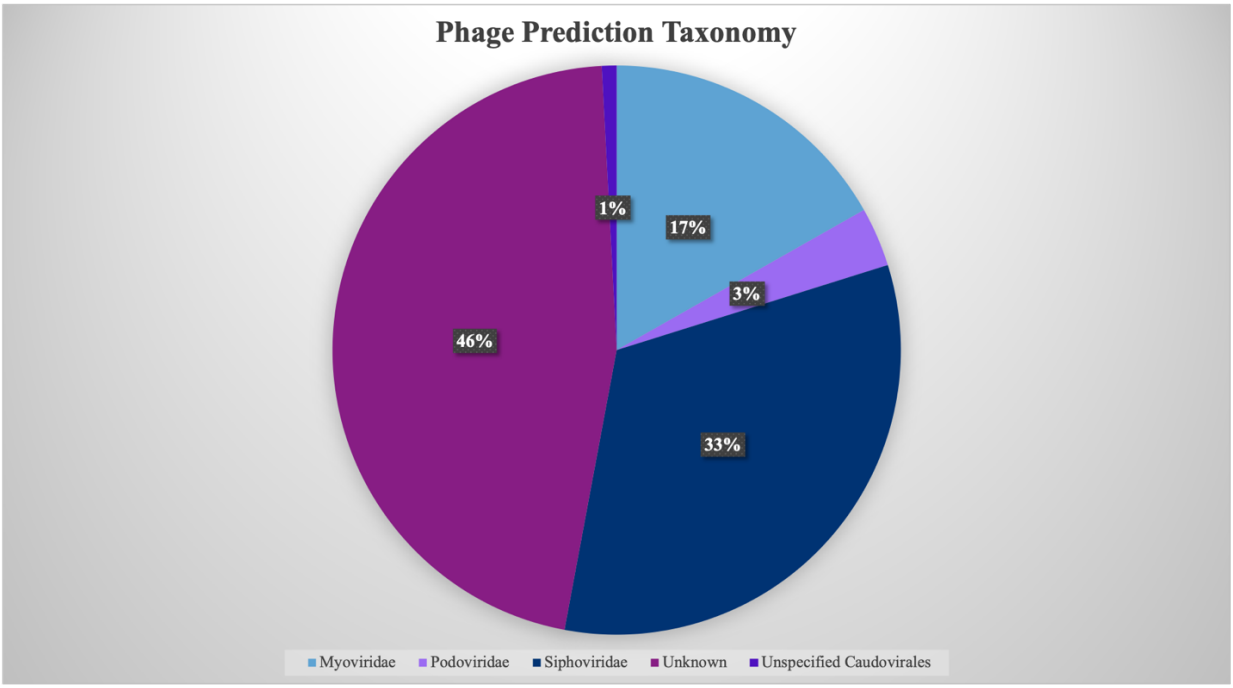


Figure 7. Predicted Phage Taxonomies. Overlapping predictions from Phaster and VirSorter were merged as one prediction for this analysis.

Many of our prophage predictions (46%, n =55) did not pass the thresholds set for the blastn query results (Figure 7) and were labeled "Unknown." Of the prophage predictions that showed significant sequence similarity to phages belonging to the Caudovirales order, 33% showed sequence similarity to the family Siphoviridae (n=39), 17% to the family Myoviridae (n=20), and 3% to the family Podoviridae (n=4). All four of the predicted podoviruses were from *E. coli* strains. 1 predicted phage exhibited sequence similarity to an unspecified Caudovirales; thus, the viral family is unknown.

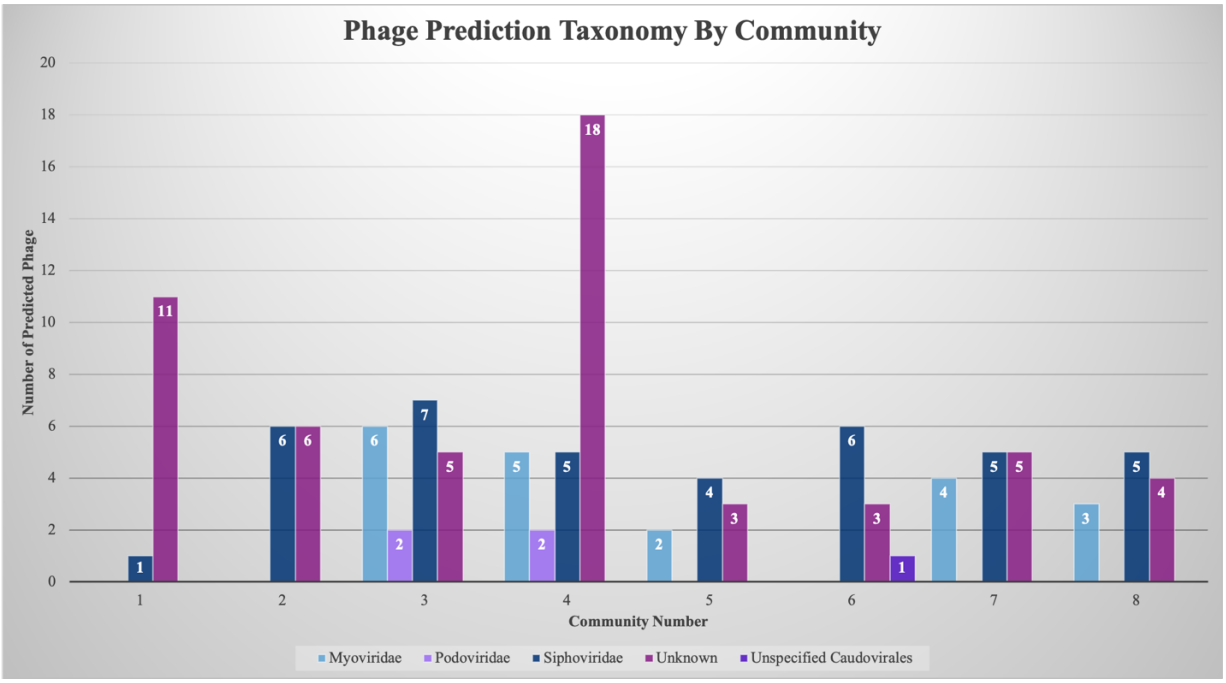


Figure 8. Predicted Phage Taxonomies by Community. Graph of taxonomy of predicted phages by community. Only Phaster predictions labeled as Intact or Questionable and VirSorter predictions in categories 1, 2, 4, and 5 were used. Overlapping predictions from the two tools were merged as one prediction for this analysis.

Taxonomic predictions were further examined by community (Figure 8). Each community has at least three predicted prophage sequences with an unknown taxonomy, with communities 1 and 4 having the most prophages of unknown taxonomic classification. The single prophage prediction classified as “Unspecified Caudovirales” belongs to Community 6. Communities 3 and 4 are the only communities to contain prophage sequences predicted to be members of the family Podoviridae. Communities 1, 2, and 6 contain no prophage sequences predicted as Myoviridae. Every community contains at least one prediction with sequence similarity to a siphovirus.

Communities 1 and 4 contain the most novel predicted prophage sequences, with 11 and 18 prophages assigned the unknown taxonomy, respectively. This points to a high number of potentially novel prophages within these two communities, relative to other communities examined here. In community 1, isolates 38 and 38.2I contain five predicted prophage sequences with unknown taxonomic classification (Appendix F). This represents all the phages predicted for these two isolates. Isolate 39.1M, also in community 1, contains one predicted prophage that cannot be assigned to a taxonomic group and one predicted prophage exhibiting sequence similarity to a Siphoviridae phage. In community 4, isolate 1284 contains the most prophage predictions, with five predicted to be representatives of Myoviridae, one predicted to be a representative of Podoviridae, five predicted to be representatives of Siphoviridae, and four predicted prophages that cannot be taxonomically classified. The prophages predicted for isolates 1295, 1296.1T, 1310, and 1310.1E did not resemble any characterized phage genomes and thus could not be assigned to any taxonomic group. Isolate 1309 contained one predicted prophage sequence exhibiting sequence similarity to a Podoviridae and three predicted prophages that could not be assigned to a taxonomic group.

Lactobacillus pH and Hydrogen Peroxide Levels

The pH and hydrogen peroxide of spent media for each of the *Lactobacillus* strains used in this study were measured in triplicate (Appendix G and H). The average of the triplicates was taken and used in further experiments. The *Lactobacillus* strains used in this project produced a range of pH and hydrogen peroxide values (Table 2). On average, the pH of spent *L. jensenii* media was 4.32, slightly lower than the *L. mulieris* average of 4.53. A Wilcoxon Test was used to test for difference between the two species; no significance was found (p-value = 0.6429). Hydrogen peroxide values between the two strains cover a wide range from 7.15 uM to 25.03 uM. *L.*

jensenii strains have values ranging from 7.15 uM to 25.03 uM, while *L. mulieris* strains range from 7.55 uM to 12.48 uM. The average hydrogen peroxide outputs from these *L. jensenii* and *L. mulieris* strains are 16.24 uM and 10.02 uM, respectively. The difference between the two species was not statistically significant (Wilcoxon Test; p-value = 0.4286). *L. jensenii* strain 1303 is the strain from this study with the lowest pH measurements and the highest hydrogen peroxide output. While there appears to be a weak negative correlation between pH and hydrogen peroxide outputs for the strains used in this study (Figure 9), data from more strains would need to be collected to make a generalization comparing the two species.

Community	***Lactobacillus* Species**	**Strain Number**	**Average pH**	**Average H_2O_2 (uM)**
1	*L. jensenii*	37	4.44	8.89
2	*L. jensenii*	847	4.25	20.26
3	*L. jensenii*	1165	4.28	16.97
4	*L. jensenii*	1303	3.85	25.03
5	*L. mulieris*	1355	4.27	12.48
6	*L. jensenii*	7766	4.69	7.15
7	*L. mulieris*	7784	4.78	7.55
8	*L. jensenii*	8489	4.40	19.11

Table 2. *L. jensenii* and *L. mulieris pH* and Hydrogen Peroxide outputs

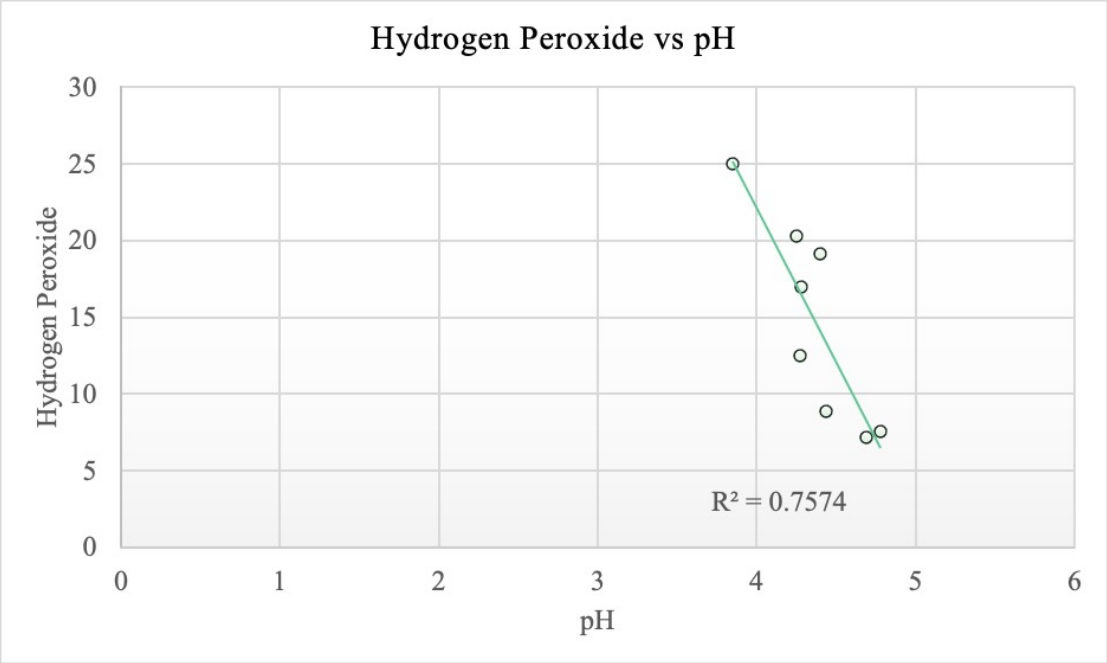


Figure 9. Hydrogen Peroxide vs pH Output

Predicted *Lactobacillus* Secondary Metabolites and Bacteriocins

***Lactobacillus* Strain Number**	**antiSMASH Predictions**	**BAGEL4 Predictions**
37	NRPS	Enterolysin_A
847	NRPS	none
1165	NRPS	none
1303	NRPS	none
1355	Lantipeptide class IV/L	Lantipeptide class IV/L
7766	NRPS	none
7784	RiPP-like: TIGR01193	Brochocin_C_BrcB, Enterocin_NKR-5-3A, Enterocin_NKR-5-3D
8489	none	Enterolysin_A

Table 3. Predicted Lactobacillus Secondar Metabolites and Bacteriocins. Predicted bacteriocins from *L. jensenii* and *L. mulieris* strains. Predictions were made by two tools: antiSMASH and BAGEL4.

In addition to changing the pH and hydrogen peroxide levels of their environment, prior studies have identified secondary metabolites and bacteriocins produced by lactobacilli that are excreted into the surrounding environment (Zacharof and Lovitt 2012). Secondary metabolites and bacteriocins were predicted from the genome sequences of the *L. jensenii* and *L. mulieris* strains using antiSMASH and BAGEL4 (Table 3). antiSMASH predicted NRPS (nonribosomal peptide synthetase proteins) in most of the *Lactobacillus* strains. BAGEL4 did not identify any products in four of these strains, *L. jensenii* 847, *L. jensenii* 1165, *L. jensenii* 1303, and *L. jensenii* 7766. antiSMASH and BAGEL4 concurred in their prediction of a class IV lantipeptide in *L. mulieris* 1355. antiSMASH predicted *L. mulieris* 7784 produces a ribosomally synthesized and post-translationally modified peptide product (RiPP)-like structure: TIGR01193. TIGR01193 (Lu *et al*. 2020) is an ABC-type bacteriocin transporter. For this same strain BAGEL4 identified three bacteriocins: Brochocin C, Enterocin NKR-5-3A, and Enterocin NKR-5-3D. Enterocin NKR-5-3A and NKR-5-3D were originally isolated from *E. faecalis* and have bactericidal effects against *Enterococcus, Lactobacillus, Bacillus* and *Listeria* (Ishibashi *et al.* 2012). Brochocin C was first classified as a bacteriocin produced by *Brochothrix campestris* (Siragusa and Cutter 1993). This bacteriocin inhibits the growth of gram-positive bacteria. BAGEL4 predicted *L. jensenii* 37 and *L. jensenii* 8489 to contain an Enterolysin A. Enterolysin A was originally characterized from *E. faecalis* and is known to degrade the cell wall of enterococci, lactococci, and lactobacilli (Nilsen *et al.* 2003).

Phage Induction by pH

In an effort to assess the occurrence of phage induction due to *Lactobacillus*-relevant pH levels, community members were grown individually in their respective media at the same acidic pH as the spent media of the *Lactobacillus* strain from their community. Serving as a control,

community members were also grown individually in their respective media without pH adjustment (pH = 7). The control is critical to ascertaining if induction occurs in response to the pH level likely to occur in a *Lactobacillus* environment or if the prophage is prone to spontaneous induction under standard laboratory conditions. Lysates from these cultures were screened for the presence of induced phages via PCR (Table 4). Phage induction was observed in four of the 29 samples grown in the adjusted media (Table 4, highlighted in green). In *A. neuii* 1295 (community 4) and *E. faecalis* 7780 (community 7), induction was observed for all three replicates tested. For *E. coli* 1354 (community 5), two different phages were detected within one replicate. There was no evidence of phage induction at the lower pH for the other two replicates. For all isolates used in this study, there were no positive PCR results to indicate phage induction in the control lines.

Community	Isolate	Primer ID	pH Group (r1/r2/r3)	Control Group (r1/r2/r3)
1	38.2I	node 9	-/-/-	-/-/-
1	38.2I	node 97	-/-/-	-/-/-
1	39.1M	node 1	-/-/-	-/-/-
1	39.1M	node 6	-/-/-	-/-/-
1	38	node 1	-/-/-	-/-/-
1	38	node 3	-/-/-	-/-/-
1	38	node 10	-/-/-	-/-/-
2	843	node 3	-/-/-	-/-/-
3	1162	node 17	-/-/-	-/-/-
3	1162	node 29	-/-/-	-/-/-
3	1162	node 2	-/-/-	-/-/-
3	1162	node 3	-/-/-	-/-/-
3	1162	node 6	-/-/-	-/-/-

3	1163	node 5	-/-/-	-/-/-
3	1163	node 24	-/-/-	-/-/-
3	1163	node 16	-/-/-	-/-/-
3	1163	node 13	-/-/-	-/-/-
3	1163	node 1	-/-/-	-/-/-
4	1284	node 33	-/-/-	-/-/-
4	1284	node 36	-/-/-	-/-/-
4	1284	node 13	-/-/-	-/-/-
4	1284	node 9	-/-/-	-/-/-
4	1284	node 26	-/-/-	-/-/-
4	1284	node 6	-/-/-	-/-/-
4	1295	node 3	+/+/+	-/-/-
4	1296.1T	node 21	-/-/-	-/-/-
4	1296.1T	node 2	-/-/-	-/-/-
4	1309	node 1a	-/-/-	-/-/-
4	1309	node 1b	-/-/-	-/-/-
4	1309	node 15	-/-/-	-/-/-
4	1310	node 3	-/-/-	-/-/-
4	1310	node 34	-/-/-	-/-/-
4	1310	node 54	-/-/-	-/-/-
4	1310.1E	node 12	-/-/-	-/-/-
5	1346	node 2	-/-/-	-/-/-
5	1346	node 3b	-/-/-	-/-/-
5	1353.1Y	node 3	-/-/-	-/-/-
5	1353.1Y	node 13	-/-/-	-/-/-
5	1354	node 2	-/-/+	-/-/-
5	1354	node 6	-/-/+	-/-/-
5	1354	node 7	-/-/-	-/-/-
6	7765	node 11	-/-/-	-/-/-
6	7765	node 1a	-/-/-	-/-/-
6	7765	node 1b	-/-/-	-/-/-

6	7768	node 3	-/-/-	-/-/-
6	7768	node8	-/-/-	-/-/-
6	7768	node 9	-/-/-	-/-/-
6	7768	node 11	-/-/-	-/-/-
7	7780	node 3	+/+/+	-/-/-
7	7780	node 9	-/-/-	-/-/-
7	7781.2F	node 7	-/-/-	-/-/-
7	7779	node 3	-/-/-	-/-/-
7	7779	node 14	-/-/-	-/-/-
7	7779	node 58	-/-/-	-/-/-
7	7779	node 67	-/-/-	-/-/-
7	7783.2Q	node 10	-/-/-	-/-/-
8	8492	node 16	-/-/-	-/-/-
8	8492	node 54	-/-/-	-/-/-
8	8492	node 57	-/-/-	-/-/-
8	8493	node 2	-/-/-	-/-/-

Table 4. Phage Induction by pH. PCR results to check for phage induction by pH. Cells highlighted in green show positive results. Cells highlighted in blue did not have positive results, but there was evidence of an induced phage(s) at the top of the PCR wells.

L. crispatus 1163, highlighted in blue, showed evidence of phage induction without a positive PCR result (Table 4). When the PCR products were run through an agarose gel, there was no band at the expected amplicon size, indicative of a positive PCR result. However, the wells where the samples were loaded fluoresced when viewed using a UV light, indicating the presence of DNA. I believe that a phage was induced, but the primers selected and prepared for this analysis failed to capture the induced phage.

Phage Induction by *Lactobacillus* Spent Media

Community members were grown in three different volumes of spent media from their corresponding *Lactobacillus* strain. After 18 hours of incubation, all of the isolates in a community at a certain concentration were pooled, filtered, and their DNA was extracted. DNA was amplified before sequencing. Sequencing results were run through a custom pipeline of python and R scripts to identify induced phages, as described in the Methods (Figure 4). Prophages that were sequenced at an abundance greater than the bacterial genome (99th percentile or greater) and sequenced evenly (90% base coverage threshold) were characterized as induced phage (Table 5). For this analysis, Phaster and VirSorter predictions from all categories were used.

Community	Community Member Number	Phage	0.5mL	1mL	2mL	Control
1	38	Node 3 (Phaster 2)	yes*		yes	yes
		Node 3 (Phaster 3)	yes	yes*		yes*
		Node 52	yes			yes*
		Node 10	yes			yes
		Node 1	yes*	yes*	yes*	yes*
	39.1M	Node 1	yes*	yes*	yes*	
2	835	Node 85	yes	yes	yes	yes*
		Node 2	yes	yes*	yes*	yes*
		Node 65				yes
	839	Node 26	yes	yes	yes	yes
		Node 1	yes	yes	yes	yes
		Node 27	yes			
		Node 4	yes			yes
	843	Node 12	yes	yes	yes	yes
		Node 3	yes	yes	yes	yes*
		Node 2	yes	yes	yes	yes

		Node 1	yes		yes	
3	1162	Node 17	yes*	yes*	yes*	yes*
	1163	Node 16		yes	yes*	
4	1284	Node 62	yes	yes	yes	yes
		Node 65	yes	yes		yes
		Node 6	yes*	yes	yes	yes
		Node 14	yes	yes		yes
		Node 67	yes	yes		yes
	1296.1T	Node 2				yes
	1309	Node 10	yes*	yes*	yes	yes
		Node 15	yes*	yes*	yes*	yes*
		Node 1 (Phaster 1)	yes*	yes*	yes*	yes*
		Node 1 (Phaster 2)	yes	yes	yes	
		Node 1 (Phaster 3)	yes	yes	yes	
		Node 1(Phaster 4)		yes*	yes	
		Node 2		yes		
		Node 3	yes*	yes*	yes	
		Node 18		yes	yes	
	1310	Node 3	yes*			
5	1346	Node 2+	yes*	yes	yes	yes*
		Node 3+	yes	yes		yes*
		Node 20	yes	yes*	yes*	yes
	1353.1Y	Node 21				yes
		Node 13	yes	yes	yes	yes*
	1354	Node 2+	yes	yes		yes*
		Node 6+	yes*	yes*	yes*	yes*
		Node 7		yes		
6	7765	Node 6	yes*	yes*	yes*	
		Node 30	yes		yes*	
		Node 11	yes		yes*	
		Node 41			yes	
		Node 1			yes	
		Node 19			yes	
	7768	Node 32	yes	yes	yes	yes
		Node 33	yes	yes	yes	yes
		Node 34		yes		

		Node 3	yes*	yes*	yes*	yes*
		Node 9		yes		
		Node 1	yes	yes		
		Node 8				yes*
		Node 14				yes
		Node 29				yes
	7769	Node 25	yes*	yes*		yes
		Node 15	yes	yes*		yes
7	7779	Node 115		yes		yes
		Node 100	yes	yes		yes
		Node 140		yes		yes
		Node 58		yes		yes
		Node 67	yes*	yes*	yes	yes*
		Node 56				yes
		Node 3				yes*
		Node 6				yes*
		Node 47				yes
		Node 11				yes
	7780	Node 28	yes	yes	yes	yes
		Node 23	yes			yes
		Node 1		yes		yes
		Node 3	yes*	yes*	yes*	yes*
		Node 9	yes	yes	yes*	yes*
		Node 10		yes		yes
		Node 21		yes	yes	yes
	7781.2F	Node 7	yes			
		Node 10	yes*			
	7783.2Q	Node 38		yes		yes
		Node 1		yes		yes
		Node 10	yes	yes*	yes*	yes*
		Node 31			yes	yes
		Node 6			yes	
		Node 19			yes	
		Node 40				yes
8	8492.1R	Node 6	yes	yes	yes	yes
		Node 5	yes	yes	yes	yes

	8493	Node 30		yes		

Table 5. Phage Induction by *Lactobacillus* Spent Media. Table of phages induced by *Lactobacillus* spent media. 0.5 mL, 1 mL, 2 mL, and Control refer to the volume of spent *Lactobacillus* media added to the culture. Isolates with no induced phage are not listed. Phage with an asterisks were considered significant after the empirical probability analysis (alpha = 0.01) . Phage with a + are potentially two predictions to different regions of the same phage predicted within a given isolate.

Overall, 23 isolates had at least one phage induced at at least one condition. In total, 106 individual phages were induced across the 23 isolates. The condition with the highest number of induced phages was the control group (n=47), followed by 1 mL (n=68), 0.5 mL (n=64), and 2 mL (n=54).

The induced phages were further examined to determine if their coverages (representative of their abundances) were significantly different than the other coverages within the same sequence data. A distribution for each isolate at each condition was created. This included any bacterial contigs as well as each of the predicted prophage sequences. The coverages for each of these sequences was used for an empirical probability test, checking for the significance of the coverage values for each putative induced phage listed in Table 5. All statistics were completed in R. An alpha of 0.01 was used to determine significance. The lower a p-value is, the more abundant the prophage was in the sample (Appendix J). Communities 3 and 6 were selected for further examination. For each isolate with phage induction found to meet our significance threshold (alpha = 0.01), the distribution was graphed. The induced phages were added to the plot based upon where their coverage fell on the distribution. Isolate 7769 from community 6 was not included in this analysis as there were no residual bacterial contigs identified in the sequencing results for the control group.

In community 3, isolate 1162 had one phage, Node 17, that met our significance threshold (alpha = 0.01) at every condition (Figure 10). While the distributions for each condition differ based upon the coverages of the residual bacterial contigs and the predicted prophage sequences, the p-values from the empirical probability analysis enable us to compare prophage abundance (prevalence of induction) across samples for the same isolate. Node 17 is the only phage from isolate 1162 that met this threshold (alpha = 0.01). Since this phage was induced at the three experimental conditions and the control, it is likely that the prophage is spontaneously induced rather than induced as a result of stress due to the *Lactobacillus* spent media.

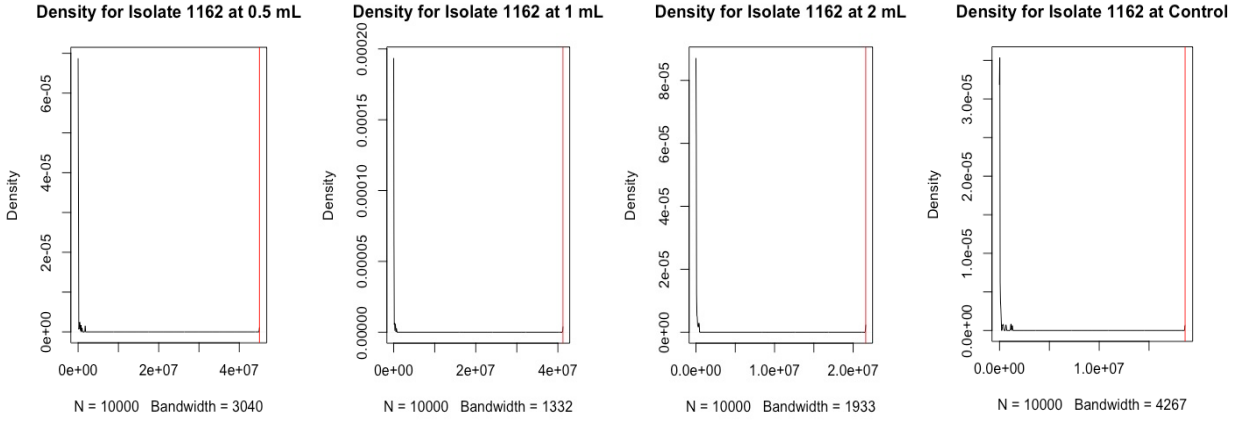


Figure 10. Density plots for isolate *E. coli* 1162 (Community 3). Phage Node 17, induced from 1162, is plotted in red.

Isolate 1163, also from Community 3, showcases a different scenario (Figure 11). One phage, Node 16, was induced from isolate 1163 at the 1 mL spent media and 2 mL spent media conditions. However, when the empirical probability analysis was applied, only the induction

event at the 2 mL spent media condition was considered significant. However, these events show a case in which the phage was not induced in the control conditions. The Node 16 phage was only induced in the presence of *Lactobacillus* spent media. However, since the phage was only significantly induced at the 2 mL spent media condition, we see that a higher volume of *Lactobacillus* metabolites are necessary to create enough stress to warrant the induction of phage Node 16 from isolate 1163.

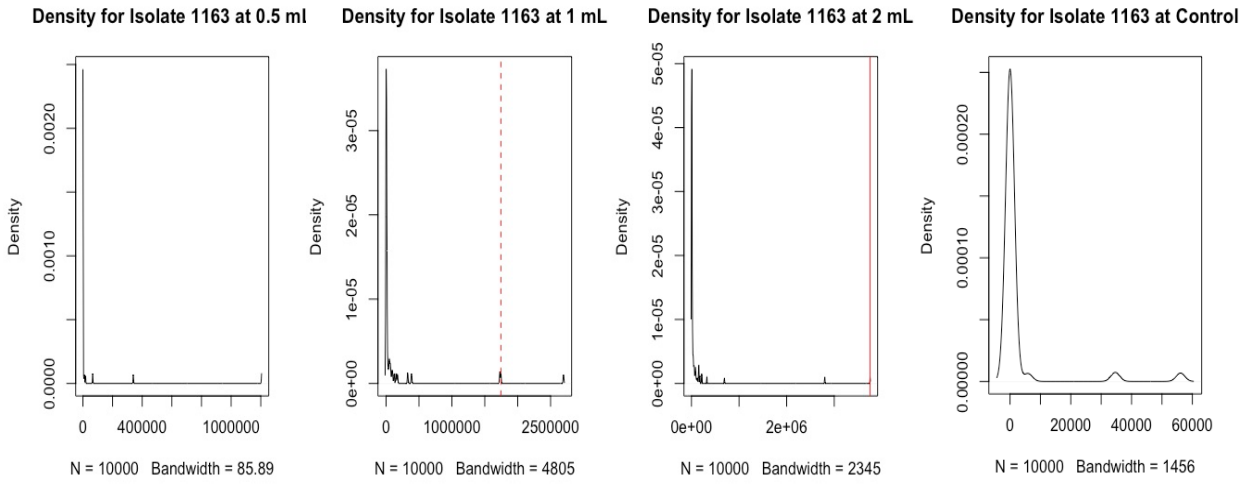


Figure 11. Density plots for isolate *L. crispatus* 1163 (Community 3). Phage Node 16, induced from 1163, is plotted in red. The solid line indicates induction that was significant after the empirical probability analysis. The dashed line indicates evidence of induction that did not pass the empirical probability analysis.

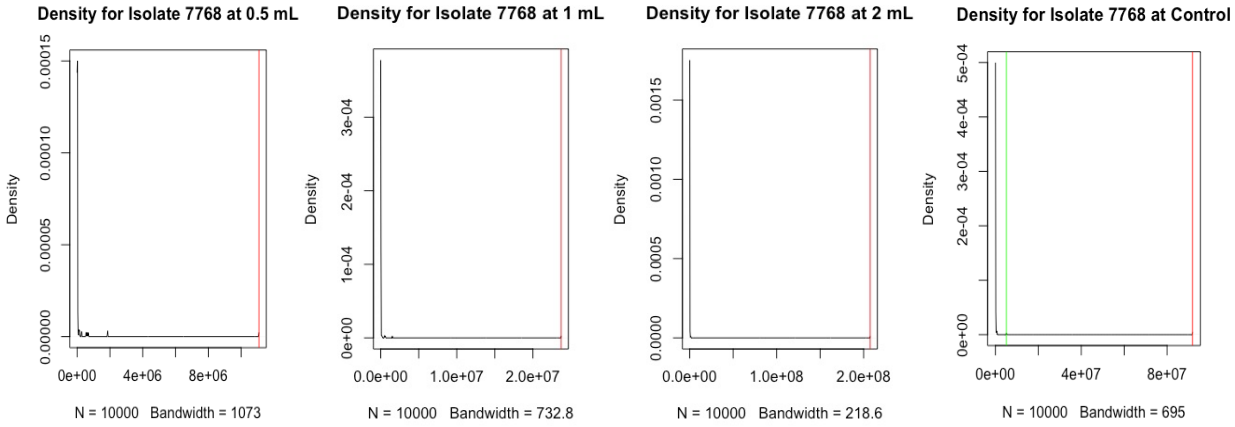


Figure 12. Density plots for isolate *S. anginosus* 7768 (Community 6). Phage Node 3 is plotted in red. Phage Node 8 is plotted in green.

Community 6 was also further examined. In isolate 7768 we saw the phage Node 3 induced at all conditions, including the control (Figure 12). Much like the phage Node 17 from isolate 1162, we believe this phage is also the product of spontaneous induction. However, from isolate 7768, we also saw phage Node 8, which only met our threshold (alpha = 0.01) and thus was only induced under the control conditions. This indicates that the conditions created by the *Lactobacillus* spent media are not stressful enough to induce this phage, but the control conditions cause stress to the host and thus trigger the prophage's induction.

In isolate 7765, we saw the opposite event occur (Figure 13). All three phages induced from this isolate were only detected in the samples treated with *Lactobacillus* spent media. Phage Node 6 was induced at all three experimental conditions, with conditions 0.5 mL and 1 mL spent media showing the same level of significance and the 2 mL spent media condition showing a higher level of significance. The additional phages, Node 30 and Node 11, were only induced at the 2 mL spent media condition. These phages require a specific stressor, only provided by the 2

mL condition, to be induced. This indicated that a critical level of metabolites is required in the environment to trigger the temperate phage to enter the lytic life cycle.

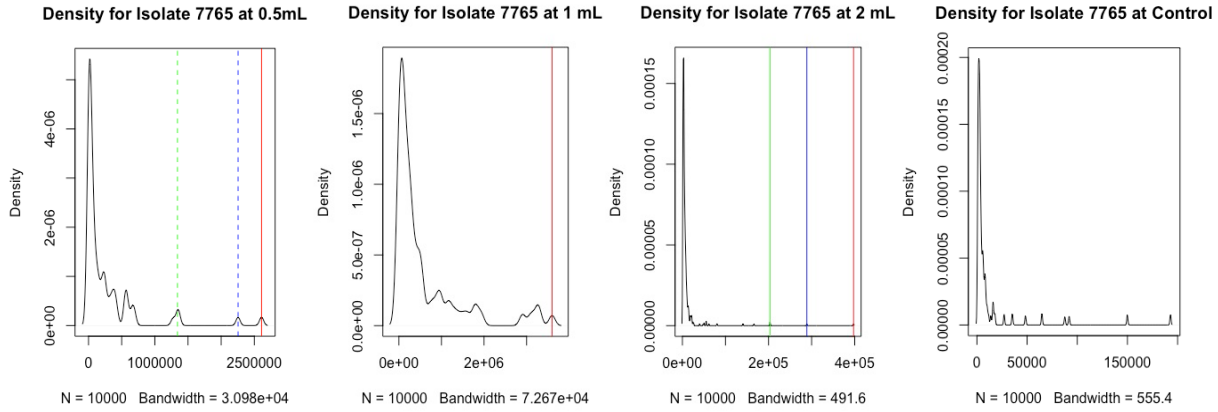


Figure 13. Density plots for isolate *S. epidermidis* 7765 (Community 6). Phage Node 6 is plotted in red. Phage Node 30 is plotted in green. Phage Node 11 is plotted in blue. The solid line indicates induction that was significant after the empirical probability analysis. The dashed line indicates evidence of induction that did not pass the empirical probability analysis.

Isolates from other communities were also examined using the empirical probability analysis (Table 5 and Appendix J). Thirty-four individual phages had an empirical probability at least one condition meeting our threshold (alpha = 0.01). Seven of these phages were induced in all four conditions, indicating spontaneous induction occurred. Another nine phages were induced only in the control, indicating that the laboratory conditions presented a stressful environment for those bacteria.

Case Study: Induction of Coliphage

To better understand which component of the spent *Lactobacillus* media was responsible for phage induction, we further tested the *E. coli* isolates from communities 3, 4, and 5. Coliphages

are well studied and characterized, and our lab has tested coliphage induction of several clinical urinary isolates with success, making the *E. coli* isolates from these communities ideal candidates for further investigation.

The four *E. coli* strains from the communities tested were grown individually at the pH, the hydrogen peroxide concentration, and a combination of the two conditions reflective of the values output by their corresponding *Lactobacillus* strain. Strains were grown in the following conditions: *E. coli* strain 1162 in pH 4.28 and hydrogen peroxide concentration of 16.97 uM, *E. coli* strain 1284 in pH 3.85 and hydrogen peroxide concentration 25.03 uM, and *E. coli* strains 1346 and 1354 in pH 4.27 and hydrogen peroxide concentration 12.48 uM. *E. coli* strains were also grown in unadjusted media to serve as a control for spontaneous phage induction. Cultures were filtered and the filtrate was spotted on naïve laboratory hosts: *E. coli* C, *E. coli* B, and *E. coli* K-12. The formation of a plaque indicates that a prophage(s) was induced from the urinary isolate and that the induced phage(s) is capable of lysing the laboratory strain (Figure 14). Induced phages showed varied in their lysis efficacy, from fully clearing the bacterial lawn to turbid plaques. Areas with any bacterial lawn clearing were recorded and harvested for further analysis (Table 6). It is important to note that phages may be induced that are unable to lyse the laboratory strain; these phages will be missed in this process.

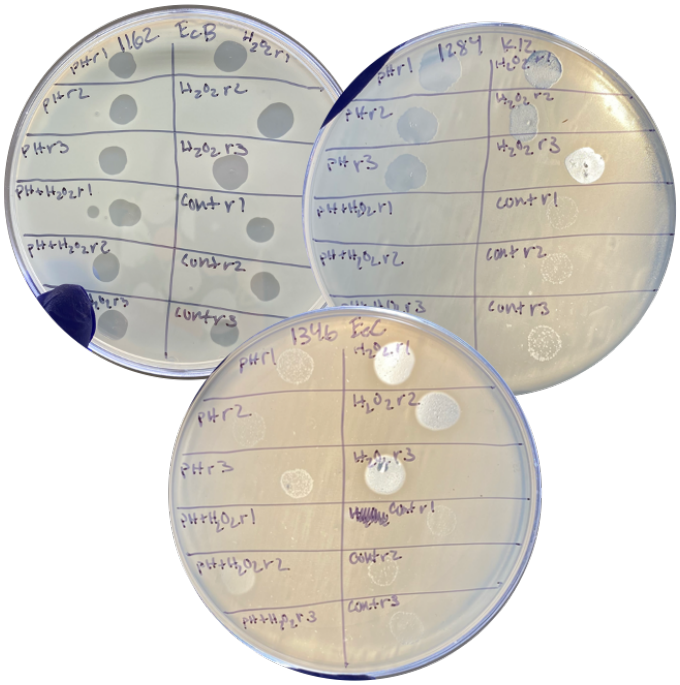


Figure 14. Coliphage Plaque Formation. Example of plaques formed on plates during a spot test for coliphage induction.

Isolate	Laboratory *E. coli* Strain	pH (r1/r2/r3)	pH + H2O2 (r1/r2/r3)	H2O2 (r1/r2/r3)	control (r1/r2/r3)
1162	EcB	+/+/+	+/+/+	+/+/+	+/+/+
	EcC	+/+/+	+/+/+	+/+/+	+/+/+
	K-12	+/+/+	-/-/-	+/+/+	+/+/+
1284	EcB	-/-/-	-/-/-	-/-/-	-/-/-
	EcC	+/+/+	-/-/-	+/+/+	+/+/+
	K-12	+/+/+	-/-/-	+/+/+	+/+/+
1346	EcB	-/-/-	-/+/-	+/+/+	-/-/-
	EcC	+/+/+	-/+/-	+/+/+	+/+/+
	K-12	-/-/-	-/+/-	+/+/+	-/-/-
1354	EcB	-/-/-	+/+/+	+/+/+	-/-/-
	EcC	-/-/-	+/+/+	+/+/+	-/-/-
	K-12	-/-/-	+/+/+	+/+/+	-/-/-

Table 6. Coliphage Plaque Formation. Results of plaque spot test of coliphages platted on laboratory *E. coli* strains B (EcB), C (EcC), and K-12 (K-12*). Plaque formation is noted with a + and the absence of formation is noted by -. Replicates for each condition and naïve host are denoted as r1/r2/r3.*

Each urinary *E. coli* strain tested induced at least one phage able to lyse one of the naïve laboratory *E. coli* strains. Additionally, each induced phage(s) displayed a unique host range and phages were induced at varying conditions across isolates (Table 6). Phages were induced from *E. coli* 1162 at all experimental conditions, as well as the control, and were able to lyse laboratory hosts.

Phage(s) induced from *E. coli* strain 1284 were unable to lyse *E. coli* B. There was no plaque formation evidence of induced phage capable of infecting the laboratory *E. coli* strains at the combination pH and hydrogen peroxide condition, but phage(s) were induced by pH and hydrogen peroxide by themselves and in the control group.

Phages induced from *E. coli* 1346 showed the widest variability in its ability to lyse laboratory hosts. From the pH only condition, phages were induced in all three replicates, and

this phage(s) was only able to lyse *E. coli* C. Phages were induced in the second replicate under the combination pH and hydrogen peroxide conditions. This phage(s) was able to lyse all three laboratory strains. All three replicates produced a phage under the hydrogen peroxide condition. This phage(s) was able to lyse all three laboratory strains. Under the control condition, phage induction was observed. However, the induced phage could only lyse *E. coli* C. The variability of the plaque formation and ability to lyse laboratory *E. coli* strains suggests that more than one phage was induced from this sample.

E. coli 1354 produced phages at all three replicates under the pH and hydrogen peroxide condition and the hydrogen peroxide condition. The induced phages can lyse all three laboratory hosts. No phage induction was observed for the pH or control conditions. This suggests that hydrogen peroxide levels triggered the induction of the observed phage.

Each plaque was harvested and filtered. Its DNA was then extracted and screened for phage via PCR using the same primers that were previously designed for the *E. coli* isolates. Through this process, we were able to identify induced phages from 10 of the 31 plaquing conditions (Table 7). Isolates 1346 and 1162 only had one phage identified by PCR, Node 3 and Node 3 (Primer Node 3b), respectively. Isolate 1354 had no phage identified by PCR, leaving all of the phages from the six plaque producing conditions unidentified. Interestingly, isolate 1284 had multiple phages identified, sometimes within the same condition. Results showed that each identified plaque contained the Node 13 phage. However, the plaque at condition pH, plated on *E. coli* K 12 also contained the Node 9 phage. Node 6, Node 13, and Node 33 phages were all identified within the hydrogen peroxide condition plated on *E. coli* C. This indicates that multiple phages are inducible from this isolate under the same conditions, although, some phages have a narrower host range. For example, both Node 13 and Node 9 were induced under the pH

condition from isolate 1284. However, only Node 13 phage was able to plaque on both *E. coli* C and *E. coli* K 12, whereas the Node 9 phage could only infect and lyse the *E. coli* K 12.

Based upon BLAST results, the taxonomies for some of these identified phages were determined. The Node 13 phage from isolate 1284 was the only identified phage with unknown homology. Of the remaining identified phages from 1284, two showed homology to myoviruses (Node 6 phage and Node 9 phage) and one showed homology to siphoviruses (Node 33 phage). The phages identified from 1162 and 1346 both showed homology to a siphovirus.

Isolate	Laboratory *E. coli* Strain	pH (r1/r2/r3)	pH + H2O2 (r1/r2/r3)	H2O2 (r1/r2/r3)	control (r1/r2/r3)
1162	EcB	No PCR ID	No PCR ID	No PCR ID	No PCR ID
	EcC	No PCR ID	No PCR ID	Node 3	No PCR ID
	K12	No PCR ID	No plaque	Node 3	No PCR ID
1284	EcB	No plaque	No plaque	No plaque	No plaque
	EcC	Node 13	No plaque	Node 6/Node 13/ Node 33	Node 13
	K12	Node 9/Node 13	No plaque	Node 13	Node 13
1346	EcB	No plaque	No PCR ID	No PCR ID	No plaque
	EcC	Node 3	No PCR ID	No PCR ID	Node 3
	K12	No plaque	No PCR ID	No PCR ID	No plaque
1354	EcB	No plaque	No PCR ID	No PCR ID	No plaque
	EcC	No plaque	No PCR ID	No PCR ID	No plaque
	K12	No plaque	No PCR ID	No PCR ID	No plaque

Table 7. Identification of Coliphages. Cells in green show coliphage that were identified via PCR. Cells in red represent phage plaques that were not identified by PCR.

CHAPTER FOUR

DISCUSSION

Isolates and Their Respective Phages

While prior studies have identified several different bacterial species within the female urinary tract (Wolfe *et al.* 2012; Siddiqui *et al.* 2011; Dong *et al.* 2011), *Lactobacillus* species are common and often dominant members of the microbiota (Ravel *et al.* 2011; Pearce *et al.* 2014; Price *et al.* 2020). Here we selected 8 communities of bacteria isolated from a single urine sample that contained either *L. jensenii* or *L. mulieris*. Both of these *Lactobacillus* species have previously been implicated as playing a role in maintaining urinary tract health (Stapleton, 2016). Furthermore, the communities examined here include common urinary taxa found within the female urinary tract. Each community is unique, containing different species and numbers of community members. Even with a small number of communities (n=8), we were able to explore the prophages within 19 different bacterial species previously documented within the female urinary microbiota.

Our analysis of the bacterial genomes from these 8 communities resulted in 119 individual, high confidence, prophage predictions. Prior studies of the urobiome as well as the gut microbiome have used similar thresholds for VirSorter and Phaster predictions (Paez-Espino *et al.* 2016; Miller-Ensminger *et al.* 2018; Camarillo-Guerrero *et al.* 2021), finding that most of these prophages are likely viable temperate phages. Only two of the urinary isolates in the communities examined here, *S. anginosus* 7782 and *C. coyleae* 8490, did not contain any high

confidence prophage predictions. This concurs with our previous report on the prevalence of lysogens in the urinary tract (Miller-Ensminger *et al.* 2018). When taking into account the low confidence predictions, every isolate is predicted to contain at least one region containing a phage coding region(s). Prior examination of urinary species for which no phage had yet to be isolated were also found to contain phage coding regions (Malki *et al.* 2016). Furthermore, this finding supports prior hypotheses that phages are the most abundant viruses in the urinary tract (Santiago-Rodriguez *et al.* 2015).

Within the communities, two *E. coli* isolates were predicted to harbor the most prophages. Coliphage are well studied and multiple different coliphages have been isolated, phenotypically characterized, and sequenced. In prior work from our lab, several different coliphages, including relatives of one of the most well studied phages, Lambda phage, were found to be prevalent in urinary *E. coli* strains (Garretto 2019). With genome repositories containing numerous coliphages representative of the diversity found in nature, it stands to reason that prophage prediction tools would be well trained to predict coliphages. For less studied organisms in which few (if any) phages have been isolated and characterized, phage prediction tools like VirSorter and Phaster are likely to overlook or predict prophage sequences with a low confidence. This is the case for several of the bacterial taxa found within the urinary tract. For instance, there are ~30 genomes for phage infectious of *Proteus* species, and very few *P. mirabilis*-infecting phages characterized (Corban and Ramsey 2021). While prior work found phage coding regions in *Gardnerella* genomes (Malki *et al.* 2016), only one phage has been sequenced and characterized for the species (Bordigoni *et al.* 2021). These are but two examples of urinary bacteria for which there is limited information about the phages that infect them.

When the predicted prophage sequences in the 8 communities were compared to other publicly available sequences, only 54% of the high confidence prophage sequences exhibited significant sequence similarity. This finding concurs with previous studies, which saw a high degree of novelty within the urobiome (Miller-Ensminger *et al.* 2018). This is largely due to the fact that phages in the urinary tract are understudied. The vast majority of our knowledge about phages of the human body relates to phages of the gut (Reyes *et al.* 2010; Minot *et al.* 2011; Ogilvie and Jones 2015; Manrique *et al.* 2016). While there have been a few studies of phages and the skin microbiome (Foulongne *et al.* 2012; Hannigan *et al.* 2015; van Zyl *et al.* 2018), oral cavity (Pride *et al.* 2012; Edlund *et al.* 2015), and vaginal microbiome (Jakobsen *et al.* 2020; da Costa *et al.* 2020), the diversity of phages within the human body has yet to be determined. Preliminary studies of the gut and urinary phage communities, however, suggests that these two niches harbor distinct and unique phages (unpublished). This parallels similar observations in the bacterial communities of these two environments (Thomas-White *et al*. 2018a). The urinary tract phage community is better studied than the vaginal phage community. Given the similar bacterial taxa found within these two niches, one would assume that increased exploration of the vaginal community would provide insight into the urinary community of phages and vice versa.

Many of the phages that have been isolated from the urinary tract are siphoviruses (Dallas and Kinsbury 1997; Brown-Jaque *et al.* 2016; Malki et al 2016; Miller-Ensminger *et al.* 2018; Johnson and Putonti 2019; Johnson *et al.* 2019; Brassil *et al.* 2020). A similar observation was made for the vaginal phage community (da Costa *et al.* 2021). The most frequently identified taxonomic family within the communities examined here was also Siphoviridae. Numerous strains from the communities harbored more than one predicted prophage exhibiting sequence

similarity to siphoviruses, including *S. anginosus* 839 (n=5), *E. coli* 1162 (n=7), *E. coli* 1284 (n=5), *S. anginosus* 7768 (n=6), and *K. pneumoniae* 8492 (n=4). The abundance of siphoviruses is not unique to this niche. In fact, the majority of classified viral sequences from the gut show homology to siphoviruses (Shkoporov and Hill 2019). As more phages within different organs of the human body are isolated, morphologically characterized, and sequenced, it will be interesting to see if the siphoviruses remain the dominant family of phages within the human microbiota.

Regardless of the environment under investigation, phages far outnumber the bacteria that they infect. Despite this, there are significantly less publicly available phage genomes than bacterial genomes. The computational tools used to predict prophage sequences in this analysis rely heavily on previously characterized phage genomes. The lack of available genomes may result in some phages being overlooked in this study. As more phages are isolated and sequenced, particularly from the urinary tract, it is important that these tools are updated to reflect this new data. It is already known that current prophage identification tools are optimized for tailed phage prediction. These tools often fail to detect the tailless inoviruses (Roux *et al.* 2019). However, these rod-shaped inoviruses have been previously recorded in the urobiome (Shapiro and Putonti 2020). As such this phage population may have been excluded from our analysis. Additionally, the methods used in this study would exclude obligately lytic phages.

Lactobacillus **Metabolites**

The *L. jensenii* and *L. mulieris* strains each produce several metabolites. Metabolites have been explored for their bactericidal effects against other bacteria. Bacteriocins could potentially induce phage by causing stress on bacterial hosts. Prophage induction when using spent *Lactobacillus* media that cannot be attributed to *Lactobacillus*-relevant pH levels or hydrogen

peroxide levels could potentially be attributed to bacteriocins. Four of the eight *Lactobacillus* strains (*L. jensenii* 37, *L. mulieris* 1355, *L. mulieris* 7784, *L. jensenii* 8489) encoded for regions that had significant hits to characterized bacteriocin sequences. These could be responsible for some of the observed phage induction. Induction was seen in isolates 39_1M (Community 1, *L. jensenii* 37), 1354 (Community 5, *L. mulieris* 1355), 7781_2F and 7783_2Q (Community 7, *L. mulieris* 7784), and 8493 (Community 8, *L jensenii* 8489) under conditions with the *Lactobacillus* spent media, but not the controls. While this induction may have been caused by pH or hydrogen peroxide, it is important to consider that it could have been caused by the bacteriocins produced by these four *Lactobacillus* strains. However, when these strains were grown in *Lactobacillus* relevant pH levels, no evidence of induction was observed. While this could be due to the limitations of PCR and our primer selection, we would need to also grow these strains in *Lactobacillus* relevant hydrogen peroxide conditions and *Lactobacillu*s produced bacteriocins in separate cultures to determine if one of these factors is responsible for induction. While strains *L. jensenii* 37, *L. jensenii* 847, *L. jensenii* 1165, *L. jensenii* 1303, and *L. jensenii* 7766 were predicted to contain NRPS regions by antiSMASH. Further work would need to be done to determine what NRPS metabolites are produced.

Hydrogen peroxide and lactic acid are metabolites produced through the *Lactobacillus* glycolytic pathway. While these measurements for these metabolites have been recorded in several *L. jensenii* strains, this is the first study to note the differences between hydrogen peroxide and lactic acid-related pH shifts between *L. jensenii* and *L. mulieris* strains. Within the last year, *L. jensenii* was reclassified into two species: *L. jensenii* and *L. mulieris* (Rocha *et al*. 2020). Due to the small sample size of this study, we are unable to make any broad conclusion

about the differences between these species. However, *L. jensenii* strains appear to have lower average pH values and higher average hydrogen peroxide values than *L. mulieris* strains. This highlights further work needed to understand the differences between these two species and the potentially effects these differences have on urinary tract health and the composition of the urinary microbiota.

Phage Induction by *Lactobacillus*-Relevant pH

To assess the specific effects of the pH environment likely to be produced by *L. jensenii* and *L. mulieris* in the urinary tract, community members were grown in media adjusted to the pH output by their specific community's *Lactobacillus* strain. These samples were then screened for phage induction via PCR. Surprisingly, minimal phage induction was observed by PCR. In fact, only three samples (*A. neuii* 1295, *E. coli* 1354, and *E. faecalis* 7780) showed positive PCR results, indicating phage induction. An additional isolate, *L. crispatus* 1163, did not contain a positive PCR results, however, DNA was observed in the wells of the agarose gel, indicating phage induction, but poor amplification through PCR. To determine if the reaction was inhibited by the growth medium, a 16S PCR was run on a turbid liquid culture of isolate 1163. The reaction did not produce a positive result, indicating that a component of the medium was likely inhibiting the PCR reactions (data not shown).

While these results indicate limited phage induction through *Lactobacill*us-relevant pH, it is important to address the limitations of PCR. In standard procedures, at least 32 genomes/uL (Purcell *et al.* 2016) are required for a PCR to amplify the region of interest. The filtrates screened as part of this work may indeed contain phages; however the phages may have been induced at such a low titer that there is not enough genomic DNA present for the reaction to

proceed. A phage's titer can be increased by growing said phage with a susceptible host, allowing for the phage to replicate and achieve high copy number in a culture. However, for most of the isolates used in this study, we do not have susceptible hosts. Determining host range for each phage induced by each isolate is infeasible. Performing PCR on filtrate rather than plaque may also produce false negative results. The PCR protocol used here includes incubating the filtrate with potential phage at 95°C for 10 minutes to burst the phage capsid that contains the genomic DNA. While this step has been successful for coliphage lysate PCRs (Miller-Ensminger *et al.* 2020a), this incubation may be too long or too short, resulting in either an unburst capsid or denaturation of the DNA after the capsid bursts. In either case, we would expect that the PCR reaction would proceed with minimal success.

Moreover, bioinformatic tools to predict phage are imperfect. They frequently predict prophage sequences based upon their homology to previous sequenced and characterized viruses. These tools may fail to predict the correct start and stop coordinates for prophage sequences within their hosts genome. Furthermore, they may predict prophage that coinfect a host as a singular phage instead of multiple. Our manual inspection of VirSorter and Phaster results found such instances in isolates 1346 and 1354. These computational pitfalls may have led to poor primer design, aiming to amplify regions outside of the true phage genome. Without trying multiple primers for each phage or sequencing the induced phage, there is no way to confirm or deny the validity of each primer pair to amplify within the true phage genome. Additionally, primers were only produced from high confidence predictions. However, we observed through growth with spent *Lactobacillus* media that several induced phages were from low confidence

categories. These induced phages would have been missed altogether in these PCR checks for induction.

Phage Induction by Spent *Lactobacillus* Media

To assess phage induction by *Lactobacillus* metabolites (relevant pH levels, hydrogen peroxide, and bacteriocins), isolates were grown with the spent media of their corresponding *Lactobacillus* strain at varying volumes (0.5 mL, 1 mL, and 2 mL of spent media). A culture with no spent media (pH = 7; no supplemental H_2O_2) served as a control to check for phage induction under laboratory conditions. Induction was observed across 23 isolates, indicating at least 105 phages had been induced.

Using empirical probability, we were able to determine which phage were induced at a significant level providing confidence in our assessment that induction was widespread throughout the bacterial culture. In total, 34 phages were induced at a significance level (less than alpha of 0.01) in at least one of the tested conditions. Seven of these phages were induced under the three tested volumes and the control condition. Nine phages were induced in only the control. The remaining 18 phages were induced at only one condition or a combination of other conditions.

These scenarios explore several hypotheses about phage induction in these isolates. First, phage that were induced at one or more of the *Lactobacillus* spent media volumes were likely induced by some component of the spent media. This suggests that *Lactobacillus* is responsible for phage induction from these isolates. However, not all isolates experienced induction at each concentration. This shows that some volumes may either be too stressful and result in bacteria dying before phage are induced and produce mature virions, however CFU and OD

measurements would be needed to confirm this, or the condition might mimic the urinary environment, causing little to no stress on the bacteria and thus no phage induction. The strains used in this study were isolated from an environment with *Lactobacillus* metabolites present. This colonization within the patient's bladder may have allowed for selection for strains that survive or thrive with *Lactobacillus* present. Future studies combining *Lactobacillus* metabolites and urinary bacteria that were not isolated from communities with *Lactobacillus* present are needed to further explore this hypothesis. Second, we have several examples where phages were induced only in the control group. We believe that this signifies that the laboratory conditions used to grow that bacteria are stressful, while the conditions for growth with spent *Lactobacillus* media may mimic the urinary tract environment, causing limited stress and no phage induction. In both of these two hypotheses, it is possible that *Lactobacillus* metabolites represent a component of the "normal" environment for these isolates and may "preserve" these species as no phage are induced and phage related predation and die off does not occur. The last scenario is when phage induction is seen at all conditions. We believe this represents spontaneous induction. Spontaneous induction occurs without the addition of an induction factor, meaning even without a trigger, phage enter into the lytic cycle. Spontaneous induction has been observed in other urinary lysogens (Brassil *et al.* 2019) and species found within the urinary tract (Baugher *et al.* 2014).

It is important to note that laboratory conditions are typically considered to provide bacteria with an ideal environment for growth with regards to nutrient availability, temperature, and oxygen levels. However, this study shows that many phages are still induced under laboratory conditions. Traditionally, this would be considered spontaneous induction. However,

the direct opposition of the original hypothesis of this study (*Lactobacillus* created conditions cause induction) indicates that some bacteria are actually stressed under the laboratory conditions, but not in the presence of *Lactobacillus* metabolites. This challenges the current dogma of what causes "stress" upon a bacteria. While some bacteria may thrive in a laboratory environment, others may require an environment that more closely mimics their original niche to be accurately studied. Additionally, spontaneous phage induction may not truly be spontaneous, or without a stressor. A component of the "ideal" environment created in a laboratory may apply enough stress to cause induction.

Coliphage Induction

To further explore what factors are the most important for phage induction, we specifically worked with the *E. coli* strains in this study. Our lab has extensive experience isolating and working with coliphage from the urinary microbiota (Malki *et al*. 2016; Miller-Ensminger *et al*. 2020a), and we have several laboratory *E. coli* strains which contain no prophages and are ideal candidates to infect with any induced coliphage. *E. coli* strains were grown in relevant pH and hydrogen peroxide conditions as well as a combination condition reflecting the pH and hydrogen peroxide of their corresponding *Lactobacillus* strain. Lysates from these cultures were plated on laboratory hosts and induced phages that could infect these hosts produced visible plaques (Table 5 and Figure 14. It is important to note that phages can be highly selective for which strains they infect, requiring certain surface proteins to attach to their host. (For a review, see Hyman 2013.) Some additional phages may have been induced during this process that were unable to infect any of the three laboratory hosts. Therefore, conditions where no plaques were observed, may not be true negatives.

We observed induction at different conditions across the four *E. coli* isolates. For example, *E. coli* 1162 had a visibly induced phage at every growth condition. This phage(s) had the ability to infect every bacterial strain, except for the phage induced in the pH/ hydrogen peroxide combination condition. This phage was unable to infect and lyse *E. coli* K-12, producing no plaque. These results match those from the growth of 1162 in spent *Lactobacillus* media. In that experiment, we identified just one phage, which was induced at every condition. This suggests that the phage induced from 1162 is the result of spontaneous induction and is not necessarily linked to a trigger from *Lactobacillus* metabolites.

Conversely, *E. coli* 1354 highlights that specific *Lactobacillus* produced conditions might be responsible for phage induction. In isolate 1354, phage induction is only observed in the hydrogen peroxide and combination pH and hydrogen peroxide conditions. No induction is visibly observed in the pH or control conditions. This highlights several interesting points. First, since induction does not occur during all conditions, it is unlikely that this induced phage is a product of spontaneous induction. Second, since the phage is only observed after growth in conditions containing hydrogen peroxide, it is likely the hydrogen peroxide output from the *Lactobacillus* strain which causing induction in this *E. coli* strain.

Using PCR, we were able to identify phage from 10 of the 31 observed plaques from the induced coliphages (Table 7). Plaques that we failed to identify could have failed for several reasons. First, many of the DNA concentrations after the extraction step were too low, containing insufficient DNA. Because of this, there may have not been enough DNA to be detected by PCR. Even before DNA extraction, the plaques were purified using a 0.22 um cellulose filter to remove any remaining bacterial cells and debris. However, some phage can adhere to the filter

and thus not pass into the filtrate. If this is the case for any of these phages, they would have been present in the extracted filtrate. Additionally, the primers used in this study do not reflect all the predicted phage for each *E coli* isolate. It is possible that some of the observed plaques were the result of a low confidence phage that was induced. If so, we do not have primers to amplify these phages and they would be missing from this analysis.

Through PCR analysis, 1162 and 1346 both had one phage identified for multiple plaques. It is possible that these two phages were responsible for all the observed plaques from their respective isolates, however, for the reasons outlined above, we may have not been able to identify them. Interestingly, 1284 had multiple phages identified. The most commonly identified phage was Node 13 which was present in each of the identified plaques. However, two plaques had more than one phage identified. The plaque of *E. coli* C from the hydrogen peroxide condition had three observed phages: Node 6, Node 13, and Node 33. When plated on *E. coli* K 12, the same lysate produced a plaque that was only shown to contain Node 13 phage. This indicates that the Node 6 and Node 33 phages may only be able to infect *E. coli* C and not *E. coli* K 12 or *E. coli* B. When plated on *E. coli* K 12, the lysate from the pH only condition produced a plaque that contained phages Node 9 and Node 13. Node 9 was not identified within the plaque harvested from *E. coli* C that was produced from the same lysate. It appears that the Node 9 phage only infects *E. coli* K 12 out of the strains used in this experiment. Nonetheless, this shows that 1284 had multiple viable phage that were inducible at several conditions, with some conditions producing multiple phages.

Moreover, half of the phage identified in this analysis showed homology to Siphoviridae. This concurs with previously reported studies, showing that Siphoviridae are common phage in

the urinary microbiome (Dallas and Kinsbury 1997; Brown-Jaque *et al.* 2016; Malki et al 2016; Miller-Ensminger *et al.* 2018; Johnson and Putonti 2019; Johnson *et al.* 2019; Brassil *et al.* 2020). Two of the remaining phages were identified as Myoviridae. This is the second largest taxonomic group of our phages in this study with homology to other known phages. Only one phage from the plaque PCR analysis had no homology to other phages, even though majority of phage in this study fall into the category of Unknown Taxonomy.

CHAPTER FIVE

CONCLUSIONS

The urinary tract is home to a diverse microbiota. However, compared to other sites in the human body, such as the gut or oral cavity, the microbes that inhabit this niche are severely understudied. A wide range of bacteria have been isolated and catalogued from urine samples and now the phages that infect these bacteria are being explored. However, the effects that one bacteria species has on the phages of another species has yet to be examined.

To address this gap in knowledge, we explored the role *L. jensenii* and *L. mulieris*, which are commonly considered to be protective species within the urinary tract, play in inducing phage from other bacterial species in the urinary tract community. In this study, we explored eight communities, each containing one *L. jensenii* or *L. mulieris* strain and at least two other community members. The community members represent the diversity of bacteria that has been found within the female urinary microbiome. In total, 29 bacteria from the eight communities were sequenced and their genomes screened for the presence of prophages. We found evidence of 119 unique, high confidence phages with the majority showing no homology to previously characterized phages. This confirms the results of previous studies, showing that the phages of the urinary microbiome share little homology with phages from other sources. The most common taxonomy for phages with homology to other characterized phages was Siphoviridae.

Lactobacilli release lactic acid, dropping the pH of their environment, and hydrogen peroxide as part of the glycolytic pathway. Because pH and hydrogen peroxide have been used in

laboratory settings to induce phage, we hypothesized that lactobacilli in the urinary tract may be causing induction in other species in the urinary tract. We measured the pH and hydrogen peroxide outputs of the lactobacilli strains used in this study. We found that pH alone had limited effect on induction when prophage sequences were detected via PCR. However, further investigation into coliphages found that the PCR assays likely underestimate the number of phage induced by altered pH as a result of low phage titers and/or limitations in exactly predicting the phage sequence.

Conversely, when community members were grown in the spent media of their corresponding *Lactobacillus* strain, sequencing results showed evidence of 105 induced phages. Community members were grown with varying concentrations of spent media from their corresponding *Lactobacillus* strain. The communities were then pooled and sequenced. For the analysis of these sequences, to determine if phage were induced, we created and used several custom python and R scripts. This analysis is the first of its kind.

This study is the first to explore the relationship between *Lactobacillus* and the phage of other urinary microbiota members. Here we found that *Lactobacillus* metabolites can induce phages in other community members. However, phages were also induced only in the control conditions. This challenges our current notions of what is a “stressor” and how spontaneous induction occurs in isolates whose natural environment does not reflect that created in a laboratory context. Additionally, the methods used to determine what phages were induced from pooled sequencing samples present a new methodology. This method allows for the pooling of multiple samples, containing different phage and bacteria components to be sequenced and analyzed for the presence of phage at the same time.

APPENDIX A

ASSOCIATED IRB STUDY NUMBERS AND PUBLICATIONS

Community	***L. jensenii / L. mulieris* Identity and Number**	**Patient ID**	**Study Name**	**Patient Symptom**	**Collection Method**	**Collection Date**	**Associated Publications**	**Associated IRB Number**
1	*L. jensenii* 37	C029	Astellas	OAB/UTI-	Transurethral Cath	11/1/13	PMID: 24371246, PMID: 26210757, PMID: 25006228, PMID: 26423260	204195
2	*L. jensenii* 847	MUM_15	MUM	no LUTS	Transurethral Cath	10/5/14	PMID: 28970961	206470
3	*L. jensenii* 1165	EQUC128	UTI-EQUC	UTI+	Transurethral Cath	6/11/15	PMID: 36962083	206469
4	*L. jensenii* 1303	EQUC145	UTI-EQUC	UTI+	Transurethral Cath	7/13/15	PMID: 36962083	206469
5	*L. mulieris* 1355	EQUC0152	UTI-EQUC	UTI+	Transurethral Cath	7/30/15	PMID: 36962083	206469
6	*L. jensenii* 7766	SD08	rUTI	RUTI-LUTS	Transurethral Cath	8/15/17	In Review.	17077AW (UCSD)
7	*L. mulieris* 7784	RUTI9	rUTI	RUTI-UTI+	Transurethral Cath	8/28/17	In Review.	17077AW (UCSD)
8	*L. jensenii* 8489	MIR068	Mirabegron	OAB+/UTI-	Transurethral Cath	7/14/18	In Prep	207102

APPENDIX B

GENOME ACCESION NUMBERS

Community Member Number	Community Member Identity	Accession Number
37	*Lactobacillus jensenii*	GCA_007785915.1
38.2I	*Micrococcus luteus*	Awaiting Accession Number
39.1M	*Staphylococcus epidermidis*	Awaiting Accession Number
38	*Proteus mirabilis*	GCA_012030315.1
835	*Staphylococcus epidermidis*	Awaiting Accession Number
839	*Streptococcus anginosus*	GCA_012030555.1
843	*Enterococcus faecalis*	GCA_012030565.1
847	*Lactobacillus jensenii*	GCA_012029675.1
1162	*Escherichia coli*	GCA_003892455.1
1163	*Lactobacillus crispatus*	GCA_012030075.1
1165	*Lactobacillus jensenii*	GCA_007786155.1
1284	*Escherichia coli*	GCA_003892355.1
1295	*Actinomyces neuii*	GCA_012030015.1
1296.1T	*Staphylococcus hominis*	Awaiting Accession Number
1303	*L. jensenii*	GCA_007786145.1
1309	*Enterococcus faecalis*	GCA_012030535.1
1310	*Proteus mirabilis*	GCA_012030515.1
1310.1E	*Corynebacterium amycolatum*	Awaiting Accession Number
1346	*Escherichia coli*	GCA_003886295.1
1353.1Y	*Streptococcus anginosus*	Awaiting Accession Number
1354	*Escherichia coli*	GCA_003886225.1
1355	*Lactobacillus mulieris*	GCA_007786095.1
7765	*Staphylococcus epidermidis*	GCA_012030625.1
7766	*Lactobacillus jensenii*	GCA_012030285.1
7768	*Streptococcus anginosus*	GCA_012030235.1
7769	*Corynebacterium aurimucosum*	GCA_012030615.1
7779	*Klebsiella pneumoniae*	GCA_012030245.1
7780	*Enterococcus faecalis*	GCA_012030205.1
7781.2F	*Staphylococcus epidermidis*	Awaiting Accession Number

7782	*Streptococcus agalactiae*	GCA_012030185.1
7783.2Q	*Aerococcus urinae*	Awaiting Accession Number
7784	*Lactobacillus mulieris*	GCA_012102935.1
8490	*Corynebacterium coyleae*	GCA_012030345.1
8492.1R	*Corynebacterium aurimucosum*	Awaiting Accession Number
8492	*Klebsiella pneumoniae*	GCA_012030275.1
8493	*Staphylococcus epidermidis*	GCA_012029805.1
8489	*Lactobacillus jensenii*	GCA_007785935.1

APPENDIX C

PRIMER SEQUENCES AND INFORMATION

Community	Isolate	Primer ID	Forward Primer Sequence	Reverse Primer Sequence	Virsorter Prediction	Phaster Prediction	Positive Control Worked?
1	38.2I	node 9	CGCGGATCAAGAAGACCAAG	GCTCAGGTCCTCGTTGTACT	none	Incomplete	yes
1	38.2I	node 97	AGCTCATCTACGCCCTGATC	TAGGGCTTGTTCTTCACGGT	Category 2	none	yes
1	39.1M	node 1	GTTTCGAACCGTTCCTAGC	GGACTTGGCCCATGTTGTTT	Category 4	Intact	yes
1	39.1M	node 6	TAAACAGCAACACCACGAGC	TCACTCTCCACCGACCAAAG	Category 6	Questionable	yes
1	38	node 1	CACCGACAACATCAACAAGG	CTTGCTGAATGCACGAAAAA	Category 5	Intact	yes
1	38	node 3	TTAAATGTCAGCTCGCAACG	CAGCACGCAGAACATTAGGA	Category 5	Questionable	yes
1	38	node 10	GCCCGTTGTTGTTGAGAAAT	CAAGAAACCGCAGAAAAAGC	Category 5	Intact	yes
2	843	node 3	GCCCGGAATTATTTTTGGTT	TAATGGTGCACAGCAATGGT	Category 5	Intact	yes
3	1162	node 17	CTTCCAGATCCAGCTTTCGC	GGCGATCTTGTCTACTCCGA	Category 2	Intact	yes
3	1162	node 29	GGCCACACTGTAGTAATGCG	ATTGGCGTGTCGGTTTATCG	Category 2	Intact	yes
3	1162	node 2	CGAGATGGAACTGCACGAAG	GCATATCAACGGCACCACAT	Category 4	Intact	yes
3	1162	node 3	GTCTCCGTGCCTTATACCGA	TTCCCCACCAGCTCGTTTAT	Category 5	Intact	yes
3	1162	node 6	CGCTTCATCGCTTTCCATCA	ACGCTGGAACTGGAATCTGA	Category 5	Intact	yes
3	1163	node 5	CGCACGATTCAGCATCTCAA	AAAGTGGTTTTGGCGAGTCC	Category 2	Intact	yes
3	1163	node 24	ATCTGCATTCAAAAGCCGCT	GTGGCTCTTGTTCCGACTTG	Category 5	Intact	yes
3	1163	node 16	GTGGCTCTTGTTCCGACTTG	GATACCAGCCGCTTGAAGTG	Category 2	Incomplete	yes
3	1163	node 13	CCATTTTCCATGACCGTGCT	AACCGTCATCTCTCCTGTGG	Category 5	Questionable	no
3	1163	node 1	CTGAGACTGCACCTGTACCA	CAACCACCAGCCAAACATGA	Category 5	Incomplete	yes
4	1284	node 33	TAACATTGGCCCCGGTAAGT	GGTAATGATTGACCGCCCAC	Category 2	Intact	yes
4	1284	node 36	GGCTCCAAGTGAAAACAGCA	CGTCAGATGAACGAAGGCTG	Category 2	Intact	yes

4	1284	node 13	TTCCACGGCATCAGTTACCT	GTCCGGTCGTTTTATGAGGC	Category 4	Intact	yes
4	1284	node 9	TCCGTCTTTCAGGGTCTTCC	CGCTGGAAATCTCGGAAGTG	Category 4	Intact	yes
4	1284	node 26	CTTCATCACTAAAGGCCGCC	CGGCGTAAATTCAGAGGTGG	Category 5	Incomplete	no
4	1284	node 6	GTTGATGTTTGCCGGTGACT	ATCCCATGAAATCGCCGTG	Category 5	Incomplete	yes
4	1295	node 3	TCGACAATCTTTTCGTGCAG	AAACGTGCCGAGCTTAAAGA	Category 5	Intact	yes
4	1296.1T	node 21	AGCTCGCCTACCCTCCTATA	CCACGTCTTGTTGTTCGCTT	Category 5	Incomplete	no
4	1296.1T	node 2	GGGGTGGTATCTAATGGCGA	TCTATGATGTGGCCCTTCCC	Category 5	Incomplete	yes
4	1309	node 1a	ACGCCTATTTTGCCAACAAC	TCGGTGTCGTGAATTTGTGT	Category 5	Questionable	yes
4	1309	node 1b	TAATGATTGCCCGTGACGTA	CTCGTTGTACGGACGGATTT	Category 5	Intact	yes
4	1309	node 15	AATAAACCGCCGTCAATCAG	TTTTGCATTAATCGCTGCTG	Category 2	Intact	yes
4	1310	node 3	CACCGACAACATCAACAAGG	CTTGCTGAATGCACGAAAAA	Category 5	Questionable	yes
4	1310	node 34	GCCCGTTGTTGTTGAGAAAT	CAAGAAACCGCAGAAAAAGC	Category 2	Intact	yes
4	1310	node 54	TTAAATGTCAGCTCGCAACG	CAGCACGCAGAACATTAGGA	Category 1	Questionable	yes
4	1310.1E	node 12	CACGGTTAATCCTCGGCAAG	GAGAAACTGGCTGGGGTACT	Category 5	Incomplete	yes
5	1346	node 2	AGTAAGTACGGGCGTCACAA	AACATAGTCAGGCGGGAACA	Category 5	Intact	yes
5	1346	node 3b	GCTGATAAGCCTGGTTGACG	CTTTCTCCGGTACATGCTGC	Category 5	Intact	yes
5	1353.1Y	node 3	GAGAAACTGGCTGGGGTACT	CCGACCGCTAGAGTTGAGAT	Category 5	Questionable	yes
5	1353.1Y	node 13	AAGATGGGTCAACGTCGAGT	ACTAGACACCCCGATTTGCA	Category 4	Intact	yes
5	1354	node 2	CTTTCTCCGGTACATGCTGC	GCTGATAAGCCTGGTTGACG	Category 4	Intact	yes
5	1354	node 6	GCGATGTTTACTGGCTCTGG	GCACGTTGATTTTCAGCAGC	Category 5	Intact	yes

5	1354	node 7	CCGACCGATGCCAAAATCAT	GGCTTTCTCGCTCGGTTTAG	Category 5	Incomplete	no
6	7765	node 11	GAGGCAATGGCATCATTCTT	AGATGCCTCGCCTGTAGAAA	Category 5	Questionable	yes
6	7768	node 3	TGTGTTCGCAGAAGCAAATC	AAATAGCTGACGCGCTCAAT	Category 5	Questionable	yes
6	7768	node8	CAGCGTTCTTCATCAGGTCA	CCGGTTAAAAGCATCACGTT	Category 2	Intact	yes
6	7768	node 9	AACCAGCCAAACAAGTCACC	TTCCAGAAATCCCTTCATCG	Category 5	Incomplete	yes
7	7779	node 3	AAAAACGATTGGCCGTGTAG	CGCCTGTCTTCCTGTTCTTC	Category 5	Incomplete	yes
7	7779	node 14	TGCTTCTCGTTTCTCGGTTT	AGGTGGTGCACAGGAAAATC	Category 5	Intact	yes
7	7779	node 58	CGTTCTTTCCGAGCTTAACG	GCGATGCTGTTGAGTTTGAA	Category 2	Questionable	yes
7	7779	node 67	GCGTTTATCCTGAGCTCGAC	AACGATACCAACATGCGTCA	Category 2	Intact	yes
7	7780	node 3	TGCATTTTCAGGCAAGACAG	CCGGCACATCACCTTTAACT	Category 4	Intact	yes
7	7780	node 9	GTAACGGCCAAAGCACATTT	AAAAAGGCAACTGCGAAGAA	Category 5	Intact	yes
7	7781.2F	node 7	AGTACCATGTCGTCACCTCC	CAACTCCTACACGACGAGCT	Category 5	Incomplete	yes
7	7783.2Q	node 10	CGAGAAGCCAACGATGTCTG	AACCAACCTTCAACGGCTTC	Category 5	Incomplete	yes
7	7783.2Q	node 31	TTTAGGACAGCGTGGAGGTT	GCTCGTTTCTACAGCGTCAG	Category 3	Incomplete	yes
8	8492.1R	node 5	AGACCTAACGGCATTCCACA	CGGCATTCTTTCCTACCTGC	Category 5	Intact	no
8	8492	node 4	AAAAACGATTGGCCGTGTAG	CGCCTGTCTTCCTGTTCTTC	Category 5	Intact	no
8	8492	node 16	TGCTTCTCGTTTCTCGGTTT	AGGTGGTGCACAGGAAAATC	Category 5	Intact	no
8	8492	node 54	CGTTCTTTCCGAGCTTAACG	GCGATGCTGTTGAGTTTGAA	Category 1	Intact	no
8	8492	node 57	AACGATACCAACATGCGTCA	GCGTTTATCCTGAGCTCGAC	Category 1	Intact	no
8	8493	node 2	CAAAACATGAATTGGCAACG	TGCAGCTTGATTACCACTGC	Category 5	Intact	yes

APPENDIX D

HYDROGEN PEROXIDE STANDARD CURVE TABLE

Concentration	0 uM	1 uM	2 uM	3 uM	4 uM	5 uM	6 uM	7 uM	8 uM	9 uM	10 uM
sd 1	17	717	1378	2029	2632	3247	3845	4453	4904	5380	5899
sd 2	5	658	1310	1978	2487	3140	3734	4242	4844	5393	5748
sd 3	5.56E-01	626	1305	1969	2543	3043	3617	4253	4819	5299	5970
sd average	7.52E+00	667	1331	2003.5	2554	3143.33333	3732	4316	4855.66667	5357.33333	5872.33333

APPENDIX E

COMPLETE PHASTER AND VIRSORTER PREDICTIONS

Community	Isolate	Prediction	VirSorter or Phaster	Prediction Category
1	38.2I	NODE 182	VirSorter	2
		Node 194	VirSorter	2
		Node 222	VirSorter	2
		Node 233	VirSorter	2
		Node 97	VirSorter	2
		Node 119	VirSorter	3
		Node 122	VirSorter	3
		Node 200	VirSorter	3
		Node 205	VirSorter	3
		Node 899	VirSorter	3
		Node 9	Phaster	Incomplete
		Node 164	Phaster	Incomplete
	39.1M	Node 1	Phaster	Intact
		Node 6	Phaster	Questionable
		Node 9	Phaster	Incomplete
		Node 1	VirSorter	5
		Node 14	VirSorter	3
		Node 6	VirSorter	6
	38	Node 1	Phaster	Intact
		Node 3	Phaster	Questionable
		Node 10	Phaster	Intact
		Node_3	Phaster	Incomplete
		Node_18	Phaster	Incomplete
		Node 9	Phaster	Incomplete
		Node 23	Phaster	Incomplete
		NODE 31	Phaster	Incomplete
		Node 31	VirSorter	2
		Node 10	VirSorter	5
		Node 1	VirSorter	5
		Node 3	VirSorter	5
		Node 52	VirSorter	3
		Node 18	VirSorter	6
		Node 2	VirSorter	6
		Node 4	VirSorter	6
		Node 9	VirSorter	5

2	835	Node 2	Phaster	Intact
		Node 6	Phaster	Intact
		Node 65	Phaster	Incomplete
		Node 2	VirSorter	2
		Node 6	VirSorter	2
		Node 65	VirsSorter	2
		Node 240	Virsorter	2
	839	Node 1	Phaster	Intact
		Node 4	Phaster	Questionable
		Node 11	Phaster	Intact
		Node 13	Phaster	Questionable
		Node 5	Phaster	Incomplete
		Node 26	VirSorter	1
		Node 27	VirSorter	2
		Node 11	VirSorter	5
		Node 13	VirSorter	5
		Node 1	VirSorter	5
		Node 4	VirSorter	5
		Node 3	VirSorter	6
		Node 7	VirSorter	6
	843	Node 3	Phaster	Intact
		Node 1	Phaster	Incomplete
		Node 1	VirSorter	5
		Node 3	VirSorter	5
		Node 12	VirSorter	3
		Node 2	VirSorter	6
3	1162	Node 2	Phaster	Intact
		Node 3	Phaster	Intact
		Node 6	Phaster	Intact
		Node 6	Phaster	Questionable
		Node 17	Phaster	Intact
		Node 18	Phaster	Intact
		Node 29	Phaster	Intact
		Node 8	Phaster	Incomplete
		Node 44	VirSorter	1
		Node 17	VirSorter	2
		Node 29	VirSorter	2

		Node 40	VirSorter	2
		Node 48	VirSorter	2
		Node 52	VirSorter	2
		Node 55	VirSorter	2
		Node 58	VirSorter	2
		Node 2	VirSorter	4
		Node 3	VirSorter	5
		Node 6	VirSorter	5
		Node 1	VirSorter	6
		Node 22	VirSorter	6
		Node 7	VirSorter	5
	1163	Node 5	Phaster	Intact
		Node 13	Phaster	Questionable
		Node_1	Phaster	Incomplete
		Node_1	Phaster	Incomplete
		Node 16	Phaster	Incomplete
		Node 60	Phaster	Incomplete
		Node 24	Phaster	Intact
		Node 117	VirSorter	2
		Node 16	VirSorter	2
		Node 5	VirSorter	2
		Node 13	VirSorter	5
		Node 1	VirSorter	5
		Node 24	VirSorter	5
		Node 12	VirSorter	3
		Node 18	VirSorter	3
		Node 43	VirSorter	3
		Node 66	VirSorter	3
		Node 96	VirSorter	3
		Node 27	VirSorter	6
		Node 29	VirSorter	6
4	1284	Node 9	Phaster	Intact
		Node 13	Phaster	Intact
		Node 22	Phaster	Questionable
		Node 22	Phaster	Questionable
		Node 33	Phaster	Intact
		Node 36	Phaster	Intact

		NODE _6	Phaster	Incomplete
		Node 14	Phaster	Incomplete
		Node 18	Phaster	Incomplete
		Node 21	Phaster	Incomplete
		Node_26	Phaster	Incomplete
		Node_28	Phaster	Incomplete
		Node 62	VirSorter	1
		Node 65	Virsorter	1
		Node 33	Virsorter	2
		Node 36	VirSorter	2
		Node 53	VirSorter	2
		Node 54	VirSorter	2
		Node 67	VirSorter	2
		Node 72	VirSorter	2
		Node 13	VirSorter	4
		Node 9	VirSorter	4
		Node 14	VirSorter	5
		Node 26	VirSorter	5
		Node 6	VirSorter	5
		Node 30	VirSorter	3
		Node 59	VirSorter	3
		Node 15	VirSorter	6
	1295	Node 3	Phaster	Intact
		Node 13	Phaster	Incomplete
		Node 13	VirSorter	2
		Node 3	VirSorter	5
	1296.1T	Node_1	Phaster	Incomplete
		Node 2	Phaster	Incomplete
		Node_21	Phaster	Incomplete
		Node 21	VirSorter	5
		Node 2	VirSorter	5
		Node 20	VirSorter	6
	1309	Node 1	Phaster	Intact
		Node 15	Phaster	Intact
		Node_1	Phaster	Incomplete
		Node_1	Phaster	Incomplete
		Node 2	Phaster	Incomplete

		Node 10	Phaster	Incomplete
		Node 15	VirSorter	2
		Node 1	VirSorter	5
		Node 1	VirSorter	5
		Node 2	VirSorter	5
		Node 10	VirSorter	3
		Node 18	VirSorter	6
		Node 3	VirSorter	6
	1310	Node 3	Phaster	Questionable
		Node 34	Phaster	Intact
		Node 54	Phaster	quesitonable
		Node_5	Phaster	Incomplete
		Node_15	Phaster	Incomplete
		Node_15	Phaster	Incomplete
		Node_25	Phaster	Incomplete
		Node 33	Phaster	Incomplete
		Node 44	Phaster	Incomplete
		Node 54	VirSorter	1
		Node 34	VirSorter	2
		Node 44	VirSorter	2
		Node 33	VirSorter	5
		Node 3	VirSorter	5
		Node 5	VirSorter	5
		Node 14	VirSorter	6
		Node 15	VirSorter	6
		Node 25	VirSorter	6
	1310.1E	Node 12	Phaster	Incomplete
		Node 12	VirSorter	5
		Node 17	VirSorter	3
		Node 24	VirSorter	3
		Node 11	VirSorter	6
5	1346	Node 2	Phaster	Intact
		Node 3	Phaster	Intact
		Node 8	Phaster	Questionable
		Node 3	Phaster	Incomplete
		Node 7	Phaster	Incomplete
		Node 2	VirSorter	5

		Node 3	VirSorter	5
		Node 8	VirSorter	5
		Node 1	VirSorter	6
		Node 20	VirSorter	6
	1353.1Y	Node 3	Phaster	Questionable
		Node 13	Phaster	Intact
		Node 1	Phaster	Incomplete
		Node 7	Phaster	Incomplete
		Node 21	Phaster	Incomplete
		Node 21	VirSorter	2
		Node 13	VirSorter	4
		Node 3	VirSorter	5
		Node 7	VirSorter	6
	1354	Node 2	Phaster	Intact
		Node 6	Phaster	Intact
		Node 2	Phaster	Incomplete
		Node 7	Phaster	Incomplete
		Node 2	VirSorter	4
		Node 6	VirSorter	5
		Node 7	VirSorter	5
		Node 1	VirSorter	6
6	7765	Node 11	Phaster	Questionable
		Node 1	Phaster	Incomplete
		Node 6	Phaster	Incomplete
		Node 11	VirSorter	5
		Node 6	VirSorter	5
		Node 31	VirSorter	3
		Node 41	VirSorter	3
		Node 19	VirSorter	6
		Node 30	VirSorter	6
	7768	Node 3	Phaster	Questionable
		Node 8	Phaster	Intact
		Node 8	Phaster	Incomplete
		Node 9	Phaster	Incomplete
		Node 14	Phaster	Incomplete
		Node 32	VirSorter	2
		Node 33	VirSorter	2

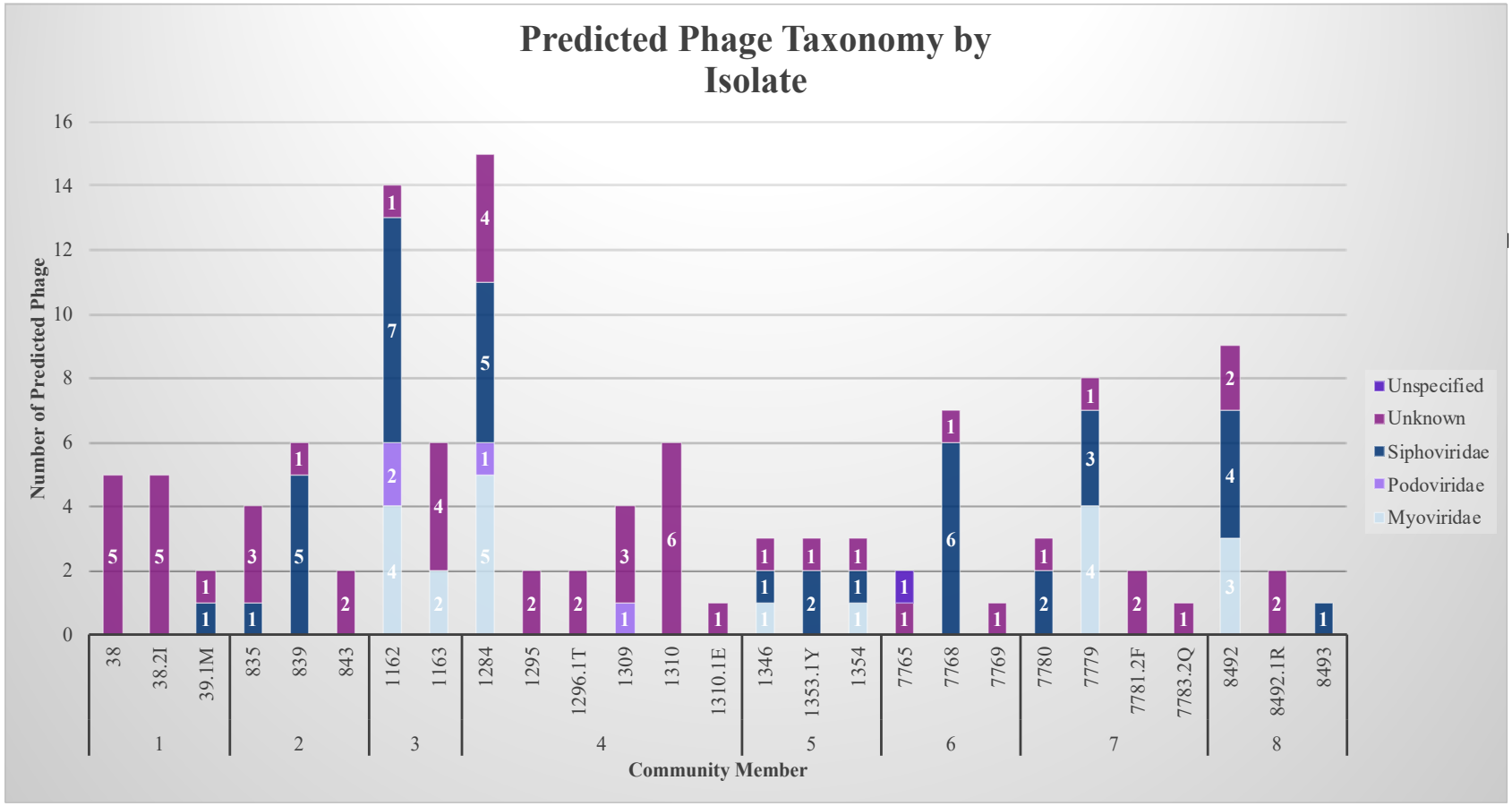
		Node 34	VirSorter	2
		Node 8	VirSorter	2
		Node 1	VirSorter	5
		Node 3	VirSorter	5
		Node 9	VirSorter	5
		Node 29	VirSorter	6
		Node 4	VirSorter	6
	7769	Node 15	Phaster	Incomplete
		Node 25	VirSorter	5
		Node 15	VirSorter	6
7	7779	Node 6	Phaster	Questionable
		Node 14	Phaster	Intact
		Node 58	Phaster	Questionable
		Node 67	Phaster	Intact
		Node 3	Phaster	Incomplete
		Node 29	Phaster	Incomplete
		Node 55	Phaster	Incomplete
		Node 56	Phaster	Incomplete
		Node 58	VirSorter	2
		Node 67	VirSorter	2
		Node 71	VirSorter	2
		Node 11	VirSorter	5
		Node 14	VirSorter	5
		Node 29	VirSorter	5
		Node 3	VirSorter	5
		Node 28	VirSorter	3
		Node 97	VirSorter	3
		Node 33	VirSorter	6
	7780	Node 3	Phaster	Intact
		Node 9	Phaster	Intact
		Node 1	Phaster	Incomplete
		Node 3	VirSorter	4
		Node 1	VirSorter	5
		Node 9	VirSorter	5
		Node 28	VirSorter	3
		Node 10	VirSorter	6
		Node 21	VirSorter	6

		Node 23	VirSorter	6
	7781.2F	Node 2	Phaster	Incomplete
		Node 7	Phaster	Incomplete
		Node 10	VirSorter	5
		Node 7	VirSorter	5
	7782	Node 5	Phaster	Incomplete
		Node 13	VirSorter	6
		Node 1	VirSorter	6
		Node 5	VirSorter	6
	7783.2Q	Node 1	Phaster	Incomplete
		Node 6	Phaster	Incomplete
		Node 10	Phaster	Incomplete
		Nod 17	Phaster	Incomplete
		Node 31	Phaster	Incomplete
		Node 10	VirSorter	5
		Node 27	VirSorter	3
		Node 31	VirSorter	3
		Node 38	VirSorter	3
		Node 40	VirSorter	3
		Node 19	VirSorter	6
8	8490	Node 2	Phaster	Incomplete
		Node 3	Phaster	Incomplete
		Node 6	Phaster	Incomplete
		Node 12	Phaster	Incomplete
		node 14	Phaster	Incomplete
		Node 39	VirSorter	3
	8492.1R	Node 5	Phaster	Intact
		Node 6	Phaster	Incomplete
		Node 5	VirSorter	5
		Node 6	VirSorter	5
	8492	Node 1	Phaster	Questionable
		Node 4	Phaster	Intact
		Node 16	Phaster	Intact
		Node 54	Phaster	Intact
		Node 57	Phaster	Intact
		Node 32	Phaster	Incomplete
		Node 47	Phaster	Incomplete

		Node 63	Phaster	Incomplete
		Node 54	VirSorter	1
		Node 57	VirSorter	1
		Node 61	VirSorter	2
		Node 11	VirSorter	5
		Node 16	VirSorter	5
		Node 19	VirSorter	5
		Node 32	VirSorter	5
		Node 4	VirSorter	5
		Node 106	VirSorter	3
		Node 29	VirSorter	3
		Node 47	VirSorter	3
		Node 92	VirSorter	3
	8493	Node 2	Phaster	Intact
		Node 4	Phaster	Incomplete
		Node 2	VirSorter	5
		Node 30	VirSorter	3
		Node 43	VirSorter	3
		Node 47	VirSorter	3
		Node 4	VirSorter	6

APPENDIX F

PHAGE TAXONOMY BY ISOLATE


Predicted Phage Taxonomy by Isolate
Number of Predicted Phage
Community Member
Unspecified
Unknown
Siphoviridae
Podoviridae
Myoviridae
38
38.2I
39.1M
835
839
843
1162
1163
1284
1295
1296.1T
1309
1310
1310.1E
1346
1353.1Y
1354
7765
7768
7769
7780
7779
7781.2F
7783.2Q
8492
8492.1R
8493
1
2
3
4
5
6
7
8

APPENDIX G

LACTOBACILLUS pH MEASURMENTS

Community	*Lactobacillus* ID	*Lactobacillus* Number	pH r1	pH r2	pH r3	average pH
1	*L. jensenii*	37	4.43	4.44	4.45	4.44
2	*L. jensenii*	847	4.51	4.25	4.41	4.25
3	*L. jensenii*	1165	4.23	4.27	4.35	4.28
4	*L. jensenii*	1303	3.8	3.82	3.92	3.85
5	*L. mulieris*	1355	4.3	4.25	4.26	4.27
6	*L. jensenii*	7766	4.74	4.79	4.53	4.69
7	*L. mulieris*	7784	4.76	4.78	4.79	4.78
8	*L. jensenii*	8489	4.48	4.4	4.34	4.4

APPENDIX H

LACTOBACILLUS HYDROGEN PEROXIDE MEASUREMENTS

Community	*Lactobacillus* ID	*Lactobacillus* number	Fluorescence Measurements			Fluorescence average	Average H_2O_2 concentration
			r1	r2	r3		
1	*L. jensenii*	37	696	654	651	667	8.89
2	*L. jensenii*	847	1283	1251	1467	1333.66667	20.26
3	*L. jensenii*	1165	1065	1216	735	1140.5	16.97
4	*L. jensenii*	1303	1592	1744	1503	1613	25.03
5	*L. mulieris*	1355	688	985	959	877.333333	12.48
6	*L. jensenii*	7766	549	582	1308	565.5	7.15
7	*L. mulieris*	7784	476	586	705	589	7.55
8	*L. jensenii*	8489	1192	1340	669	1266	19.11

APPENDIX J

PHAGE INDUCED BY *LACTOBACILLUS* METABOLITES WITH EMPIRICAL PROBABILITY P-VALUES

Community	Isolate	Phage	.5mL	1mL	2mL	Control
1	38	Node 3 (Phaster 2)	0.0081623		0.0120622	0.0121122
1		Node 3 (Phaster 3)	0.01121222	0.01041224		0.01881203
1		Node 52	0.02276193			0.03311167
1		Node 10	0.01441214			0.06436089
1		Node 1	0.00151246	0.00226244	0.00276243	0.00461239
1	39.1M	Node 1	0.00861229	0.00731232	0.0080623	
2	835	Node 85	0.03271168	0.01786205	0.01946201	0.00906227
2		Node 2	0.01086223	0.00626234	0.00676233	0.00306242
2		Node 65				0.0160121
2	839	Node 26	0.1493588	0.09911002	0.10131	0.05131122
2		Node 1	0.02026199	0.02041199	0.02066198	0.01751206
2		Node 27	0.1910577			
2		Node 4	0.1059599			0.08371041
2	843	Node 12	0.02281193	0.06256094	0.1228094	0.02201195
2		Node 3	0.0039124	0.01346216	0.0400615	0.0039124
2		Node 2	0.01326217	0.03861153	0.2064073	0.01251219
2		Node 1	0.03256169		0.2970051	
3	1162	Node 17	0.00656234	0.0079123	0.00891228	0.00746231
3	1163	Node 16		0.02491188	0.0041624	
4	1284	Node 62	0.04171146	0.1038894	0.2011065	0.05856104
4		Node 65	0.07886053	0.1127584		0.1063098
4		Node 6	0.00916227	0.0199196	0.03823448	0.01176221

4		Node 14	0.09781005	0.16587		0.1304092
4		Node 67	0.05896103	0.1045617		0.08241044
4	1296.1 T	Node 2				0.07141071
4	1309	Node 10	0.00521237	0.00456239	0.01111222	0.01006225
4		Node 15	0.00301243	0.00281243	0.00881228	0.00591235
4		Node 1 (Phaster 1)	0.00091248	0.00096248	0.00311242	0.00196245
4		Node 1 (Phaster 2)	0.01151221	0.01051224	0.03151171	
4		Node 1 (Phaster 3)	0.01391215	0.01291218	0.04856129	
4		Node 1(Phaster 4)		0.00831229	0.02611185	
4		Node 2		0.01716207		
4		Node 3	0.00721232	0.00641234	0.020112	
4		Node 18		0.01506212	0.03701157	
4	1310	Node 3	0.00528781			
5	1346	Node 2+	0.00751231	0.01841204	0.01296218	0.00136247
5		Node 3+	0.03956151	0.05016125		0.0039124
5		Node 20	0.02331192	0.00666233	0.00431239	0.01021224
5	1353.1 Y	Node 21				0.02036199
5		Node 13	0.02636184	0.05466113	0.03946151	0.00736232
5	1354	Node 2+	0.02486188	0.01716207		0.0041124
5		Node 6+	0.00841229	0.00551236	0.0042124	0.00136247
5		Node 7		0.05126122		
6	7765	Node 6	0.00676233	0.00636234	0.00131247	

6		Node 30	0.0428614 3		0.0068623 3	
6		Node 11	0.019862		0.0040124	
6		Node 41			0.0119122	
6		Node 1			0.0145121 4	
6		Node 19			0.0189120 3	
6	7768	Node 32	0.0818604 5	0.0415114 6	0.0703107 4	0.0543111 4
6		Node 33	0.0711607 2	0.0356616 1	0.0617609 6	0.0487612 8
6		Node 34		0.0475113 1		
6		Node 3	0.0063623 4	0.0026124 4	0.0039624	0.0026624 3
6		Node 9		0.0148121 3		
6		Node 1	0.0528611 8	0.0205119 9		
6		Node 8				0.0084122 9
6		Node 14				0.0206619 8
6		Node 29				0.0592610 2
6	7769	Node 25	0.0031124 2	0.0019624 5		0.1052099
6		Node 15	0.0101122 5	0.0061123 5		0.3078048
7	7779	Node 115		0.2178571		0.0349616 3
7		Node 100	0.0232619 2	0.0282117 9		0.0128621 8
7		Node 140		0.1348091		0.0221119 5
7		Node 58		0.0470613 2		0.0157621 1
7		Node 67	0.0082622 9	0.0089122 8	0.0132121 7	0.0066623 3
7		Node 56				0.0271618 2

7		Node 3				0.0042623 9
7		Node 6				0.0015124 6
7		Node 47				0.0246618 8
7		Node 11				0.0108622 3
7	7780	Node 28	0.0272618 2	0.0169120 8	0.0237119 1	0.0237119 1
7		Node 23	0.0375615 6			0.0298117 5
7		Node 1		0.0571110 7		0.0416114 6
7		Node 3	0.0049123 8	0.0034124 2	0.0030124 3	0.0030124 3
7		Node 9	0.0159121	0.0101622 5	0.0094622 6	0.0094622 6
7		Node 10		0.0491112 7		0.0356616 1
7		Node 21		0.0243618 9		0.0167120 8
7	7781.2 F	Node 7	0.0184620 4			
7		Node 10	0.0060623 5			
7	7783.2 Q	Node 38		0.0538111 5		0.0292117 7
7		Node 1		0.0434614 1		0.0245118 9
7		Node 10	0.0297617 6	0.0050123 8	0.0045123 9	0.0021124 5
7		Node 31			0.0372615 7	0.0104122 4
7		Node 6			0.0149621 3	
7		Node 19			0.0266618 3	
7		Node 40				0.0364615 9
8	8492.1 R	Node 6	0.3751031	0.3759531	0.1873078	0.1906577
8		Node 5	0.1226094	0.0669108 3	0.0633109 2	0.568057

8	8493	Node 30		0.0371115 7		

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VITA

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