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Investigations of *Methanobrevibacter* spp. Culture to Enable Archaea Virus Propagation, and Comparison of the Efficacy of Individual Bacteriophages and their Cocktails against *Escherichia coli* O157

by

Jane Madeline Fletcher

A THESIS

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Abstract

Viruses that target prokaryotes are a growing area of interest for control of pathogens and other detrimental microorganisms. Archaeal viruses were identified as an option for the mitigation of methane produced by *Methanobrevibacter ruminantium*, which lives in ruminants such as cattle. In this study we tested three protocols to culture *Methanobrevibacter spp.* While a PCR was able to confirm the presence of *M. ruminantium* in these cultures, we could not achieve replicable growth. Unfortunately, an anti-methanogen virus could not be isolated from rumen fluid, and the culture of the archaea was found to be challenging.

Another microbial target, *Escherichia coli*, was also explored in this study. Phages are used to control pathogenic *E. coli*, however, while phages can be used as support or as an alternative to antibiotic therapy, pre-existing or induced anti-phage mutations in the targeted bacteria can reduce the efficacy of this approach. Phage cocktails have been used as a strategy to reduce the likelihood that phage resistance would develop. However, in some cases, individual phages were found to be more effective at lysing bacteria and at reducing the frequency of resistance development. Therefore, we tested individual (T1, T4, T5, and rV5) and cocktail phage treatments against six strains of *E. coli* O157, including EDL933, R508N, CO281-31N, E32511, H4420N, and E318N. EDL933 was selected for further experiments as this is a well characterized human pathogenic strains. Our results showed that T5 and cocktail T1+T4+T5+rV5 treatments were effectively lysing bacteria, however, we have strong indications that phage resistance developed in both individual and cocktail-exposed EDL933. Cocktail T1+T4+rV5 was more effective at lysing EDL933 compared to T1, T4, or rV5 treatments. Initially the concentration of T1 in T1+T4+rV5, and T5 in cocktail T1+T4+T5+rV5 decreased before increasing to the levels of replication observed in individual treatments. T1 in T1+T4+rV5 decreased over time until it was not detectable. Sequencing of exposed strains and the phages is needed to reveal the genetic basis of resistance. More research into strategic combinations of phages, based on receptor usage or lytic activity, is required to increase the susceptibility of pathogenic *E. coli* to phages or antibiotics.

Keywords: ***Methanobrevibacter spp.*, Methane, Viruses, *E. coli* O157, Bacteriophages, Cocktail, Cattle.**

Preface

Research conducted in this thesis was performed in the Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada. Work described herein was carried out by me, Jane Madeline Fletcher, from September 2018 through December 2021 under the supervision of Dr. Frank van der Meer and Dr. Dongyan Niu. Dina Moinul, Kat Smith, Vivienne Berger, Akeel Faizal, and Dr. Emmanuel Bumunang assisted me with different experiments, including culture setups, *E. coli* plating, and phage plating.

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Finally, I would like to thank all of my friends and family for listening to me talk about archaea, bacteriophages, and viruses for far too much time, and never once complaining about it. Thank you all for your support and love.

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Abbreviations

AAF	Alberta Agriculture and Forestry
AAFC	Agriculture and Agri-Food Canada
ATP	Adenosine triphosphate
Phages	Bacteriophages
CFU	Colony-forming units
CRISPR	Clustered regular interspaced short palindromic repeats
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
GHG	Greenhouse gas
LD50	Lethal dose to kill 50% of the population
M. ruminantium	*Methanobrevibacter ruminantium*
M. smithii	*Methanobrevibacter smithii*
MNA	Modified nutrient agar
MOI	Multiplicity of infection
mTSB	TSB containing 10 mM $MgSO_4$
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming units
PSI	Pounds per square inch
RM02	Rumen medium 2
RPM	Rotations per minute
rV5	*Vequintavirus*
STEC	Shiga toxin-producing *Escherichia coli*
T1	*Rogunavirus*
T4	*Tequatrovirus*
T5	*Tequintavirus*
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VFA	Volatile fatty acids

Chapter 1

1 Literature Review

This chapter reviews current information available about the microbiome of cattle, and the viral influences on both the rumen and the lower digestive tract. This chapter also covers current mitigation methods for methane emissions and control of pathogenic *E. coli*, including phage therapy.

1.1 The bovine microbiome and influences

1.1.1 The bovine microbiome

Bovines are domesticated ruminant herbivores, also referred to as cattle, are a vitally important animal to humans. Currently, there are around one billion head of cattle present in the world, and they hold high value for humans due to their use for food production, fertilizer (manure) and labour [1]. Cattle are capable of digesting low-quality forages and producing high-quality products for human consumption and use, including milk, beef, and leather[2]. Cattle, which are undeniably one of the most important animals in human history, have undergone a long history of selection, and as we move further into the 21st century, that selection extends towards the manipulation of the commensal microorganisms living within many animals[3,4].

A complex microbiome exists within cattle, with bacteria, protozoa, fungi, archaea, and viruses living in and on sites all over the animal, including the rumen and the upper and lower digestive tract[5]. The microbiome of the rumen has a complex suite of interactions, with many end products of certain microbial processes being utilized by other microbes. Bacteria are the most common members of the rumen microbiome, can be both free floating in the rumen fluid (30%), and attached to feed particles, other microbes, or epithelial cells (70%). Bacteria digest cellulose in the concentrates and forages that cattle consume, and convert the otherwise indigestible cellulose to fatty acids and utilize the energy from fatty acids to build amino acids[3,6]. Protozoa contribute to fermentation by producing hydrolytic enzymes to degrade components of feed, and predate upon bacteria in the rumen and produce hydrogen gas. Defaunation (removal of protozoa from the rumen) can lower methane production, but can also result in a less stable fermentation[7,8]. Ruminal fungi are comprised of both yeasts and molds, with molds being identified as more important to ruminal digestion. Ruminal fungi can produce enzymes required for the breakdown of feed, including hemicellulases, pectin lyases, amylases, and proteases[1]. The only archaea identified in the rumen thus far are methanogens. These methanogens utilize hydrogen and formate as energy sources, which are both products of digestion by other microorganisms. Methanogens are the producers of methane, which is a major contributor to anthropogenic methane emissions, leading to atmospheric warming[9]. Finally, viruses are numerous in the rumen, infecting all microbial populations, although the bacterial viruses are the best characterized. Viruses are important to the maintenance of the microbial population by lysing infected microorganisms, thus ensuing nutrient cycling among the microbes[10].

The lower digestive tract of cattle is also populated with a diverse microbiome. Cattle are a reservoir of *Escherichia coli (E. coli),* of which several strains can cause severe disease in humans. In particular a zoonotic infection of the cattle-derived *E. coli* O157:H7, also known as Shiga toxin-producing *E. coli* (STEC), can result in symptoms ranging from diarrhea and nausea to renal disease or even death. A human STEC infection can result from consuming infected water or food such as beef, vegetables, fruit, and flour, or from contact with another infected individual[11].

1.1.2 Viruses of prokaryotes

Viruses are obligate intracellular parasites that can only reproduce by infecting host cells and utilizing their replication machinery to produce progeny viruses. Viruses that target prokaryotes, also known as bacteriophage or simply 'phages', and archaea viruses are the most numerous biological entities on earth, with estimates of 10^{30} phages in the oceans alone[12]. Phages are also present in high numbers in any location where bacteria can be found[13]. Phages and archaeal viruses are also a major factor in microbial turnover, which is the process whereby nutrients are returned to the environment for subsequent use by other microbes [14,15].

Phages can have two life cycles, lytic and lysogenic (Figure *1.1*), however, not all phages have a lysogenic component to their life cycle. Phages that are capable of lysogeny are also termed temperate phages. The lytic cycle occurs when a phage attaches to a host cell, injects the viral genetic material into the host cell, prevents the host cell from synthesizing host proteins, utilizes host cell machinery to create proteins for new virions, assembles the proteins into the protein coat encapsulating the viral DNA, and lyses the host cell to release the progeny. The lytic cycle results in the death of the host cell and is important for host population control and biogeochemical cycling[14,16]. The lysogenic cycle occurs when instead of replicating, the viral genetic material that is injected into the host inserts itself into the host genome which results in a prophage, which is the phage genome integrated into the bacterial chromosome. The prophage is copied in subsequent daughter cells and remains in the host genome until an event induces exit of the prophage leading to replication of phage progeny. This replication event can be induced through temperature changes, oxidative stress, antibiotic use, chemical presence, UV exposure, or anything else that damages the host genome. Once the induction event occurs, the phage resumes the lytic cycle as described[17].

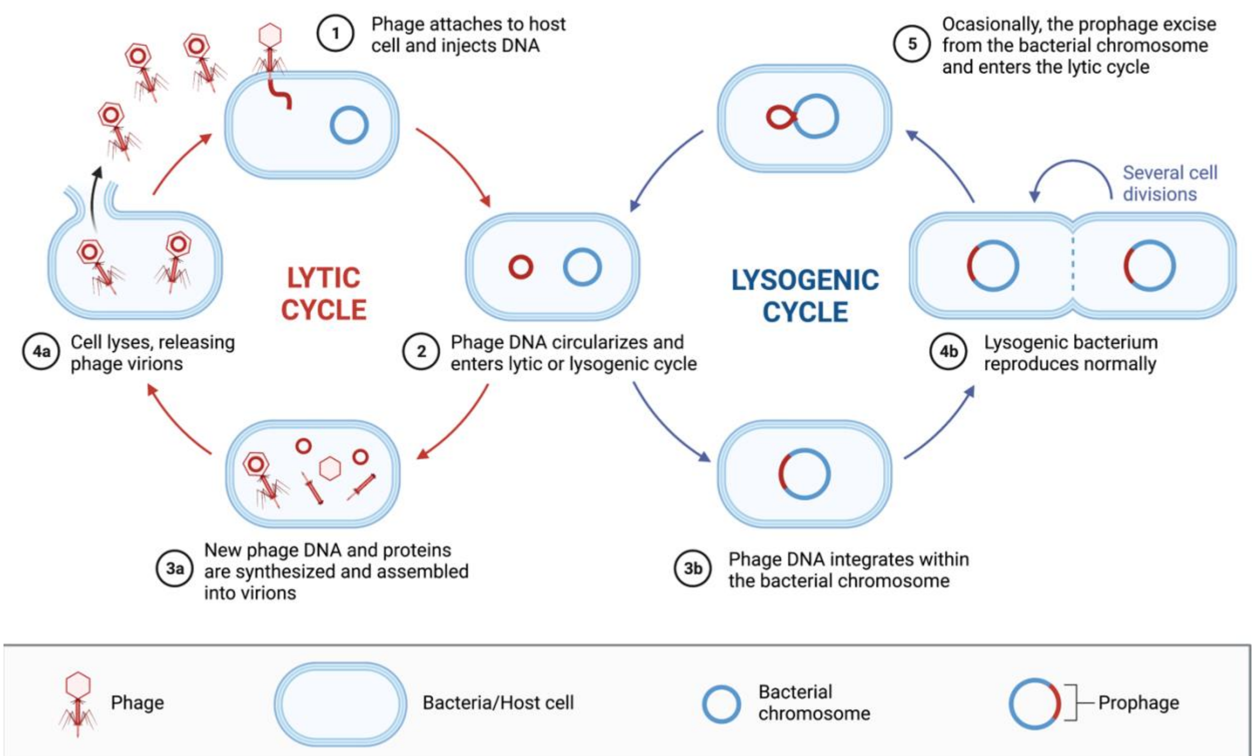


Figure 1.1: Lytic and lysogenic cycle of bacteriophage replication

Phages were identified more than a century ago, when Fredrick Twort noted that his bacterial colonies were turning transparent and died from infection with what he theorized was an "ultra-microscopic virus"[18]. Since then, phage therapy has been utilized in several ways, although its use was largely overshadowed by the growing popularity of antibiotics. Antibiotics were cheaper, more easily obtained, and exhibited a broader range in bacterial targets. Phage therapy for humans was still in use in the former Soviet Union countries, especially Georgia, throughout the 20th century, but in Western countries it largely fell out of use in favour of antibiotics. However, as antibiotic resistance becomes a growing concern, more researchers are studying phage therapy for human, veterinary, and agricultural use[19].

An advantage of using phages is that, unlike antibiotics, phages are often highly specific to a single bacterial host. This specificity means that treatments can be targeted specifically to a singular microbe species instead of causing damage to a whole microbial community. Extensive antibiotic use may result in opportunistic infections with, for example, *Clostridium difficile*[20]. The use of phages also does not contribute to antibiotic resistance, and as the demand for antibiotic-free food grows, phages may be seen as a more natural alternative by the public[21]. Phages and antibiotics utilize different killing mechanisms, and as such, they potentially can be used against antibiotic resistance bacteria[23]. Additionally, while one main advantage of phage therapy was thought to be the concept of "autodosing", where it is thought that only one round of treatment with phages is required, as the phages, when coming into contact with the target bacteria, would lyse the bacterial cells to produce more phage particles, thus eliminating the need to add more phages to the system[24,25]. However, there is debate as to the actuality of this phenomenon, as phage amplification has not been found in some experimental trials, with phage titres generally decreasing after dosing when applied to *Listeria monocytogenes*. This is thought to be due to the random nature of the interactions between phages and their target bacteria, and as the bacterial concentration decreases due to lysis, the likelihood of a phage particle encountering a bacterial cell decreases[26,27].

One disadvantage to phage therapy is the difficulty in finding a specific phage that is well-suited for therapy. The ideal therapeutic phage is lytic, not temperate, stable enough for long-term storage, safe for use in the desired animal or human and easy to produce in large quantities[28]. As the insertion of certain prophages can result in the bacterial production of endotoxins, it is very important to both select a phage lacking any virulent elements, and only utilize purified phage preparations[29]. A temperate phage is not advantageous for a biocontrol strategy, as an induction event is required to finally lyse and kill the target bacteria[29]. The specific nature of phages requires the identification of the target to be successful[30]. In addition, although phage preparations are generally considered to be safe, when all necessary precautions are taken[29], the unfamiliarity with phage treatments in Western countries of a treatment consisting of a virus may result in public resistance, despite an increasing resistance to antibiotic-treated food[21].

Phages are common in bacterial-dense environments such as sewage and cattle feedlots, and after collection, the phages can often be isolated if the host bacteria are culturable[31]. Phages can be isolated from the environment through a series of filtration and centrifugation to separate the viruses from bacteria and other microorganisms before being cultured in the host bacteria[32,33].

Although the use of phage therapy in human disease is rare in Western countries, phage therapy is already in use to improve food safety[34]. EcoShield for example, a commercial phage cocktail containing three separate phages, reduces the amount of *E. coli* O157:H7 when used on contaminated tomatoes, broccoli, spinach, ground beef, lettuce, and cantaloupe[35,36]. Other commercial phage cocktails in use include SalmoFresh for treating *Salmonella spp.* colonization of chicken breasts and skin[37,38] and Listex for treating *Listeria monocytogenes* colonization of cheese, milk, raw fish, and vegetables[39,40].

1.2 Archaea

Archaea is one of the three domains of life, with the other two being Eukaryota and Bacteria. Although archaea and bacteria have multiple similarities, including their size, unicellularity, lack of structured cell organelles, and reproduction methods, the domains Archaea and Eukarya are phylogenetically more similar to each other than to Bacteria[41]. Archaea share a circular chromosomal structure with Bacteria but have similar transcription and translation processes to Eukarya. Archaea also utilize ether-linked lipids in cell membranes, whereas both Bacteria and Eukarya utilize ester-linked lipids[42]. There are currently no known archaeal pathogens, and many archaea have symbiotic relationships with other organisms[43].

Up until 1990, archaea were considered to be a type of bacteria under the prokaryotes, but a case was made that there should be a new classification system based on molecular similarities and differences[44]. This system classified cells based on their ribosomal RNA (rRNA) genes using 16S ribosomal sequencing. The rRNA characteristics of Archaea include both a hairpin loop bulge shared with Eukaryota, a characteristic prokaryotic rRNA structure, and another rRNA structure seen only in Archaea. By defining differences based on molecular structure rather than microscopically visible characteristics, Woese and his colleagues suggested three separate domains of life[44]. This newly created domain system altered the previously classified five Kingdoms of life, by separating Bacteria and Archaea.

Although archaea are known for their presence in extreme environments of which halophiles in salt ponds and the hyperthermophiles in hot springs – best known as the source of Taq polymerase from *Thermophilus aquaticus* – are examples, many archaea were discovered in moderate environments[45]. One difficulty with identifying members of Archaea is that many are not easily culturable *in vitro*, leaving rRNA sequencing as the best option for identification.

1.2.1 Methanobrevibacter spp.

The group of archaea of interest for this project is the genus *Methanobrevibacter*, which are methanogens, members of Euryarchaeota. *Methanobrevibacter ruminantium* is a methanogen present in the foregut, specifically the rumen of ruminants. Feed breakdown in the rumen occurs through multiple microorganisms, almost all of which have close and influential interactions with each other in the rumen. Plant material consumed by the ruminant goes through several cycles of mastication and fermentation (known colloquially as “chewing the cud”). As the animal regurgitates and chews rumen contents, plant material is broken down into smaller pieces. Various bacterial species metabolize the carbohydrates present in plant material and turn those into volatile fatty acids (VFAs). VFAs are utilized by the animal as an energy source, whereby carbon dioxide is produced[46]. Protozoans predate upon these bacteria and produce hydrogen gas[8].

M. ruminantium is one of the endpoint metabolizers, as it utilizes hydrogen gas to reduce carbon dioxide to methane to produce ATP, and this methane is excreted by the ruminant mainly through the process of eructation, or belching[47].

This methane is released into the environment, and is a contributor to climate change as a greenhouse gas (GHG). Methane in particular is a potent GHG that has 28 times the radiative forcing potential of carbon dioxide[48]. Enteric methane from domesticated ruminant fermentation accounts for 87-97 teragrams (Tg) of methane per year[49], therefore research is conducted which aims to reduce these emissions. With the global demand for livestock products doubling in the past 40 years and expected to continue to increase over the next 20 years[50], a need for reduction of the environmental impacts of livestock production is required.

In our project we also explored the use of *Methanobrevibacter smithii,* this is a common methanogen present in the human digestive tract[51]. Although *M. smithii* is usually the major methane producing organism within the human gut microbiome, the amount is negligible compared to methane produced by cattle[52]. *M. smithii* fills a similar niche within the human digestive tract, removing hydrogen gas from the microbiome and producing methane[53].

1.2.2 Methane mitigation

Methods that were explored for methanogenesis mitigation include: defaunation, where protists are removed from the rumen[54]; vaccination against methanogens present in the rumen[55,56], probiotics, to alter rumen composition[57], antibiotic additives to feed such as ionophores[57], plant extracts such as tannins and seaweeds addition to feed[58–60], chemical additives such as 3-nitrooxypropanol[61,62], and alteration in feed type, both forages and concentrates[63,64]. These strategies have had mixed success, as a key element of methane mitigation is to lower emission without adversely impacting rumen function. Altering the rumen microbiome is difficult, as the numerous complex interactions between the bacteria, protists, fungi, archaea, and viruses result in tightly-held niches that often have multiple redundant members. As a result, elimination of a particular type of microbe often results in its vacated niche being filled by counterparts within the population[3,6].

Archaeal viruses, while likely as ubiquitous in the environment as their archaeal hosts, are not nearly as well studied as bacteriophages[65,66]. This is partly due to the difficulty of culturing archaea in the laboratory, as the inability to culture viral hosts makes it challenging to isolate the virus. One review described nearly 6300 prokaryotic viruses, of which 6196 were bacteriophages, compared to only 88 archaeal viruses[67].

The investigation into the use of archaeal viruses to combat methane production from ruminants became of interest recently, especially since the sequencing of *M. ruminantium* revealed evidence of a prophage gene within its genome[47]. Since its discovery, attempts were made to use a viral enzyme to reduce *M. ruminantium* in the rumen[68]. Utilizing an existing biocontrol method such as a whole virion or a component of a virus has the advantage of not introducing a foreign element into the animal.

However, there are obstacles to working with methanogens. One of the main difficulties is that fastidious nature of *M. ruminantium* and the unique methods needed to culture it within the laboratory. *M. ruminantium* is an obligate anaerobe, which presents difficulties in a laboratory setting. The degree of anaerobicity required for *in vitro* culture exceeds the level possible when an anaerobic chamber is used. Consequently, as described below, specific culturing techniques are required to successfully culture archaea.

1.3 *E. coli*

Escherichia coli, or *E. coli,* is a common member of the mammalian gut microbiome, but is also found in the hindgut of reptiles, fish, and birds, as well as in soil, water, and plants. *E. coli* is one of the most thoroughly studied organisms in existence, as the growth is typically consistent and rapid, with a wide range of application as a model organism[69,70].

E. coli exists as a commensal microorganism within the human lower digestive tract, and produces essential vitamins such as K and B12[71,72]. *E. coli*, as a key member of the gut microbiome, contributes to the exclusion of pathogenic bacteria by occupying a niche, generally the thin mucosal layer that lines the gut epithelia[73].

However, *E. coli* can also be pathogenic in certain circumstances. *E. coli*, when introduced into a human host outside of the gut, can result in opportunistic infections, where an otherwise benign microbe causes infection when given the chance. This infection can be caused by a variety of routes, including oral consumption, intestinal punctures, urinary tract contamination. Immunocompromised individuals have a higher probability of becoming infected with *E. coli* due to the bacteria taking advantage of a weakened immune system[74].

1.3.1 Pathogenic *E. coli*

E. coli also exists in a more pathogenic form when virulence factors are introduced into the cell. There are six categories of pathogenic intestinal *E. coli*: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC)[75]. The six categories have different methods of colonizing intestinal epithelial cells, including producing different toxins, entry into the host cell, and generally resulting in the death of the host cell (Figure *1.2*)[75]. The focus of this thesis was on EHEC, infection with which can cause serious disease and death in humans.

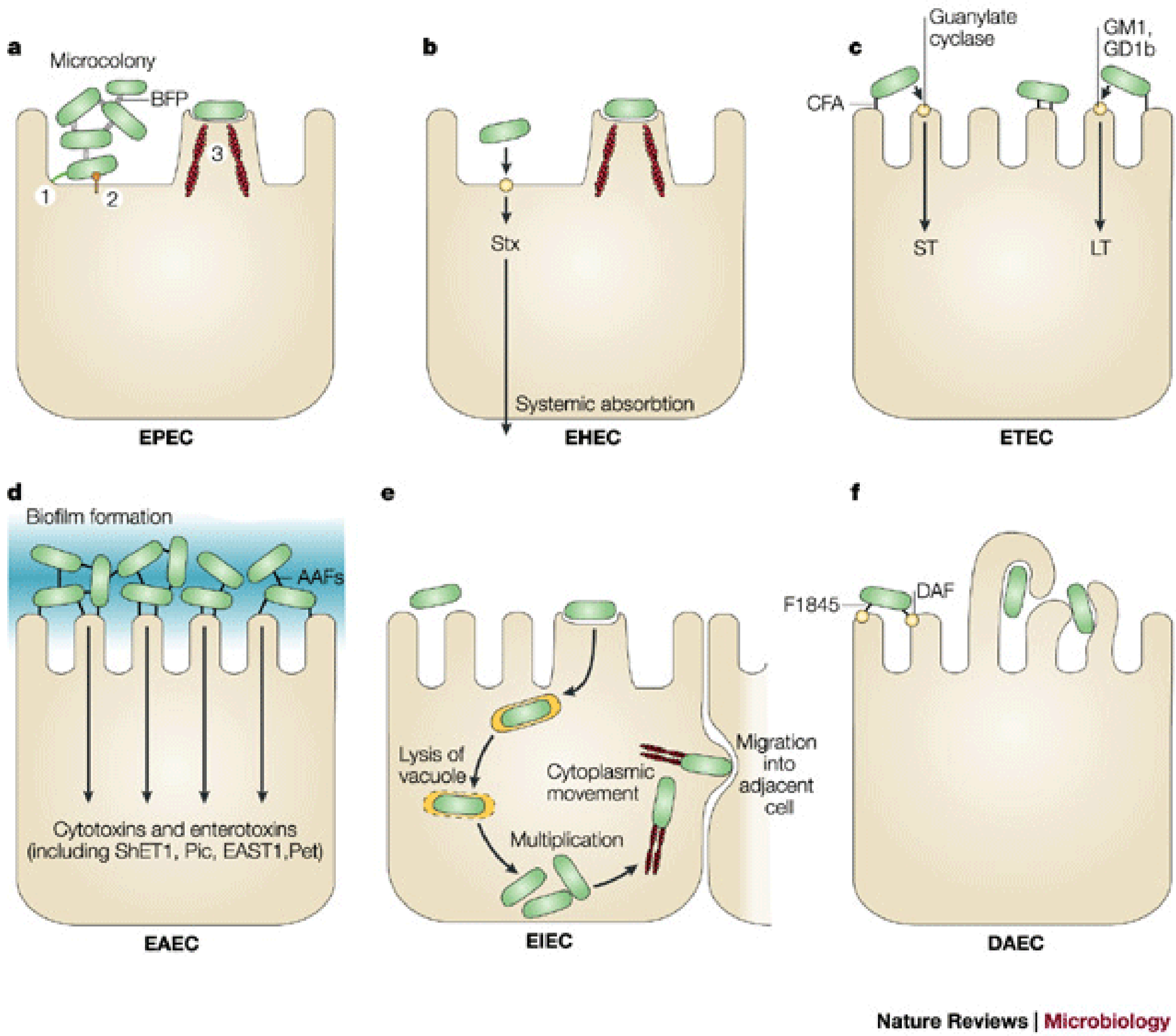


Figure 1.2: The effect of six types of pathogenic intestinal *E. coli* on intestinal cells. Image from Pathogenic *Escherichia coli*, Kaper, 2004[75]

EHEC is also commonly referred to as Shiga-toxin producing *E. coli* (STEC), which was known as *Shigella spp.* until genetic sequencing revealed it was *E. coli* with certain virulence factors[76]. Non-pathogenic *E coli* can become an STEC strain through the process of transduction of virulent genetic elements through a prophage carrying the virulence factors. Transduction is a type of horizontal gene transfer where genetic material from one bacterial cell is transferred to another, naïve cell. This genetic material can be integrated into the genome of the new bacterial cell, remain separate as a plasmid, or can be lost. This virulence factor can be replicated with the bacterial chromosome during bacterial reproduction and passed down to future generations of bacteria[77].

One of the key virulence factors of STEC is the production of Shiga toxin. Shiga toxin is a holotoxin, i.e. a protein toxin comprised of more than one subunit, with the subunits divided into two main subtypes, Stx1 and Stx2 [77]. Both types are encoded on lysogenic phage that have integrated as a prophage into the bacterial chromosome. The two types of Shiga toxin have the same mode of action, which is to prevent protein synthesis in the host cell by destroying the 28S rRNA, leading to apoptosis[78].

STEC lives within the cattle gut microbiome as part of the normal gut flora. As cattle lack the intestinal receptors for Shiga toxin, STEC is not considered to be pathogenic within the cattle host[79]. However, when humans acquire STEC, they can develop severe disease and potentially die. STEC infection can result in haemorrhagic colitis as intestinal cells are killed, which can lead to intestinal necrosis and perforation. Shiga toxin can also enter the circulatory system from the intestinal epithelial cells, where it can travel to the kidneys and damage renal epithelial cells. This damage can result in haemolytic uremic syndrome (HUS) where the kidney blood vessels become damaged and inflamed, kidney failure, and death. The conditions arise from both direct damage from Shiga toxin, and from cytokine production from the immune system, which results in inflammation of the local tissues[74].

In the US alone, there are over 175, 000 cases of STEC-caused food poisoning per year[80]. As the Shiga toxin gene is encoded by the late prophage gene, any damage to the genome can result in increased expression of Shiga toxin. This means that treatment of STEC infection in humans is difficult, as the presence of antibiotics at levels required to inhibit bacterial growth, increases Shiga toxin production up to 140 fold[81]. Generally, treatment consists of symptom management, including IV rehydration to address fluid loss from diarrhea, and peritoneal dialysis to mitigate early cases of HUS, as HUS can lead to other complications such as blood clots in the kidney blood vessels, and can result in kidney failure[82].

STEC has multiple well-characterized serogroups, identified by the different molecular composition of the O antigen[83]. While there are at least 184 recognized O serogroups of *E. coli*, with several in particular that are known for causing disease in humans, including O26, O103, O111, O121, O45, O145, the O157 serotype remains the most commonly detected pathogen in outbreaks of STEC in the Western world[84].

1.3.2 Mitigation methods

As mentioned above, STEC infection in humans is difficult to treat and as such, a method of interest is to remove STEC from food production systems before it comes in contact with consumers. This can be accomplished through the use of pasteurization, various chemicals, ultraviolet radiation, gamma radiation, and filtration, but all methods of disinfection are not suitable for all foods – for example the inability to heat fresh vegetables and still preserve the same quality, or being unable to filter non-liquid foods – leading to the need for additional methods of removing STEC[85].

One method being investigated to control STEC is the use of phages within food production systems. There is growing interest on the use of phages to control STEC both before it enters, and while it is present in the food production system. One method of using phage to control STEC is to add phages to disinfect products such as vegetables with a phage spray, or milk by adding phages[34,86]. There are also studies into disinfection of the immediate environment of production animals such as chickens through the use of phage sprays[34]. More experimental research is being done to use phage therapy within cattle, as a means of eliminating these pathogenic bacteria at the primary reservoir[87]. While there were successes with the use of phages against *E. coli* O157, multiple studies of the use of phages as a biocontrol method found that phage cocktails were effective at initially reducing the population of bacteria, regrowth was common[88] and reinfection was observed[89]. In another publication, a three-phage cocktail was evaluated for the control of *E. coli* O157 on beef, and was effective in the majority of cases. In many cases bacteria became insensitive to these phages, which resolved generally within 50 generations[90].

While phage are currently being used in food production systems to control certain pathogens such as *E. coli*, one major issue with the use of phage against bacteria is that bacteria can not only rapidly acquire mutations that confer resistance against phages, but also have existing defence mechanisms against infection by phages, such as the protection of external receptor proteins by exopolysaccharide structures[91], or systems such as CRISPR (clustered regularly interspaced short palindromic repeats) Cas9, a prokaryotic defence system that stores fragments of DNA from phages that have infected the bacterial cell previously, and Cas9 enzyme degrades any recognized phage genomic sequences[92]. Certain outer membrane proteins on bacteria are utilized by phages to attach and enter into the cell, such as O and K antigens, or type 1 fimbriae among others. Mutations of these receptors, such as OmpC in O157:H7, can reduce the ability of phages to attach and start the infection process[93].

Bacteria, including *E. coli,* can also produce outer membrane vehicles that consist of outer membrane lipid and outer membrane protein and share several receptors with the bacterial cell. These outer membrane vehicles can act as decoys for phage attachment, leaving fewer phages to infect the bacterial cells[94].

One approach to combat this bacterial resistance is the use of phage cocktails, or multiple phages within a single treatment, with the aim that the presence of multiple phages may hinder the development of resistance. Phage cocktails have advantages and disadvantages when compared to individual phages. Individual phages may not always be as effective as a cocktail of phages[95], but are simpler to produce, and there are individual phages that are highly lytic[96]. Phage cocktails require a higher cost to produce and maintain a diverse phage bank[96]. While cocktails were generally considered to be more effective at lysing bacteria, recent research suggests that the lytic nature of phages may result in antagonistic effects when presented together in a cocktail leading to phage interference and, as a consequence, reduced lysis[97]. Phage interference may result through a number of mechanisms, including abortive infections, where a bacterial cell infected by a phage particle will self-lyse before the phage particle has produced mature progeny phage or by certain phages outcompeting others in growth[98]. Superinfection exclusions have been found to occur with T4 phages, where infection by one phage can produce proteins that block entry of additional phage DNA into the host cell[99]. Phage can even block their own receptor, as found in a T5 infection of *E. coli* where a lipoprotein was produced that blocked the ferrichome-iron receptor utilized by T5 and prevented superinfection[100]. Other publications have explored the use of phage cocktails against *E. coli* O157, but have encountered difficulties with the development of anti-phage resistance and a lack of protection against reinfection[88–90]. This has led to our exploration of the efficacy of phage cocktails and how rapidly anti-phage resistant mutant bacteria develop.

The phage selected for our experiments were AKFV33 (*Tequintavirus*, T5), AHP24 (*Rogunavirus*, T1), wV7 (*Tequatrovirus*, T4), and AHP24S (*Vequintavirus*, rV5). All four phage have demonstrated lytic activity against STEC O157:H7[97]. Previously, among the 11 phage cocktails of combinations of the 4 phages, the phage cocktail T1+T4+rV5 was most effective against *E. coli* O157 at 37°C in-small scale broth cultures[97].

1.4 Statement of rationale

While antibiotics remain highly valuable tools for both the prevention and the treatment of infection, there is a growing need for alternative options. Antibiotic resistance is an increasing threat, rendering certain antibiotics less effective over time. In cases of STEC infection, antibiotics are not recommended for use, further increasing the need for different mitigation methods and treatments. A promising avenue of research is the use of phages and other viruses against unwanted microorganisms and could potentially be utilized as a supplement or even a replacement for traditional antibiotic therapy.

M. ruminantium is the primary source of methane emissions from domesticated cattle and other ruminants. As knowledge of the environmental impacts of methane emission increases, a growing area of research is in methane mitigation. Feed additives, supplements, and vaccines have all been promising areas of research, as well as the focus of my project, viruses that target these methanogens. Reducing the amount of methane produced by cattle has potential for positive environmental impact, and utilizing an existing virus to reduce the number of methanogens has the advantage of not introducing a foreign element into the animal.

Domesticated cattle are a major source of *E. coli* O157, a pathogen that can cause severe disease and death in humans. Phages demonstrating lytic activity against *E. coli* O157 were identified, and commercial treatments are already in use against *E. coli* O157, but there are issues with phage treatments that require further work to address. Exposure of bacteria to phages can result in anti-phage mutations, which can render the phage treatment less effective, and the interactions that take place between phages are not fully understood. Examining the efficacy of cocktail treatments of phages has the potential to broaden our understanding of interactions between phages and bacteria, and among phages themselves.

1.5 Research questions

1. Can existing protocols be adapted to reliably culture *Methanobrevibacter spp. in vitro*?
2. Can a virus that lyses *Methanobrevibacter spp.* be isolated from rumen fluid?
3. Will the application of phage cocktails to *E. coli* O157 prove advantageous over single phage treatments?
4. How does phage amplification differ between cocktail and individual phage treatments?

1.6 Hypotheses

1. An archaeal virus isolated from rumen fluid can be used to reduce the number of *Methanobrevibacter spp.* present in an in vitro culture.
2. Phage cocktails are not necessarily more effective at suppressing growth of *E. coli* O157 than individual phages at 37°C.
3. Phage amplification in a phage cocktail will be decreased compared to phage amplification of individual phages.

1.7 Objectives

1. To develop a system to reliably culture *Methanobrevibacter spp.*
2. To examine different methods of measuring growth of *Methanobrevibacter spp.*

3. To compare anti-O157 effectiveness between phage cocktails and individual phages in a broth culture system.
4. To compare phage amplification between individual and cocktail phage treatments.

Chapter 2

2 Culture of *Methanobrevibacter spp.*

2.1 Introduction

The growing interest in the use of phages to lyse undesirable microbes has led to potential applications beyond targeting pathogenic bacteria. Methane is a major GHG that has a severe environmental impacts, with the main source of methane from agricultural emissions being a product of methanogenic archaea that live within the rumen[49]. There have been recent investigations into the use of components such as the lytic enzyme of an archaeal virus to target the methanogenic archaea within the rumen[68], and to study rumen archaea, culture protocols for *Methanobrevibacter spp.* need to be adapted for use in the local laboratory, including reliable methods of measuring growth.

2.2 Materials and methods

2.2.1 Culture and measurement of *Methanobrevibacter spp.* growth

We evaluated 3 different culture media that were described in literature or used in the Agriculture and Agri-Food Canada Lethbridge (AAFC, Lethbridge, Canada) and the AgResearch laboratories for *M. ruminantium* and *M. smithii in vitro* culture: Deutesche Sammlung von

Mikroorganismen (DSMZ, Braunschweig, Germany) 141[101], modified DSMZ 141, and rumen medium 2 (RM02)[102], these are detailed below.

Culture media was made according to the DSMZ 141 instructions[101], as described on the DSMZ website (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf). All chemicals and other components were purchased from VWR (Edmonton AB, Canada). The protocol is fully detailed in the appendices (5.1: DSMZ 141 media), but briefly: the components are combined and oxygen is removed through the use of CO_2 sparging (bubbling gas through the media) and the addition of cysteine, a reducing agent. An alteration was made to the original protocol (Jay Yanke at the Lethbridge AAFC, personal communication) therefore 100% CO_2 gas was used for sparging instead of the indicated 80% H_2 and 20% CO_2, while heating the media to improve oxygen removal, this avoid the use of highly flammable H_2. The media was dispensed under anaerobic conditions to Hungate-type tubes (VWR) and autoclaved for 20 min at 121°C and 15 PSI. The Hungate tubes had both a rubber stopper to form an airtight seal, and a plastic screw cap that covered the rubber stopper (Figure 2.1).

A variation on the DSMZ 141 media according to Mr. Yanke was also evaluated, here referred to as Jay's media. The alterations for 1000 mL of Jay's media were as follows:

DMSZ media (5.1) with the addition of:

- 60 mL of a volatile fatty acid mixture (5.2)
- 50 mL of clarified rumen fluid (rumen fluid autoclaved at 121°C and 15 PSI for 20 minutes, followed by centrifugation for 20 minutes at 10,000g at 4°C to remove particulate)

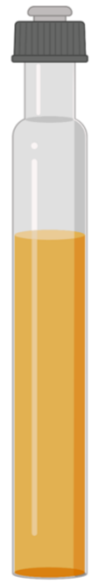


Figure 2.1: Hungate-style tube with rubber stopper and plastic screw cap. Image created with BioRender.com

To prepare Jay's media, all media ingredients except for bicarbonate, vitamins, and cysteine, were combined in a round-bottomed flask. Concentrated NaOH was used to adjust the pH to 6.5. Bicarbonate was added, and the media was heated using a Bunsen burner. Once the media was boiling, cysteine was added to help remove oxygen while being sparged with 100% CO_2. A resazurin indicator was used to monitor the presence of oxygen in the media, which is pink when oxygenated and colourless when not oxygenated. With the addition of the clarified rumen fluid, the final media was a pale, clear yellow fluid. Nine mL of the media was dispensed as described above.

To briefly summarize the use of the anaerobic setup with multiple gas cannulas (Figure *2.2*), the small gas cannulas (A/B) exclude atmospheric air from entering the Hungate tube, and a serological pipette (C) was used to continuously sparge the media with CO_2 while preparing and dispensing the media, and the valve dispenser (D) was used to draw 9.5 mL of media and dispense it as described above. Once the tubes cooled down, 0.5 mL of vitamin solution (which had been separately filter sterilized and sparged with N_2 gas) was added to each tube. The tubes were pressurized with anaerobic CO_2 and stored at 4°C until used.

Another type of media was used according to instructions from Dr. Peter Janssen of AgResearch in Palmerston North, New Zealand[102]. The full protocol of RM02 can be found in appendix (5.3: Rumen Medium 2 (RM02)), briefly: the media components were combined and oxygen was removed by boiling and sparging with 100% CO_2. The media was dispensed under anaerobic conditions to Hungate-type tubes and autoclaved for 20 minutes at 121°C and 15 PSI. A rumen fluid vitamin mix was prepared by centrifuging and autoclaving rumen fluid before adding $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ to precipitate colloidal materials, and subsequently adding a vitamin mix. This rumen fluid vitamin mix was added to the RM02 base media post-autoclave at a 5% addition.

To grow the archaea, the Hungate tubes were anaerobically inoculated with frozen archaea cultures that had been partially defrosted. Once the tubes were sealed, a gas mixture of 80% H_2 and 20% CO_2 was used to vent and pressurize the tubes to 12psi using a sterile needle inserted through the rubber stopper of the tube. As the methane production results in a reduction of pressure within the sealed tube, over pressurization is required to avoid a vacuum forming within the tube.

The tubes were first incubated for 24 h in a 39°C stationary incubator, followed by incubation in a shaking incubator at 50 RPM and 39°C, while periodically growth was monitored. All cultures were incubated in the dark, using aluminium foil wraps, to prevent light exposure and mimic the conditions of the rumen. The shaking facilitated gas penetration into the media.

Growth was measured using two separate methods. Initially, while at the Lethbridge Agricultural and Agri-Food Research Centre, gas chromatography was used to determine CH_4, CO_2, H_2, and O_2 percentages using the Agilent 490 Gas Chromatograph (Santa Clara CA, USA). Samples were drawn from the headspace of the Hungate tubes using a sterile syringe and injected into 5 mL vacutainers, which were used for chromatography. The tubes were repressurized using a gas mixture of 80% H_2 and 20% CO_2.

While at the laboratory at the University of Calgary, to estimate archaea growth turbidity was tracked using the Genesys 30 Visible Spectrophotometer (Thermo-Fisher Scientific, Waltham MA, USA). A tube of uninoculated media was used as the blank.

Initially *M. ruminantium* 5229A from the Lethbridge Agricultural and Agri-Food Research Centre was cultured in DSMZ 141 and Jay's media, and growth was monitored using the chromatograph to measure methane. The initial frozen culture was partially thawed and added directly to a Hungate tube of DSMZ 141 media. The media from this tube was subsequently used to inoculate other tubes of both DSMZ 141 media and Jay's media.

The next batch of *M. Ruminantium* 5229A was cultured in RM02 and growth was monitored using the spectrophotometer. The inoculate (1 mL) was taken from the tubes with the highest turbidity and were used to inoculate 9 mL of fresh media. The inoculum sources can be found in Table *2.1*.

The next batch of *M. ruminantium* and *M. smithii* were ordered from DSMZ and used to inoculate RM02 media, which was measured using the spectrophotometer. *M. smithii* was added to determine if another closely related methanogen could be cultured in the same media.

2.2.2 PCR evaluation of the presence of *M. ruminantium*

DNA was extracted from rumen fluid from pelleted material and supernatant, and a culture of *M. ruminantium* from DSMZ (both pelleted and the supernatant) using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). A PCR (polymerase chain reaction)[103] was used to detect DNA from *M ruminantium*, with the DNA derived from the cultures aas described above used as a positive control. Briefly, primers targeting the *nifH* gene of *M. ruminantium* (Mrnif-f: 5′-AATATTGCAGCAGCTTACAGTGAA-3′; Mrnif-r: 5′-TGAAAATCCTCCGCAGACC-3′) were used. Amplification reaction mixtures consisted of (20 uL) of 1× PCR buffer, 200 μM deoxynucleoside triphosphate (dNTP), 1 U Taq polymerase, 25-50 μM of MgCl, 0.5 μM of each primer, 0.2 uL of bovine serum albumin, and DNA template. The PCR protocol was: 2 minutes at 92°C, followed by 30 cycles of 30 seconds at 92°C, 15 seconds at 62°C, and 30 seconds at

72°C, and 6 min at 72°C, using a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules CA, USA). The resulting product was run on a 1% agarose gel at 120 V for 30 minutes and imaged using a Bio-Rad VersaDoc MP 4000 Molecular Digital Imaging System (Bio-Rad) and visualized using Quantity One software (Bio-Rad).

M. ruminantium and *M. smithii* cultures were also preserved using DSMO solutions. The full protocol is available in the appendices (5.4: *Methanobrevibacter spp.* preservation solutions), briefly, DSMO, salt solutions, and resazurin are boiled together and sparged with 100% CO_2, dispensed into Hungate tubes, and autoclaved for 20 minutes at 121°C at 15 PSI. One mL of the 50% DMSO solution was anaerobically added to 9 mL of *Methanobrevibacter spp.* culture, cooled at -20°C for 24 h, and stored at -80°C. To use these cultures, the frozen Hungate tubes were thawed in cold water, and 1 mL was added to 9 mL of fresh RM02 media. The cultures were then incubated as described above.

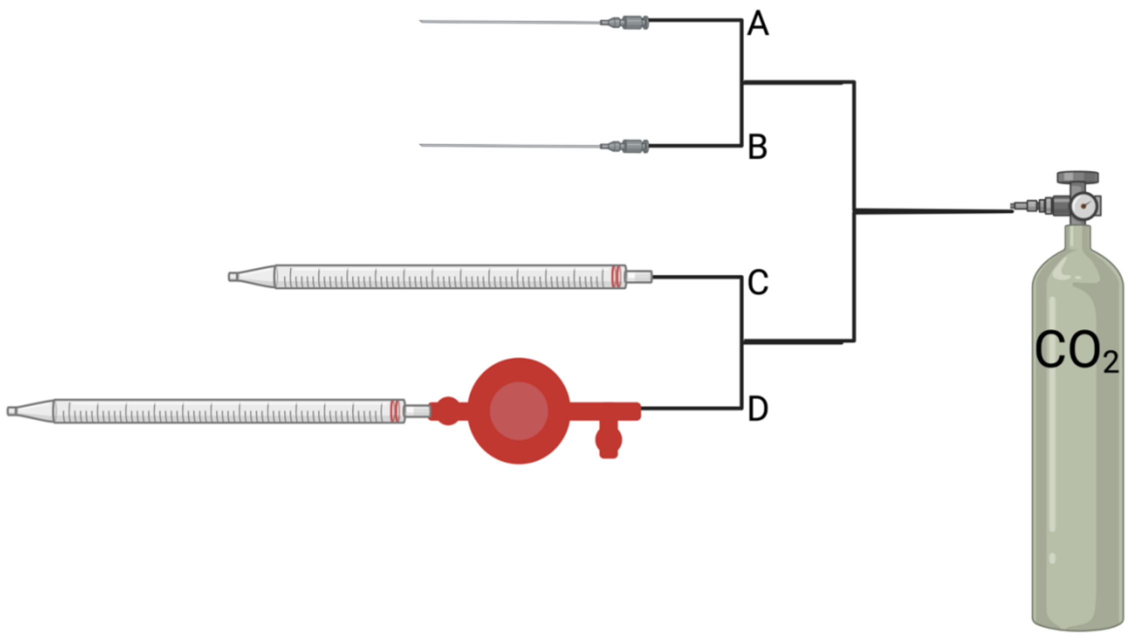


Figure 2.2: Anaerobic setup for *Methanobrevibacter spp.*

Labelled as follows: A/B: 12-gauge needles for ambient air exclusion while dispensing media; C: glass pipette for sparging with CO_2 while boiling media; D: rubber pipetting bulb for anaerobically dispensing media to Hungate tubes. Image created with BioRender.com

Culture Tube	Species	Source
0	*M. ruminantium*	Lethbridge frozen culture
1	*M. ruminantium*	Tube 0
2	*M. ruminantium*	Tube 0
3	*M. ruminantium*	Tube 0
4	*M. ruminantium*	Tube 0
5	*M. ruminantium*	Tube 0
6	*M. ruminantium*	Tube 1
7	*M. ruminantium*	Tube 1
8	*M. ruminantium*	Tube 1
9	*M. ruminantium*	Tube 1
10	*M. ruminantium*	Tube 1
11	*M. ruminantium*	Tube 1
12	*M. ruminantium*	Tube 7
13	*M. ruminantium*	Tube 7
14	*M. ruminantium*	Tube 7
15	*M. ruminantium*	Tube 11
16	*M. ruminantium*	Tube 11
17	*M. ruminantium*	Tube 11
18	*M. ruminantium*	DSMZ
19	*M. ruminantium*	DSMZ
20	*M. ruminantium*	Tube 18
21	*M. ruminantium*	Tube 18
22	*M. ruminantium*	Tube 18
23	*M. ruminantium*	Tube 18
24	*M. smithii*	DSMZ
25	*M. smithii*	DSMZ
26	*M. smithii*	Tube 24
27	*M. smithii*	Tube 24
28	*M. smithii*	Tube 24
29	*M. smithii*	Tube 24
30	*M. ruminantium*	Tube 21
31	*M. ruminantium*	Tube 21
32	*M. ruminantium*	Tube 21
33	*M. ruminantium*	Tube 21
34	*M. ruminantium*	Tube 21
35	*M. smithii*	Tube 26
36	*M. smithii*	Tube 26

37	*M. smithii*	Tube 26
38	*M. smithii*	Tube 26
39	*M. ruminantium*	Tube 10 (frozen)
40	*M. ruminantium*	Tube 10 (frozen)
41	*M. ruminantium*	Tube 10 (frozen)
42	*M. ruminantium*	Tube 10 (frozen)
43	*M. ruminantium*	Tube 33 (frozen)
44	*M. ruminantium*	Tube 33 (frozen)
45	*M. ruminantium*	Tube 33 (frozen)

Table 2.1: Inoculum source for *Methanobrevibacter spp.* culture tubes

2.2.3 Statistical analysis

Statistical analyses were carried out using GraphPad Prism (GraphPad, San Diego CA, USA) and Microsoft Excel (Microsoft, Redmond WA, USA). Growth of the archaea cultures was estimated using Excel to calculate slopes. As growth of the archaeal cultures did not follow a standard logarithmic curve, and with no clear growth pattern, we used a linear model to determine whether the archaea cultures showed growth over time. Growth was significant when $P \leq 0.05$.

2.3 Results

2.3.1 Archaeal growth measured by methane production

The culture of *M. ruminantium* was in DSMZ 141 media and Jay's media (Figure *2.3*). Of the four tubes, tubes 1 and 2 contained DSMZ 141 media, and tubes 3 and 4 contained Jay's media (5.2: Jay's media volatile fatty acid mixture). In tube 1, the initial inoculum which contained 1 mL of the thawed *M. ruminantium* from storage in Lethbridge AAFC was incubated in DSMZ 141 media, 20% methane presence was detected. This culture was used to inoculate Tube 2 and 3. In Tube 3 5.67% methane was detected in the headspace. Tubes 2 and 4 did not have any detectable methane after nine days of incubation.

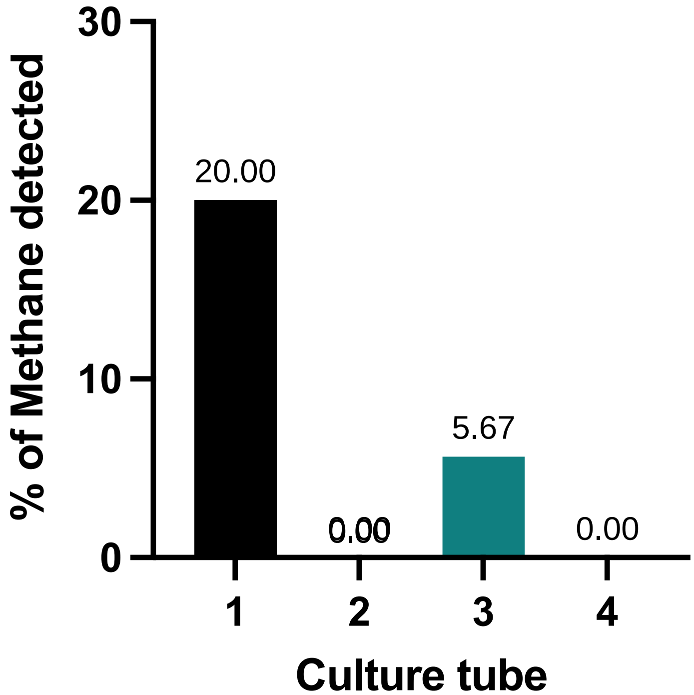


Figure 2.3: Amount of methane present in cultures of *M. ruminantium*. Tubes 1 and 2 contained DSMZ 141 media, and tubes 3 and 4 contained Jay's media.

The second round of cultures grown at the AAFC laboratory in Lethbridge were inoculated with material derived from the Tubes 1 and 3 (Figure *2.4*). Culture tubes 1, 2, 3, and 4 are the same as culture tubes 1, 2, 3, and 4 in Figure *2.3*, but were repressurized with 80% H_2 and 20% CO_2. Cultures in Tubes 5, 6, and 7 contained DSMZ 141 media, and cultures in tubes 8, 9, and 10 contained Jay's media. After 9 days of incubation only 0.05% methane was detected in tube 4, and only 0.09% methane was detected in tube 6.

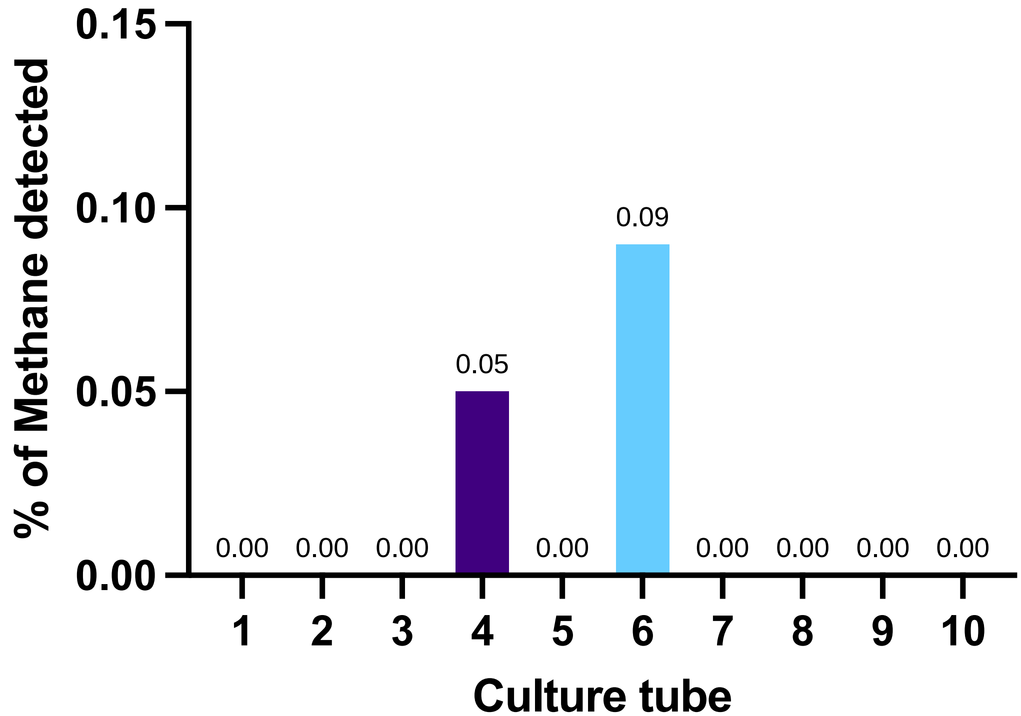


Figure 2.4: Amount of methane present in cultures of *M. ruminantium.* Cultures in Tubes 5, 6, and 7 contained DSMZ 141 media, and cultures in tubes 8, 9, and 10 contained Jay's media

*Please note the different y-axis scale as compared to Figure *2.3*

2.3.2 Archaeal growth measured by optical density

M. ruminantium and *M. smithii* had large variations in growth over time (Figure *2.5*, Figure *2.6*, Figure *2.7*, Figure *2.8*, Figure 2.9, Figure *2.10*, Figure *2.11*, and Figure *2.12*). Slopes of each individual culture was calculated to determine if growth could be detected (Table *2.2*).

In the first batch of *M. ruminantium* cultures, in Tubes 0, 1, 3, and 4, growth could be detected (Figure *2.5*). In the second batch of cultures of *M. ruminantium*, in tubes 6, 8, 9, 10, and

11 growth was detected (Figure *2.6*). In the third batch of *M. ruminantium* cultures, tubes 13, 15, and 17 growth could be detected (Figure *2.7*). The fourth batch of cultures was comprised of the *M. ruminantium* cultures from DSMZ and the dilutions from those cultures, in none of the cultures growth could be detected at any timepoint (Figure *2.8*), this was the same for the fifth batch of cultures (Figure 2.9). In the sixth batch of cultures, only in tube 34 containing a *M. ruminantium* culture inoculated from tube 21, growth could be detected (Figure *2.10*). In the seventh batch of cultures, the cultures of *M. smithii* in tubes 36 and 38 growth could be detected (Figure *2.11*). In the eighth and final batch of cultures, no growth was detected in any *M. ruminantium* culture.

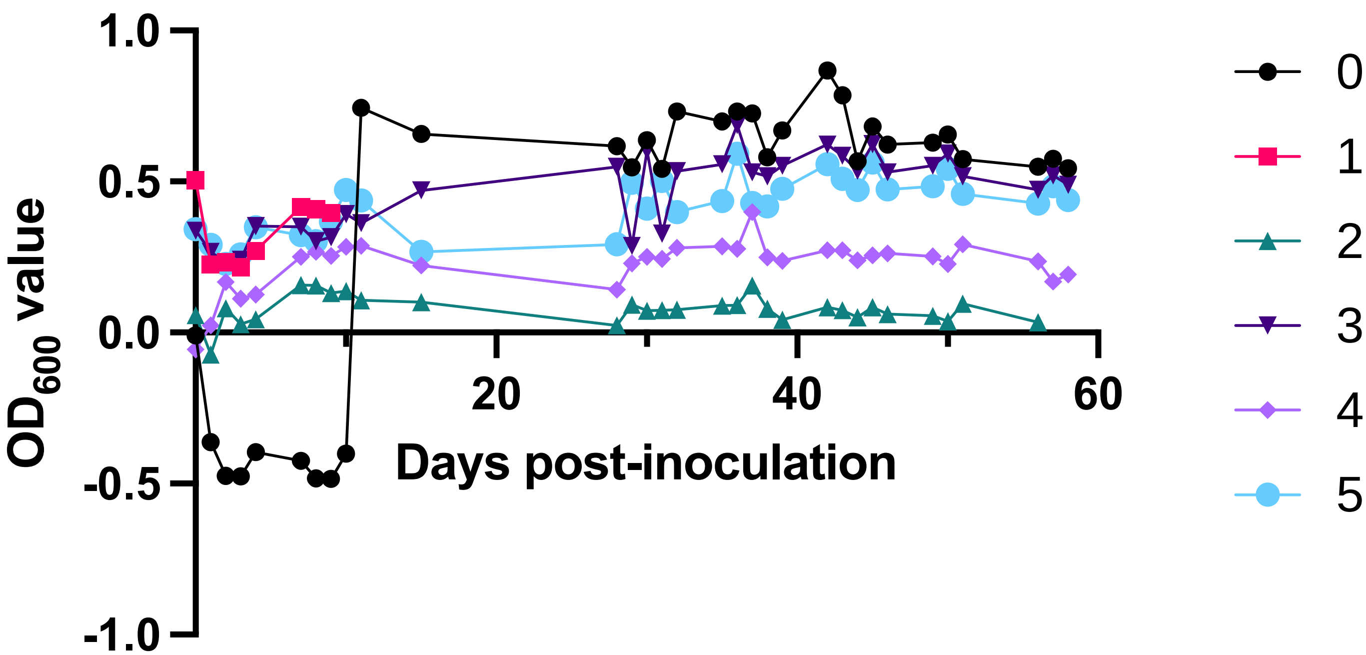


Figure 2.5: Change in optical density of *M. ruminantium* grown in RM02 media

Tube 0 was the original culture tube, with replicate tubes 1, 2, 3, 4, and 5 containing 1 mL from Tube 0 and 9 mL of fresh media

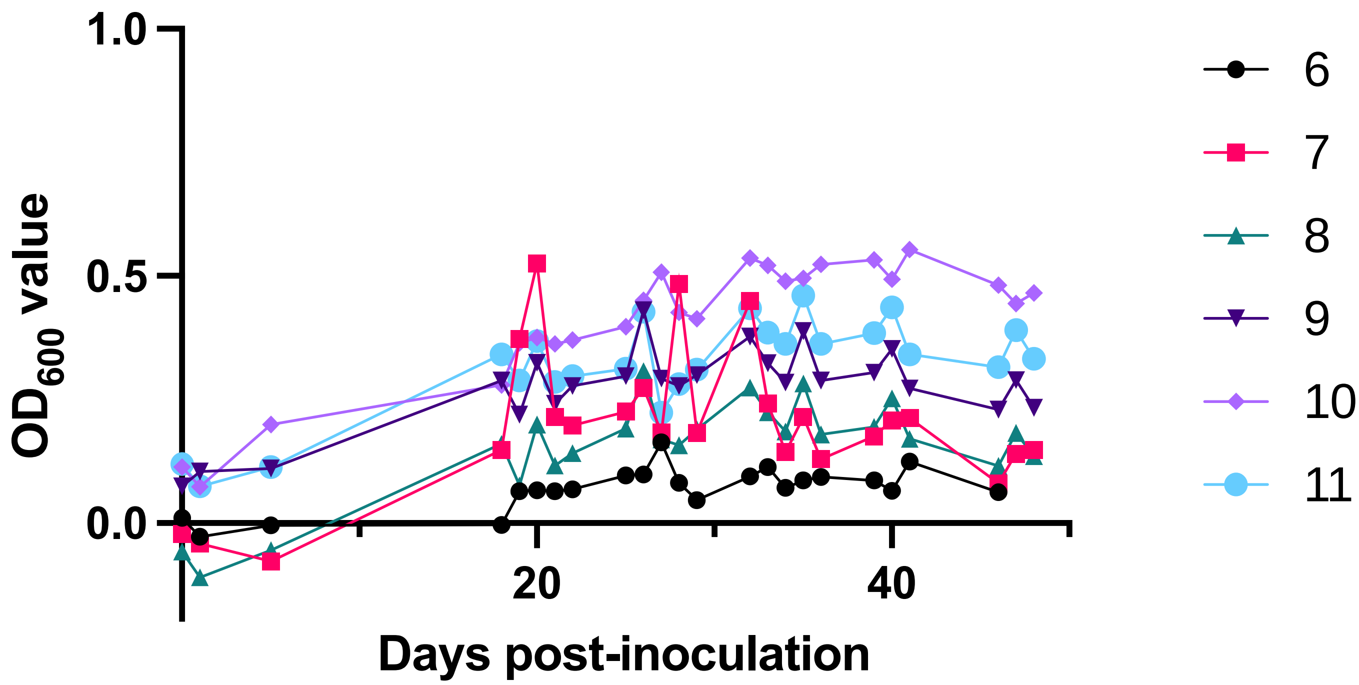


Figure 2.6: Change in optical density of *M. ruminantium* grown in RM02 media

Replicate tubes 6, 7, 8, 9, 10, and 11 contained 1 mL from Tube 1 and 9 mL of fresh media

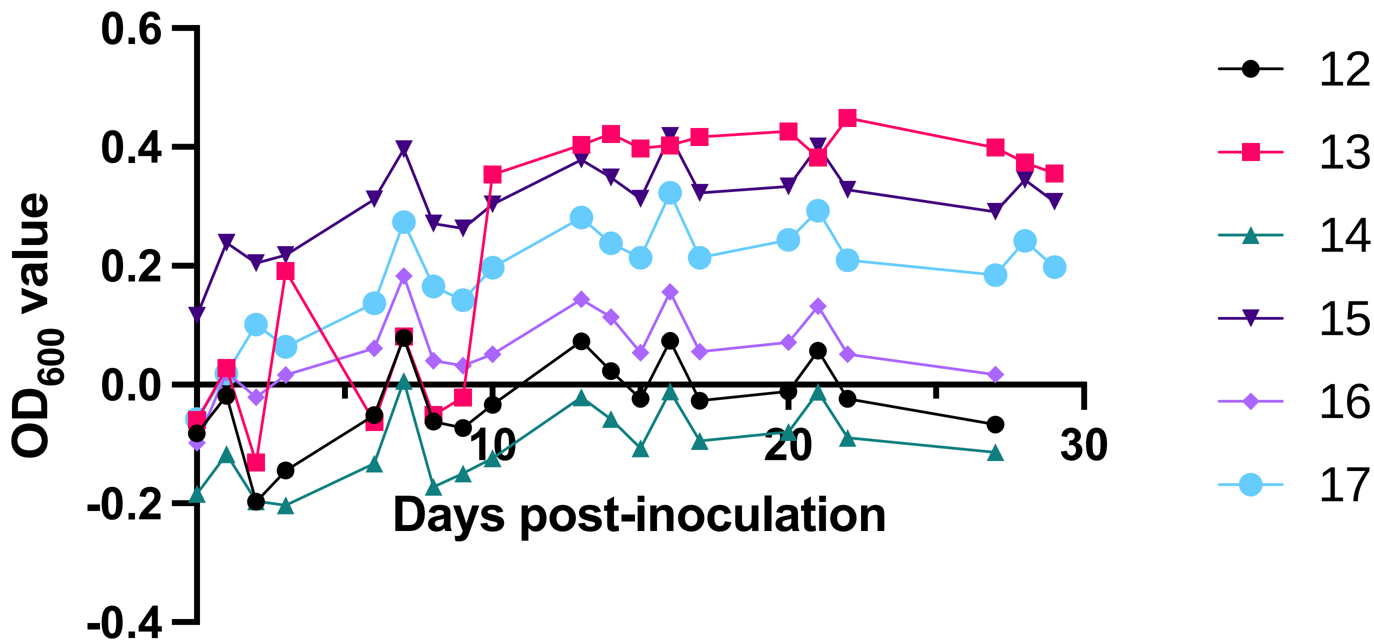


Figure 2.7: Change in optical density of *M. ruminantium* grown in RM02 media

Tubes 12, 13, and 14 contained 1 mL from Tube 7 and 9 mL of fresh media, and 15, 16, and 17 contained 1 mL from Tube 11 and 9 mL of fresh media

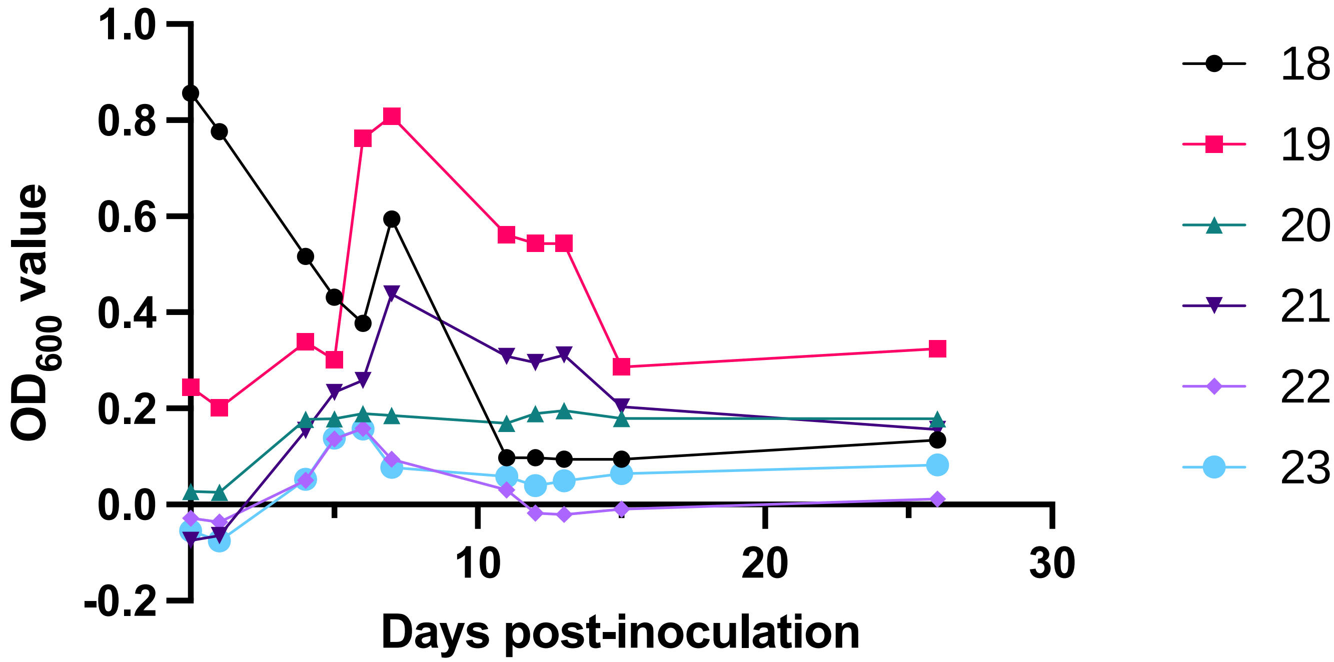


Figure 2.8: Change in optical density of *M. ruminantium* grown in RM02 media

Tubes 18 and 19 were *M. ruminantium* cultures ordered from DSMZ. Replicate tubes 20, 21, 22, and 23 contained 1 mL from Tube 18 and 9 mL of fresh media

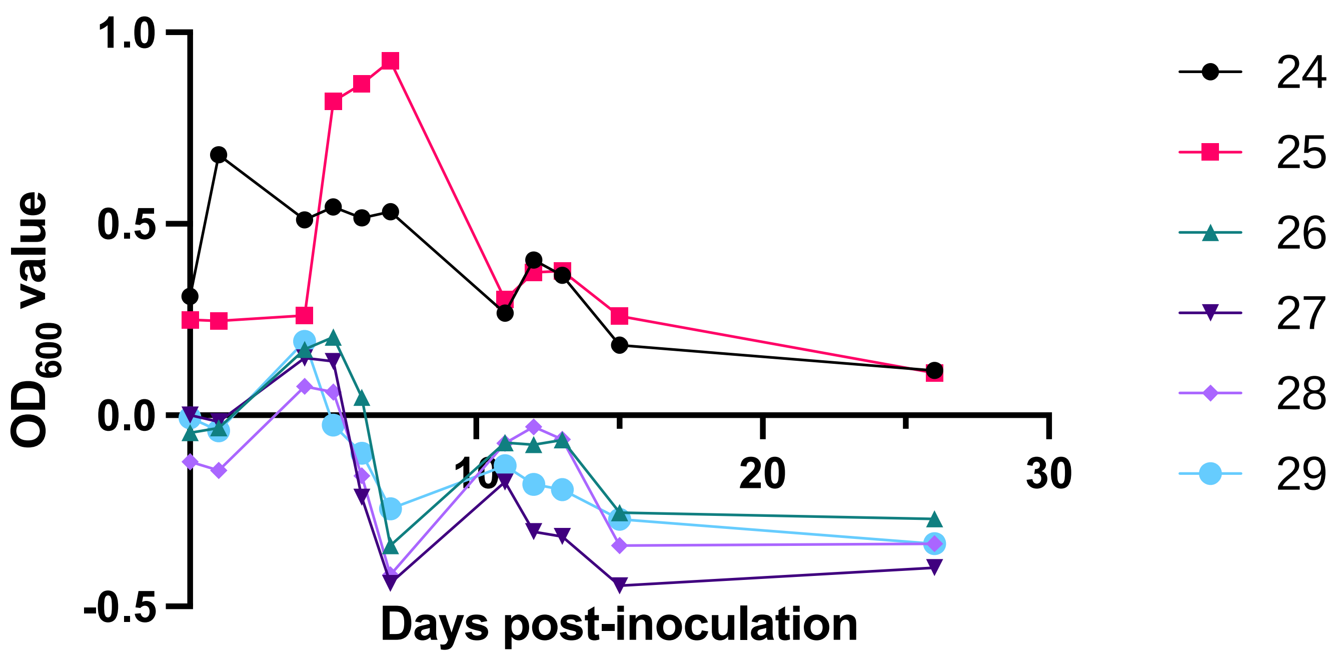


Figure 2.9: Change in optical density of *M. smithii* grown in RM02 media

Tubes 24 and 25 were *M. smithii* cultures ordered from DSMZ. Replicate tubes 26, 27, 28, and 29 contained 1 mL from Tube 24 and 9 mL of fresh media

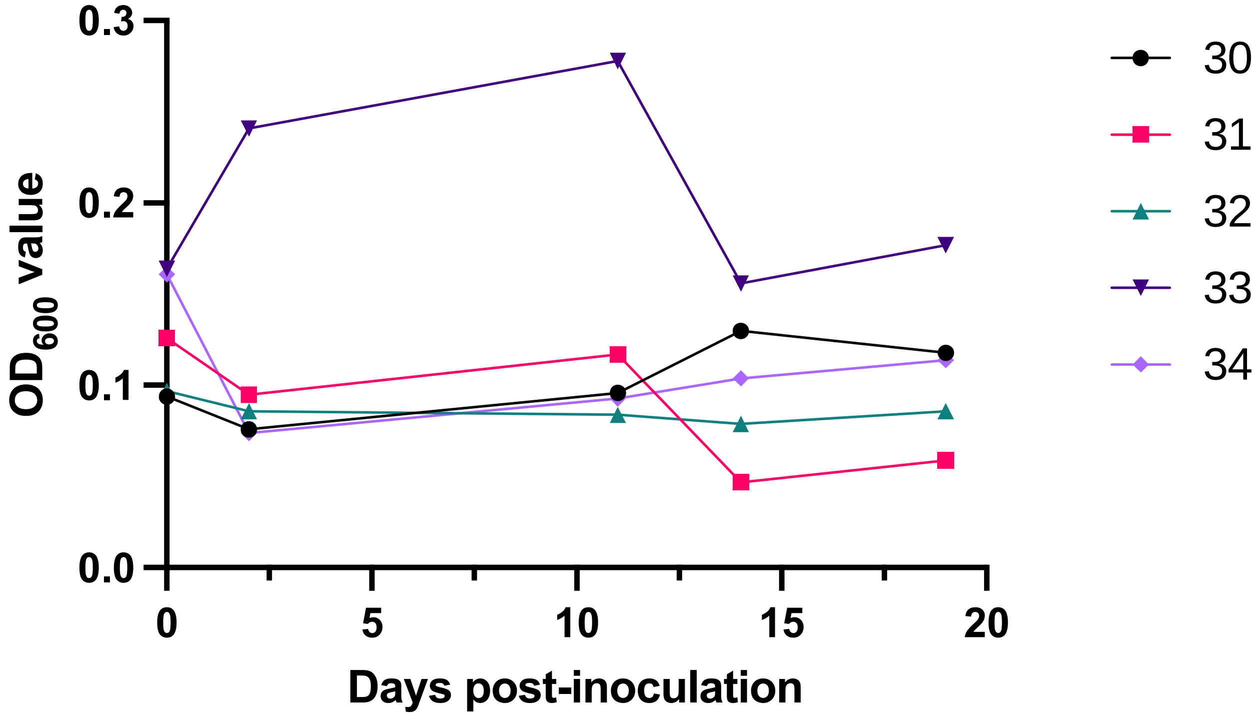


Figure 2.10: Change in optical density of *M. ruminantium* grown in RM02 media

Replicate tubes 30, 31, 32, 33, and 34 contained 1 mL from Tube 21 and 9 mL of fresh media

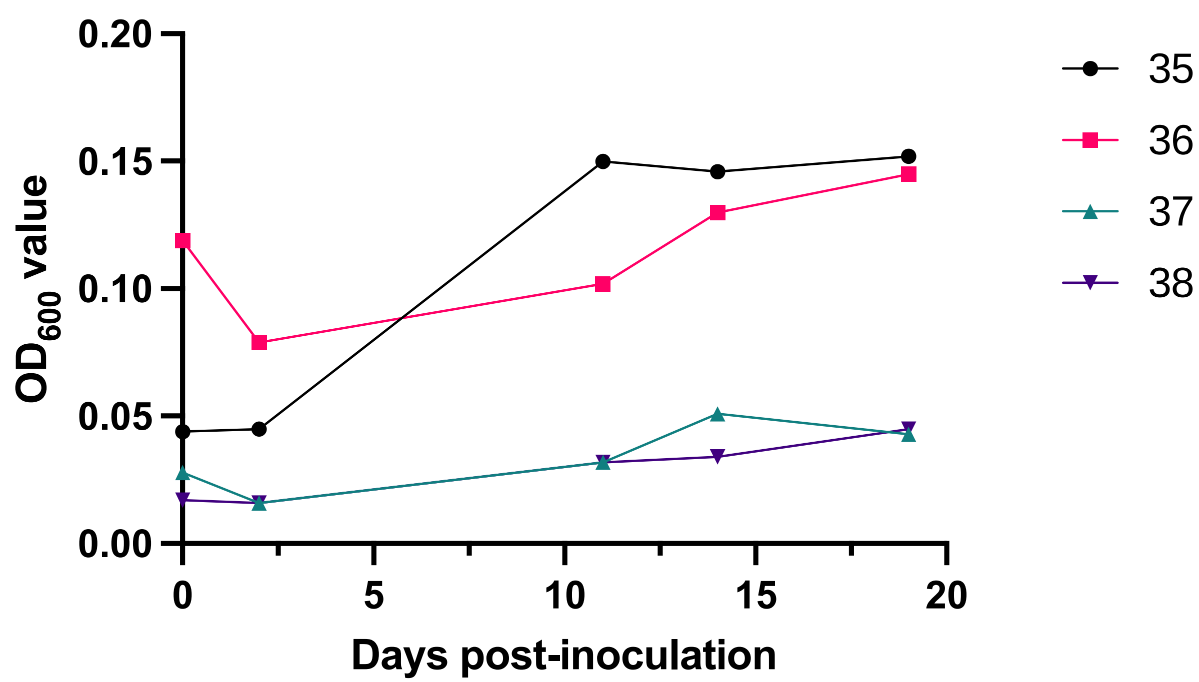


Figure 2.11: Change in optical density of *M. smithii* grown in RM02 media

Replicate tubes 35, 36, 37, and 38 contained 1 mL from Tube 26 and 9 mL of fresh media

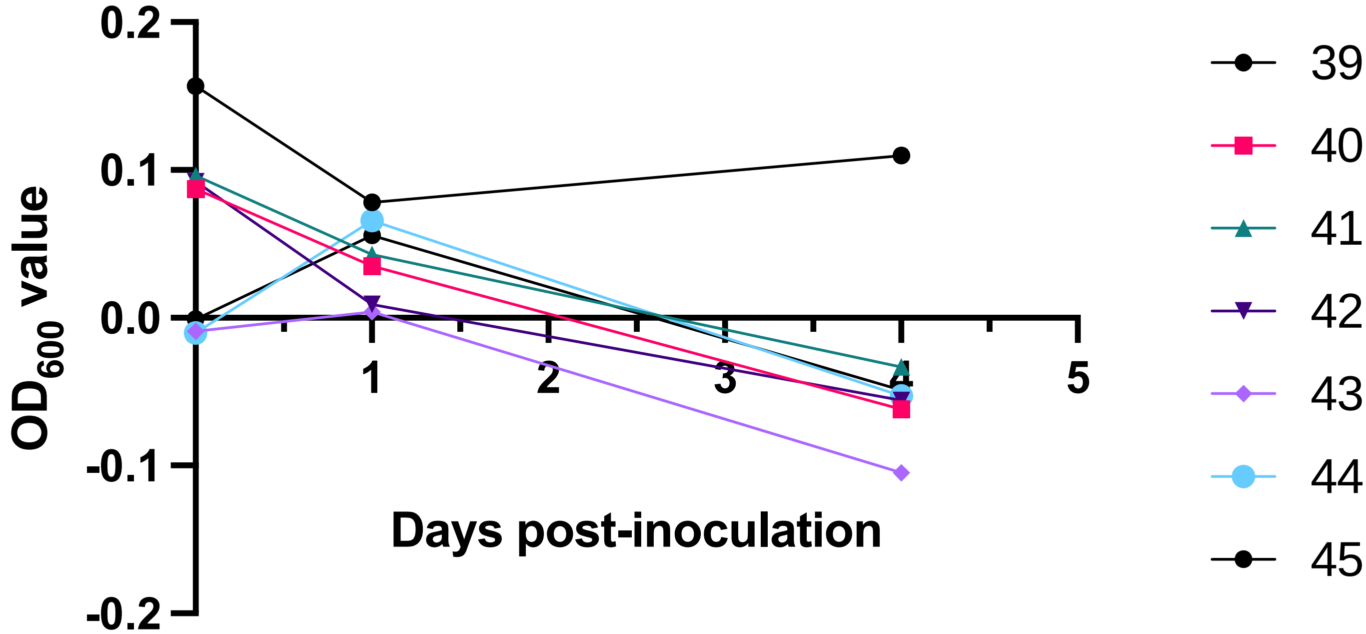


Figure 2.12: Change in optical density of *M. ruminantium* grown in RM02 media

Replicate tubes 39, 40, 41, and 42 contained 1 mL from Tube 10 (frozen) and 9 mL of fresh media, and replicate tubes 43, 44, and 45 contained 1 mL from Tube 33 (frozen) and 9 mL of fresh media

Culture Tube	**Species**	**Slope**	**R squared**	***P* value**	**Significant ?**
0	*M. ruminantium*	0.00086736	0.60834069	$P < 0.01$	yes
1	*M. ruminantium*	0.02810437	0.89105032	$P < 0.01$	yes
2	*M. ruminantium*	-0.0002629	no growth		
3	*M. ruminantium*	0.00523584	0.56354292	$P < 0.01$	yes
4	*M. ruminantium*	0.00130566	0.12383042	$P = 0.052$	no
5	*M. ruminantium*	0.00371342	0.50354944	$P < 0.01$	yes
6	*M. ruminantium*	0.00247689	0.41067224	$P < 0.01$	yes
7	*M. ruminantium*	0.00144881	0.01521558	$P = 0.57$	no
8	*M. ruminantium*	0.00467111	0.3591216	$P < 0.01$	yes
9	*M. ruminantium*	0.00288336	0.21951401	$P < 0.01$	yes
10	*M. ruminantium*	0.00802187	0.71293663	$P < 0.01$	yes
11	*M. ruminantium*	0.00530294	0.47526076	$P < 0.01$	yes
12	*M. ruminantium*	0.00382252	0.14896417	$P = 0.13$	no
13	*M. ruminantium*	0.01753022	0.55551821	$P < 0.01$	yes
14	*M. ruminantium*	0.00392021	0.21576763	$P = 0.06$	no
15	*M. ruminantium*	0.00318454	0.21766184	$P < 0.01$	yes
16	*M. ruminantium*	0.0018343	0.05967055	$P = 0.34$	no
17	*M. ruminantium*	0.00516785	0.33113385	$P < 0.01$	yes
18	*M. ruminantium*	-0.0266022	no growth		
19	*M. ruminantium*	-0.0013689	no growth		
20	*M. ruminantium*	0.00317821	0.21374139	$P = 0.18$	no
21	*M. ruminantium*	0.00371408	0.04148018	$P = 0.57$	no
22	*M. ruminantium*	-0.0029688	no growth		

23	*M. ruminantium*	0.0018257 4	0.0454778 3	*P* = 0.55	no
24	*M. smithii*	-0.0225972	no growth		
25	*M. smithii*	-0.0180345	no growth		
26	*M. smithii*	-0.0144932	no growth		
27	*M. smithii*	-0.0203491	no growth		
28	*M. smithii*	-0.0103365	no growth		
29	*M. smithii*	-0.0154275	no growth		
30	*M. ruminantium*	0.0026844 4	0.7086557 9	*P* = 0.16	no
31	*M. ruminantium*	-0.0024978	no growth		
32	*M. ruminantium*	-8.444E-05	no growth		
33	*M. ruminantium*	-0.0044267	no growth		
34	*M. ruminantium*	0.0022711 1	0.9882524 4	*P* < 0.01	yes
35	*M. smithii*	0.0061111 1	0.7701669 2	*P* = 0.12	no
36	*M. smithii*	0.0038044 4	0.9444908 5	*P* = 0.03	yes
37	*M. smithii*	0.0017155 6	0.7208321 2	*P* = 0.15	no
38	*M. smithii*	0.0015866 7	0.9908571 4	*P* < 0.01	yes
39	*M. ruminantium*	-0.0065769	no growth		
40	*M. ruminantium*	-0.0361154	no growth		
41	*M. ruminantium*	-0.0306538	no growth		
42	*M. ruminantium*	-0.0334615	no growth		
43	*M. ruminantium*	-0.0268462	no growth		
44	*M. ruminantium*	-0.0174231	no growth		
45	*M. ruminantium*	-0.0173077	no growth		

Table 2.2: Slopes of *Methanobrevibacter spp*. culture tubes

Culture batch tubes	Media	Culture source	Number of cultures showing positive growth
0-5	RM02	AAFC laboratory (frozen culture)	3/6
6-11	RM02	Tube 1	5/6
12-14	RM02	Tube 7	1/3
15-17	RM02	Tube 11	2/3
18-19	RM02	DSMZ	0/2
20-23	RM02	Tube 18	0/4
24-25	RM02	DSMZ	0/2
26-29	RM02	Tube 24	0/4
30-34	RM02	Tube 21	1/5
35-38	RM02	Tube 26	2/4
39-42	RM02	Tube 10 (frozen culture)	0/4
43-45	RM02	Tube 33 (frozen culture)	0/3

Table 2.3: Summary of *Methanobrevibacter spp.* growth

2.3.3 PCR confirmation of the presence of *M. ruminantium* in cultures

Samples from both rumen fluid and *M. ruminantium* cultures from DSMZ were subjected to PCR as described above in the methods section (2.2.1), a 400 bp product was detected (Figure *2.13*). The DNA extracted from rumen fluid was negative. While the lack of a band does not necessarily indicate a lack of *M. ruminantium* in the rumen fluid, this might indicate that the method to extract or amplify the DNA was not successful.

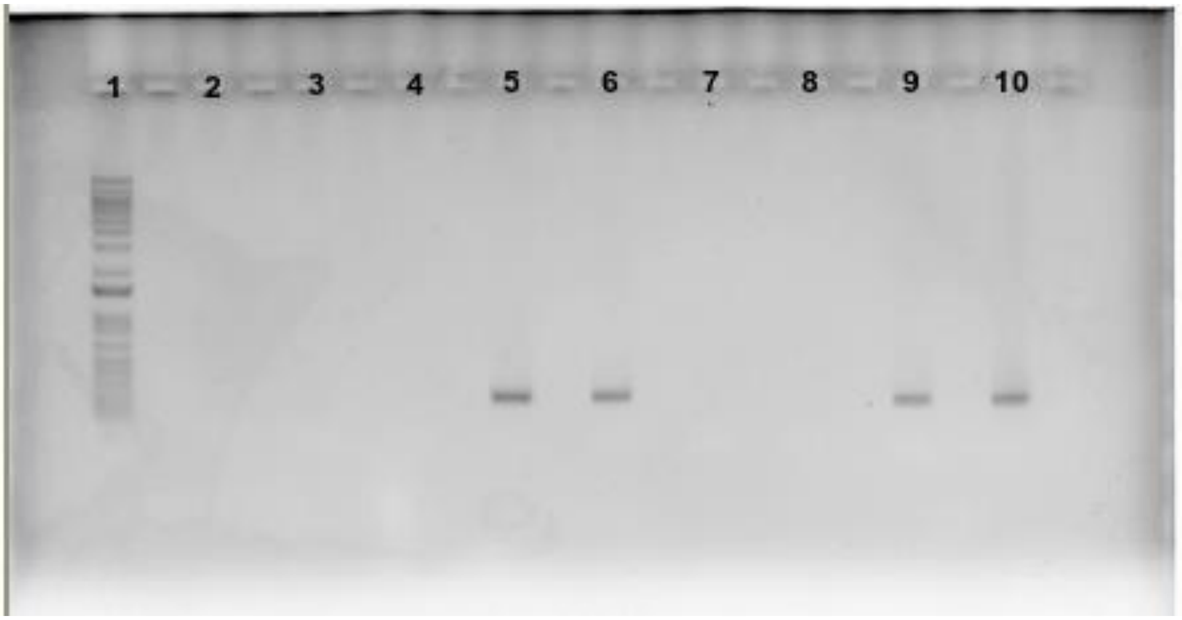


Figure 2.13: Amplification of DNA from rumen fluid and *M. ruminantium* cultures.

Lanes: 1: 1kb+ ladder; 2: negative water control; 3: rumen fluid supernatant with 50 μM MgCl; 4: rumen fluid pellet with 50 μM MgCl; 5: M. ruminantium supernatant with 50 μM MgCl; 6: M. *ruminantium* pellet with 50μM MgCl, 7: rumen fluid supernatant with 25μM MgCl; 8: rumen fluid pellet with 25μM MgCl; 9: *M. ruminantium* supernatant with 25μM MgCl; 10: *M. ruminantium* pellet with 25μM MgCl. Expected product is 400 bp.

2.4 Discussion

2.4.1 Archaeal growth measured by methane production

The methane testing completed at the AAFC laboratory in Lethbridge suggested that methanogenesis occurred in some of the sample tubes, but growth of *M. ruminantium* in DSMZ 141 media was inconsistent, with only two of the four initial culture tubes containing a detectable amount of methane after nine days of incubation. In only two out of ten subcultures a low concentration of methane was detectable after 14 days of incubation.

However, in the first culture, tube 1, a much higher concentration of *M. ruminantium* was added compared to the subcultures. As such, tube 1 contained a higher starting amount of cells than the (diluted) tubes containing the subcultures.

Due to the uncertainty of the exact volume of gas tested in our experiment, it is difficult to estimate methane production, in another study methane production could be 80 μmol/tube in the late logarithmic phase[104].

2.4.2 Archaeal growth measured by optical density

Although existing techniques for the culture of *Methanobrevibacter spp.* were utilized, we were unable to obtain the similar results. Based on our discussions with Dr. Janssen from AgResearch in Palmerston North, New Zealand, the growth curves were expected to reach peak growth between 40-50 hours, however, there was no definitive peak seen in our cultures. Additionally, even when replication was detected in the sample tubes, there was little consistency between replicates, and subcultures did not appear to have similar levels of growth as the source tubes.

With some cultures, such as the *Methanobrevibacter spp.* sourced from DSMZ (tube 18), a sharp drop in OD_{600} could be detected immediately following the start of the culture. This may indicate that the media as used in these experiments was not suitable to sustain methanogen replication.

2.4.3 PCR confirmation of *M. ruminantium* presence

The PCR presented challenges in replication. DNA from rumen fluid and *M. ruminantium* culture proved to be difficult to isolate and amplify, requiring multiple rounds of alterations to the procedure to obtain a positive result, including altering the amount of MgCl and bovine serum albumin. Laboratory shutdowns meant that sequencing was not available for the PCR products to verify if we amplified *M. ruminantium* genomic material, as such, the visible bands could not be definitively confirmed to be the expected gene product. If the assumption is made that the observed band is indeed *M. ruminantium,* then we were able to isolate target DNA from cultures of *M. ruminantium,* but not from rumen fluid.

2.4.4 Limitations

The inconsistent growth of *Methanobrevibacter spp.* means that subsequent experiments using this culture system would be difficult to interpret. When changes in growth conditions are made or the use of lytic archaeal virus experiments are performed, differences in growth or the archaea could be either due to the introduced variable or the existing inconsistencies in the culture.

The culture systems had to be adapted to the local laboratory, and that required some minor alterations of existing procedures. These alterations, including adaptations due to lack of machinery such as a pressurization machine or a specific anaerobic culturing setup, were reviewed with Dr. Janssen from AgResearch as any alterations to procedures could result in dissimilar growth. Even in laboratories that have extensive experience with *M. ruminantium* cultures, achieving consistent culture results is difficult, only 90% of the cultures are generally successful (Dr. Janssen personal communication). The slow replication of *Methanobrevibacter spp.* meant that any change in culture conditions took several weeks to evaluate.

The rumen fluid was obtained from other experiments that were being conducted by AAFC laboratories in Lethbridge, which added another variable to the growth media. Rumen fluid from a single source may have been beneficial to obtain reproducible results.

The use of a spectrophotometer to track growth is not very accurate. Although inexpensive and fast, spectrophotometers do not distinguish between live target cells and anything else present in the media that may block the light going through the media to the sensor.

Chapter 3

3 Comparing the efficacy of individual and cocktail bacteriophage treatments against *E. coli* O157

3.1 Introduction

While phages were discovered more than a century ago[18], antibiotics quickly became the treatment of choice, due to their ease of use, broad spectrum activity, and efficacy against pathogenic bacteria. However, with increasing concerns over antibiotic resistance, interest is being renewed in using phage to control bacterial pathogens, especially as a focus on supplementing or replacing antibiotics[105,106].

While the use of phages against bacteria has advantages due to their high level of specificity, there are some barriers to the widespread use of phages in both agricultural and medical applications[21,22].

Bacteria have systems in place that facilitate rapid development of anti-phage resistance, allowing escape from infection by phage. Although there is often an arms race between the phages and the bacteria, phages have also evolved a number of mechanisms to overcome bacterial resistance[107], these bacterial mutations can cause significant issues in the short term. One method of interest of mitigating these anti-phage mutations has been to utilize a phage cocktail, with multiple phages displaying lytic activity against the target bacteria. However, there has been recent research indicating that phage cocktails may be less effective than previously thought, potentially due to phage-phage interference[97].

To examine the dynamics of phage cocktails, we challenged *E. coli* O157 with individual phages and phage cocktails.

3.2 Materials and methods

3.2.1 Phage stock preparation

Phages with lytic activity against *E. coli* O157:H7 were isolated previously from cattle feedlots [108–113]: Two members of family *Siphoviridae:* T5 (*Tequintavirus*, AKFV33), and T1 (*Rogunavirus*, AHP24)*;* and two members of family *Myoviridae:* T4 (*Tequatrovirus*, wV7) and rV5 (*Vequintavirus*, AHP24S). Experimental stocks were propagated from their working stocks with *E. coli* O157:H7 R508 as a host[111].

The full protocol is available in the appendices (5.2: SOP-901: Phage research stock preparation of pathogenic *E. coli* infecting phage), but to briefly describe: *E. coli* O157:H7 R508 was plated on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD), and incubated for 16 to 18 h at 37°C. A single clonal colony was selected and inoculated into 10 mL of tryptic soy broth (TSB, Difco, Ottawa, ON, Canada, pH = 7.4) and incubated for 20 h at 37°C. From this overnight culture, 1 mL was added to 9 mL TSB for each phage being propagated and incubated for 90 minutes at 37°C and 170 RPM until in the mid-log phase of growth (OD_{600} = 0.5-0.6, ~10^8 CFU/ml). The log culture was serially diluted to 1×10^{-6} and 1×10^{-7} and 100 µl was plated on

TSA plates, which were incubated for 16 to 20 h at 37°C. Colonies were counted to determine CFU/ml.

Phage dilutions of the following multiplicities of infection (MOI, the ratio of the number of phage particles to the number of bacterial host cells) were prepared: T5: 0.0001 MOI; T1: 0.0001 MOI; T4: 0.01 MOI; and rV5: 0.0001 MOI. 200 µl of phage dilution were added to 6 mL of mid -log-phase culture of R508, as measured by a spectrophotometer, and incubated in shaking incubator for 15 minutes at 37°C and 130 RPM. This culture was vortexed and added to 300 mL of TSB with 10 mM $MgSO_4$ (mTSB) in a sterile culture flask and the flask was incubated for 5 hours at 37°C and 170 RPM. Visual evaluation and OD_{600} turbidity measurements were taken every hour to monitor the turbidity of the culture. The turbidity of the culture would increase as the bacterial cell count increased, and the turbidity would decrease as the phages infected and lysed the bacterial hosts. Once lysis was complete (marked by a distinct decrease in visible culture turbidity and the presence of bacterial debris and precipitate), the culture was centrifuged at 4800 × g for 45 minutes (Beckmann swing rotor) at 4°C. The supernatant containing the phages was poured off and filter sterilized through a 0.2 µm bottle-top filter ((Nalgene Filtration 0.2 µm SFCA membrane filter, VWR, 28199-677(250ml);28198-505(500ml); 289199-698(1000ml))), and stored at 4°C for up to three months.

3.2.2 Phage stock titration

The phage stocks propagated in the previous section were titrated using a double agar overlay plaque assay, using *E. coli* O157:H7 R508 as a host.

E. coli O157:H7 R508 was prepared as above in the phage stock preparation, and log-phase host culture was used.

The full protocol is available in the appendices (5.6: Enumeration of pathogenic *E. coli* infecting bacteriophage: double agar overlay plaque assay), but to briefly describe: The Modified Nutrient Agar plates (Enumeration of pathogenic *E. coli* infecting bacteriophage: double agar overlay plaque assay) (MNA**,** Dalynn, PN88-11041) was taken out from 4°C storage to room temperature 1-2 days prior to titration to dry. The MNA plates should have small wrinkles present on the surface if dried correctly, which is important to plaque formation.

For each sample that was titrated, 450 µl of Lambda diluent (SOP-901: Phage research stock preparation of pathogenic *E. coli* infecting phage) was pipetted into a row of an assay block, representing dilutions from 10^{-1} to 10^{-7}. Serial dilutions of each phage stock were prepared by pipetting 50 µl down the row to make 10-fold dilutions. All four phage stocks were titrated at 10^{-5}, 10^{-6}, and 10^{-7} dilutions, in duplicate. Phage-bacteria preparations were made by mixing 100 µl of phage dilution and 100 µl of mid log-phase bacterial culture in a 5 mL polypropylene tube. Controls used were agar, Lambda diluent, host culture, and a combination of Lambda diluent and host culture. Three mL of molten (56°C) 0.6% UltraPure agarose with 10 mM $MgSO_4$ was added to each 5 mL tube, inverted twice, and poured onto a dried MNA plate. The agar mixture was gently swirled to coat the entire top surface of the MNA plate, and allowed to harden at room temperature before incubating for 18-24h at 37°C.

3.2.3 Bacterial stock preparation

The full protocol is available in the appendices (5.7), but to briefly describe: Each strain of *E. coli* O157:H7 was plated on TSA and incubated for 16-20 h at 37°C. A single colony was selected and inoculated into 10 mL of TSB and incubated for 18 h at 37°C at 170 RPM. The concentration of bacteria in the overnight culture was estimated by OD_{600} using a spectrophotometer and enumerated using 100 µl serial dilutions (10^{-6} and 10^{-7}) on TSA plates, which were incubated for 16 to 20 h at 37°C. The colonies were counted to determine CFU/ml.

3.2.4 Phage *in vitro* lysis kinetics in a 10 mL broth culture

To evaluate the general growth patterns of phage-treated *E. coli* O157:H7, six strains of *E. coli* O157:H7 were cultured together with the four individual phage and two combination cocktails at an MOI of 1000. The *E. coli* O157:H7 strains selected were EDL933, R508N, CO281-31N, E32511, H4420N, and E318N. CO281-31N, E32511, and E318N were isolated from humans, and R508N and H4420N were isolated from cattle, with EDL933 being isolated from ground beef[114]. All six strains are known to cause disease in humans. All six strains were kindly provided by Agriculture and Agri-Food Canada (AAFC). EDL933, R508N, E32511, and E318N were cultured for 24 h, with optical density measurements at 0, 2, 4, 6, 8, 10, 12, and 24 hours, and repeated for 24 hours with measurements at 12, 14, 16, 18, 20, 22, and 24 hours. CO281-31N and H4420N were cultured for 24 hours with measurements at 12, 14, 16, 18, 20, 22, and 24 hours. These repeats were hoped to give an accurate picture of the growth over 24 h. As EDL933 was selected for further exploration, CO281-31N and H4420N were only cultured once, not twice.

Each strain of *E. coli* O157:H7 was prepared as described previously (3.2.3).

The laboratory phage stocks were diluted in mTSB to prepare the desired MOI of 1000, and the two cocktail combinations were prepared containing an equal amount of the component phages. The phage treatments were as follows: T5, T1, T4, rV5, and combination cocktails T1+ T4+rV5, and T1+T4+rV5+T5. 9 mL of 10^5 CFU/ml *E. coli* O157 broth culture was inoculated with 1 mL of each phage or combination at an MOI of 1000, with the negative control culture having 1 mL of mTSB added in place of the phage. Each treatment and the controls were carried out in duplicate. The cultures were incubated for 24 h at 37°C at 170 RPM.

The experiment was run over two separate time ranges, first testing the initial 12 hours post-infection, and a subsequent experiment testing the latter 12 hours post-infection using uninoculated mTSB as a blank. EDL933, R508N, E32511, and E318N were cultured for 24 hours, with optical density measurements at 0, 2, 4, 6, 8, 10, 12, and 24 hours, and again for 24 hours with measurements at 12, 14, 16, 18, 20, 22, and 24 hours. CO281-31N and H4420N were cultured for 24 hours with measurements at 12, 14, 16, 18, 20, 22, and 24 hours.

3.2.5 Enumeration of bacteria and phages from phage lysis experiments

The phage lysis kinetics experiments were run similarly to the OD_{600} experiments above, but with additional samples and measurements taken. The phage lysis kinetics experiment was repeated three times. The selected strain of *E. coli* O157:H7 was EDL933 to represent a human isolated strain of *E. coli* O157:H7. At 0, 4, 7, 10, 19, and 24 h post-infection, OD_{600} measurements were taken, using uninoculated mTSB as a blank.

At 7, 19, and 24 h post-infection, 1.8 mL samples were taken from each tube. The samples were centrifuged at 10 000 × g for 10 minutes at 4°C to pellet the bacteria in the sample. The supernatant containing each of the phages was separated and filter sterilized using a 0.2 µm filter to remove any bacteria, and stored at 4°C for subsequent phage titration. The pellet was resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS, pH=7.4) and cooled at 4°C for immediate bacterial enumeration.

The bacterial enumeration was completed directly after the sample collection, with the serial dilutions (Table 5.1) estimated based on the OD600 measurements. The resuspended pellet was diluted using PBS and 100 µl was plated on TSA. The plates were incubated for 16 to 20h at 37°C. The colonies were counted to determine the average CFU/ml. The colonies showing visible morphological changes, such as atypical colony formation, were retained for further evaluation.

The phage titrations were performed using the same method as the phage stock preparation detailed above. The dilutions (Table *5.2*) were based on both bacterial enumeration from the broth experiments and on previous phage titration completed in the laboratory.

3.2.6 Comparison of phage-exposed colonies to uninfected original colonies to determine resistance

The TSA plates retained from the bacterial enumeration stage of the broth cultures were tested further to determine if the phenotypically changed colonies retained resistance to the phage treatments. Any colony seen on the TSA plates that had an atypical appearance as compared to the non-phage-treated control was inoculated on a new TSA plate and incubated for 18 to 24h at 37°C. A single colony was selected and inoculated into 10 mL of TSB and incubated for 18h at 37°C at 170 RPM.

To test for resistance against phage treatments, a microplate virulence assay was used[112]. The full protocol is available in the appendices (SOP-903: Pathogenic *E. coli* microplate phage virulence assay: 5.9) but to briefly describe: Individual phages and phage cocktails were prepared in the same manner as in the lysis kinetics method detailed above. 20 µl of each phage preparation was serially diluted in 180 µl of mTSB down the columns of a sterile 96-well flat-bottomed microplate for each bacterial culture being tested in duplicate, resulting in a dilution of ~10^8-10 PFU/mL vertically. 20 µl of the respective bacterial cultures being tested were added to each well in a 1:9 dilution. The plate was incubated for 5h at 37°C, and visually examined to evaluate turbidity within each well. The wells were marked as positive, indicating complete lysis; partial, indicating partial lysis; or negative, indicating fully turbid culture appearing the same as the control. Each of the bacterial samples collected at 24 h were tested, in addition to any atypical colonies.

3.2.7 Statistical analysis

Statistical analyses were carried out using GraphPad Prism (GraphPad, San Diego, California, United States) and Microsoft Excel (Microsoft, Redmond, Washington, United

States). All *E. coli* and phage growth data were log-transformed prior to analysis. 2-way ANOVAs were conducted to determine differences in growth in the *E. coli* trials.

3.3 Results

3.3.1 Phage stock preparation

The phage stock preparation was repeated several times, as the working phage stock had an expiry of three months. The concentrations of the phage stocks were considered acceptable if above 1×10^8 PFU/mL (Table *3.1*). The plaques were classified into three size categories f: small: T4 and rV5; medium: T5; and large: T1 (Figure *3.1*).

Trial	**Phage**	**PFU/mL**
Preliminary OD	T1	2.70×10^{9}
Preliminary OD	T4	1.12×10^{8}
Preliminary OD	T5	5.10×10^{9}
Preliminary OD	rV5	5.50×10^{8}
Phage lysis kinetics trials 1 and 2	T1	1.45×10^{9}
Phage lysis kinetics trials 1 and 2	T4	1.25×10^{9}
Phage lysis kinetics trials 1 and 2	T5	2.24×10^{10}
Phage lysis kinetics trials 1 and 2	rV5	7.05×10^{9}
Phage lysis kinetics trial 3	T1	3.00×10^{8}
Phage lysis kinetics trial 3	T4	4.35×10^{9}
Phage lysis kinetics trial 3	T5	1.38×10^{10}
Phage lysis kinetics trial 3	rV5	3.50×10^{9}

Table 3.1: concentrations of phage stock preparations

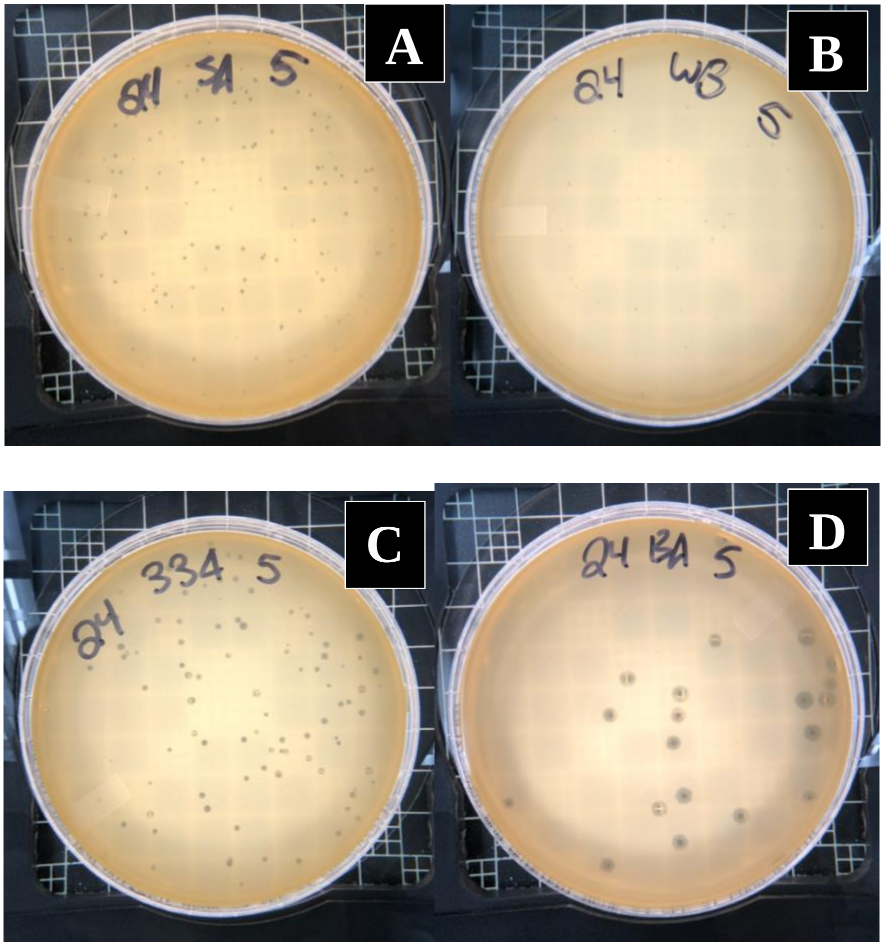


Figure 3.1: phage plaques of the four experimental phages.

Phage plaques: A: rV5 (small), B: T4 (small), C: T5 (medium), D: T1 (large)

3.3.2 Preliminary exploration of *E. coli* O157 strains treated with phage using optical density

The most effective phage treatments in most of the preliminary optical density trials was the combination of T1+T4+T5+rV5, which was significantly more effective ($P \leq 0.0001$) at reducing turbidity in comparison to the control cultures at 24 h in all experiments, and T5, which was significantly more effective ($P \leq 0.0001$) at reducing turbidity compared to the untreated control culture in 9 of the 10 trials. The time point at which a significant increase in turbidity as measured by optical density was noted, as well as if the turbidity was statistically significantly lower ($P \leq 0.05$) from the control at the 24 h time point (Table *3.2*, Table *3.3*)

O157 strain	Phage treatment	Statististically significant growth time point (hours)	*P* value	Difference in OD600 compared to control at 24 h	*P* value
EDL933 Trial 1	T4	11	0.0142	-0.251	≤ 0.0001
EDL933 Trial 1	T1	7	≤ 0.0001	-0.5075	≤ 0.0001
EDL933 Trial 1	rV5	9	0.0395	-0.356	≤ 0.0001
EDL933 Trial 1	T5	no growth	-	-1.32	≤ 0.0001
EDL933 Trial 1	T1+T4+rV5	24	≤ 0.0001	-0.6025	≤ 0.0001
EDL933 Trial 1	T5+T1+T4+rV5	no growth	-	-1.318	≤ 0.0001
EDL933 Trial 1	Control	4	≤ 0.0001	-	-
E32511 Trial 1	T4	24	0.0002	-0.7415	≤ 0.0001
E32511 Trial 1	T1	11	0.0157	-0.1345	0.8566
E32511 Trial 1	rV5	24	≤ 0.0001	-0.112	0.9349
E32511 Trial 1	T5	no growth	-	-1.26	≤ 0.0001
E32511 Trial 1	T1+T4+rV5	24	0.0113	-0.8595	≤ 0.0001
E32511 Trial 1	T5+T1+T4+rV5	no growth	-	-1.26	≤ 0.0001
E32511 Trial 1	Control	7	≤ 0.0001	-	-
R508N Trial 1	T4	no growth	-	-1.217	≤ 0.0001
R508N Trial 1	T1	9	≤ 0.0001	-0.1525	≤ 0.0001
R508N Trial 1	rV5	24	≤ 0.0001	-0.267	≤ 0.0001
R508N Trial 1	T5	no growth	-	-1.22	≤ 0.0001
R508N Trial 1	T1+T4+rV5	no growth	-	-1.214	≤ 0.0001
R508N Trial 1	T5+T1+T4+rV5	no growth	-	-1.216	≤ 0.0001
R508N Trial 1	Control	5	0.03	-	-
E318N Trial 1	T4	24	≤ 0.0001	-0.724	≤ 0.0001
E318N Trial 1	T1	24	≤ 0.0001	-0.203	0.2982
E318N Trial 1	rV5	24	≤ 0.0001	-0.1075	0.9015
E318N Trial 1	T5	no growth	-	-1.284	≤ 0.0001
E318N Trial 1	T1+T4+rV5	no growth	-	-1.283	≤ 0.0001
E318N Trial 1	T5+T1+T4+rV5	no growth	-	-1.286	≤ 0.0001
E318N Trial 1	Control	7	≤ 0.0001	-	-

Table 3.2: Increase in optical density of cultures Increase in optical density of four strains of phage-treated *E. coli* O157 over a period of 24 hours, with measurements taken for 0-12 hours and at 24 hours.

O157 strain	Phage treatment	Statististically significant growth time point (hours)	*P* value	Difference in OD600 compared to control at 24 h	*P* value
EDL933 Trial 2	T4	22	0.0345	-0.1865	0.9957
EDL933 Trial 2	T1	12	0.0462	-0.262	0.9745
EDL933 Trial 2	rV5	18	0.0429	-0.134	0.9993
EDL933 Trial 2	T5	no growth	-	-1.074	0.0125
EDL933 Trial 2	T1+T4+rV5	no growth	-	-0.663	0.3052
EDL933 Trial 2	T5+T1+T4+rV5	no growth	-	-0.9265	0.047
EDL933 Trial 2	Control	12	0.0043	-	-
E32511 Trial 2	T4	no growth	-	-0.9085	0.0152
E32511 Trial 2	T1	12	0.019	-0.142	0.9978
E32511 Trial 2	rV5	18	0.0136	-0.377	0.7651
E32511 Trial 2	T5	no growth	-	-1.235	0.0003
E32511 Trial 2	T1+T4+rV5	no growth	-	-1.377	≤ 0.0001
E32511 Trial 2	T5+T1+T4+rV5	no growth	-	-1.365	≤ 0.0001
E32511 Trial 2	Control	12	0.0004	-	-
R508N Trial 2	T4	no growth	-	-0.5955	0.4305
R508N Trial 2	T1	14	0.039	-0.0265	≥ 0.9999
R508N Trial 2	rV5	16	0.0379	-0.0415	≥ 0.9999
R508N Trial 2	T5	no growth	-	-1.168	0.0049
R508N Trial 2	T1+T4+rV5	no growth	-	-1.049	0.0156
R508N Trial 2	T5+T1+T4+rV5	no growth	-	-1.172	0.0047
R508N Trial 2	Control	12	0.0266	-	-
E318N Trial 2	T4	12	≤ 0.0001	0.1545	0.9843
E318N Trial 2	T1	12	0.0002	0.1735	0.9717
E318N Trial 2	rV5	12	0.0033	0.113	0.997
E318N Trial 2	T5	16	0.0382	0.099	0.9986
E318N Trial 2	T1+T4+rV5	no growth	-	-0.981	0.0001
E318N Trial 2	T5+T1+T4+rV5	no growth	-	-0.9925	0.0001
E318N Trial 2	Control	18	0.0009	-	-
C0281-31N Trial 1	T4	12	≤ 0.0001	-0.2415	0.1357
C0281-31N Trial 1	T1	12	≤ 0.0001	-0.401	0.0011
C0281-31N Trial 1	rV5	12	≤ 0.0001	-0.1495	0.6604
C0281-31N Trial 1	T5	12	≤ 0.0001	-0.2825	0.0479
C0281-31N Trial 1	T1+T4+rV5	14	0.0043	-1.237	≤ 0.0001
C0281-31N Trial 1	T5+T1+T4+rV5	no growth	-	-1.513	≤ 0.0001
C0281-31N Trial 1	Control	12	≤ 0.0001	-	-
H4420N Trial 1	T4	no growth	-	-0.7055	0.0146

H4420N Trial 1	T1	12	≤ 0.0001	-0.0285	≥ 0.9999
H4420N Trial 1	rV5	12	≤ 0.0001	-0.001	≥ 0.9999
H4420N Trial 1	T5	no growth	-	-1.285	≤ 0.0001
H4420N Trial 1	T1+T4+rV5	no growth	-	-0.97	0.0002
H4420N Trial 1	T5+T1+T4+rV5	no growth	-	-1.291	≤ 0.0001
H4420N Trial 1	Control	12	0.0003	-	-

Table 3.3: Increase in optical density of cultures of four strains of phage-treated *E. coli* O157 over a period of 24 hours, with measurements taken for 12-24 hours.

For EDL933 cultured for 24 hours, with measurements at 0, 2, 4, 6, 8, 10, 12, and 24 hours (Figure *3.2*), the most effective phage treatments that resulted in the least amount of turbidity of the media at 24 h were T5 (-1.3) and T1+T4+T5+rV5 (-1.3), in both tubes there was no measurable growth at any time point. The combination T1+T4+rV5 resulted in a 0.60 ($P \leq 0.0001$) reduction in OD_{600} at 24 h, and was more effective at lysis than T4 ($P \leq 0.0001$) and rV5 ($P \leq 0.0001$) individually at 24 h, but did not differ ($P = 0.1648$) from T1 at 24 h. All six phage treatments reduced the growth ($P \leq 0.0001$ for all phages) of EDL933 at 24 h as compared to the control. A significant increase in OD_{600} of EDL933 in the T1(+0.45 OD_{600}, $P \leq 0.0001$), T4 (+0.92 OD_{600}, $P \leq 0.0001$), rV5 (+0.19 OD_{600}, $P = 0.0001$) and T1+T4+rV5 (+0.72 OD_{600}, $P \leq 0.0001$) cultures was seen between 12 and 24 h.

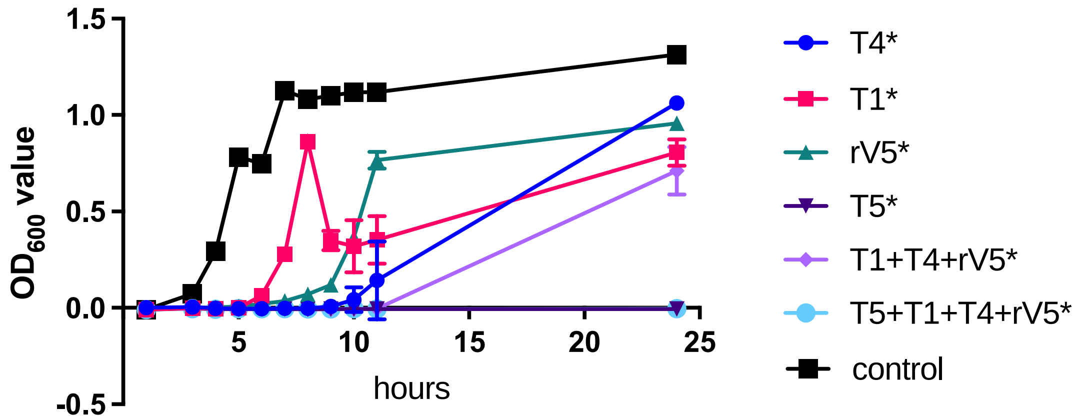


Figure 3.2:: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 EDL933

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For EDL933 cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.3*) the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T5 phage treatment, with a 1.1 reduction in OD_{600} (P = 0.0125), and combination T1+T4+T5+rV5 with a 0.9 reduction in OD_{600} (P = 0.0470) in comparison to untreated control. Combination T1+T4+rV5, T1, T4, and rV5 phage treatments did not result in a significant reduction ($P \leq 0.05$) in turbidity at 24 h in comparison to untreated control.

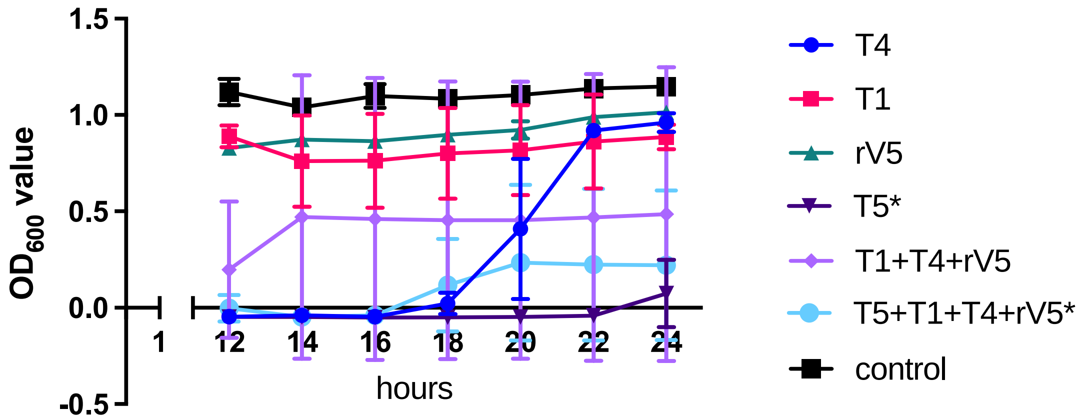


Figure 3.3: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 EDL933.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

In the growth experiment with the *E. coli* strain E32511, the most effective phage treatments that resulted in the least amount of turbidity at 24 h was T5, resulting in a reduction of 1.3 OD_{600} ($P \leq 0.0001$) and the T1+T4+T5+rV5, resulting in a reduction of 1.3 OD_{600} ($P \leq 0.0001$) (Figure *3.4*), both of which had no measurable growth (mean OD_{600}=0.00) at any time point, and were equally effective (0 difference, $P \leq 0.9999$) at lysing E32511. At the 24 h time point, the next most effective treatment was the combination of T1+T4+rV5 phages, resulting in a 0.86 OD_{600} ($P \leq 0.0001$) reduction compared to untreated control, followed by the T4 treatment, which resulted in a 0.74 OD_{600} ($P \leq 0.0001$) reduction in comparison to control. The additions of the T1 treatment and the rV5 treatment individually to the E32511 *E. coli* O157 culture did not alter OD from that observed for the control at 24 h.

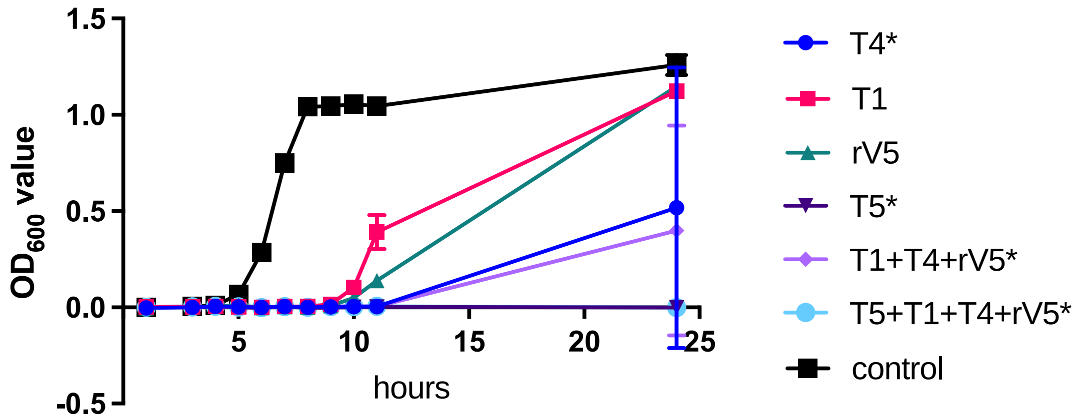


Figure 3.4: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 E32511.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For E32511 cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.5*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the combination T1+T4+T5+rV5 treatment with a reduction of 1.4 OD_{600} ($P \leq 0.0001$) and the combination T1+T4+rV5 treatment with a reduction of 1.4 OD_{600} ($P \leq 0.0001$), followed by the T5 phage treatment with a reduction of 1.2 OD_{600} ($P = 0.0003$)in comparison to untreated control. T4 was the next most effective treatment, with a reduction of 0.91 OD_{600} ($P = 0.0152$) in comparison to untreated control at 24 h. T1 and rV5 phage treatments did not have a significant difference from the untreated control at 24 h.

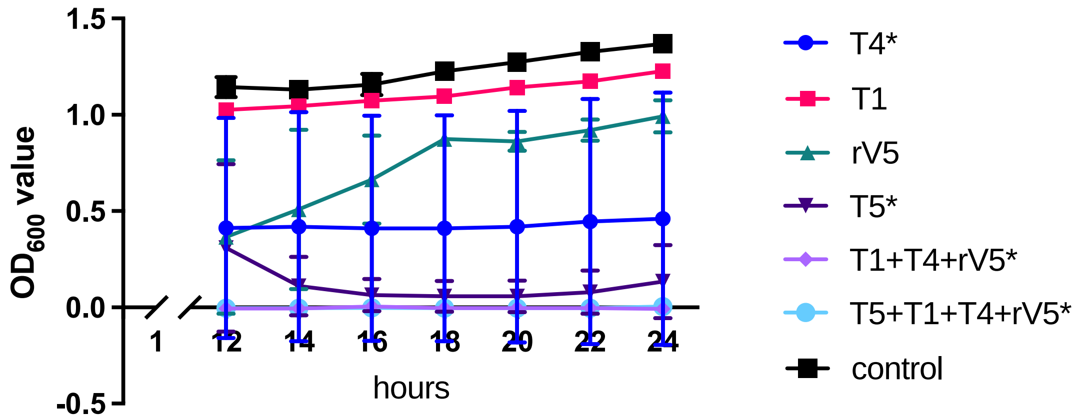


Figure 3.5: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 E32511.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

R508N cultured for 24 hours with measurements at 0, 2, 4, 6, 8, 10, 12, and 24 hours (Figure *3.6*) was one of the most susceptible to the phage treatments of all the tested strains of O157. The following phage treatments maintained no measurable bacterial growth over the 24 h incubation: T5 phage treatment, resulting in a reduction of 1.2 OD_{600} ($P \leq 0.0001$), the combination T1+T4+T5+rV5 resulting in a reduction of 1.2 OD_{600} ($P \leq 0.0001$), the combination T1+T4+rV5 resulting in a reduction of 1.2 OD_{600} ($P \leq 0.0001$), and T4 resulting in a reduction of 1.2 OD_{600} ($P \leq 0.0001$) in comparison to control. In addition, rV5 completely inhibited bacterial growth for 11 hours, and resulted in a significant reduction of 0.27 OD_{600} ($P \leq 0.0001$) in comparison to untreated control, and T1 completely inhibited bacterial growth for 8 hours, and resulted in a significant reduction of 0.15 OD_{600} ($P \leq 0.0001$) in comparison to untreated control.

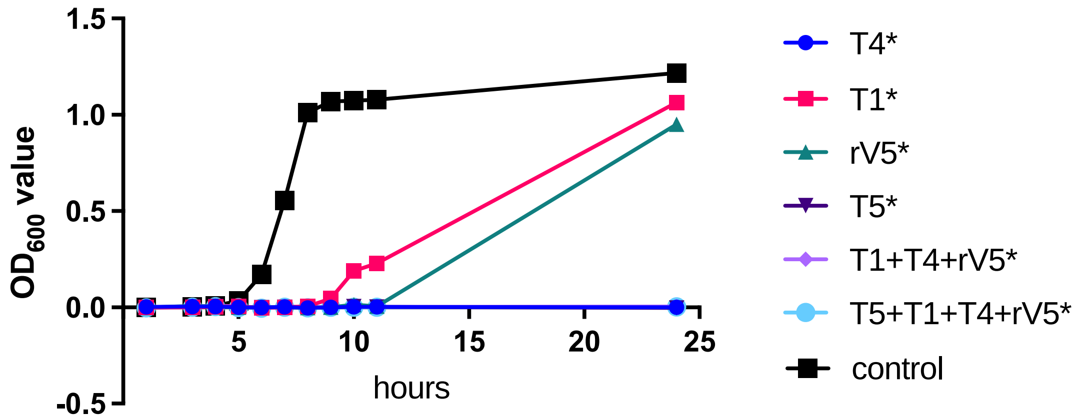


Figure 3.6: Preliminary exploration of O157 strains: Increase in optical density at 600nm of cultures of phage-treated *E. coli* O157 R508N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For R508N cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.7*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T5 phage treatment, resulting in a reduction of 1.2 OD_{600} (P = 0.0049), combination T1+T4+T5+rV5 treatment resulting in a reduction of 1.2 OD_{600} (P = 0.0047) , and combination T1+T4+rV5 treatment resulting in a reduction of 1.0 OD_{600} (P = 0.0156) in comparison to untreated control. T1, T4, and rV5 phage treatments did not result in a significant reduction in turbidity at 24 h in comparison to untreated control.

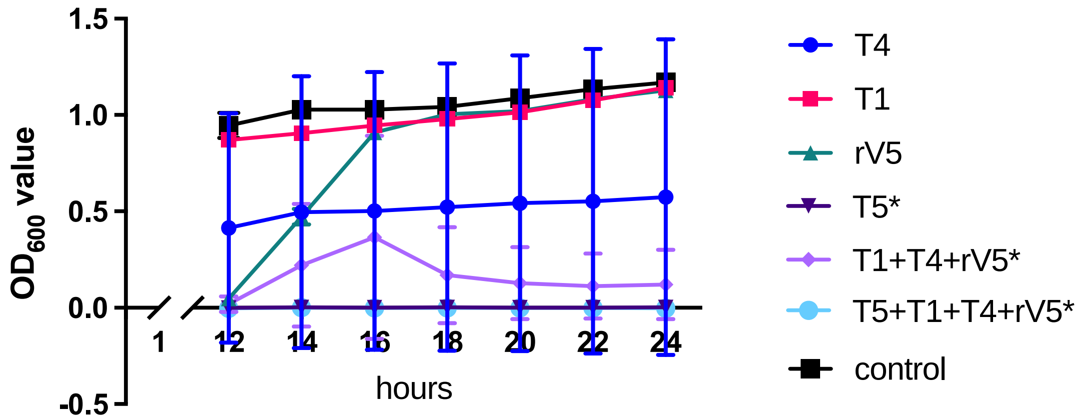


Figure 3.7: : Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 R508N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For E318N cultured for 24 hours, with measurements at 0, 2, 4, 6, 8, 10, 12, and 24 hours (Figure *3.8*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T5, combination T1+T4+T5+rV5, and combination T1+T4+rV5 phage treatments, all resulting in a reduction of 1.3, OD_{600} ($P \leq 0.0001$) in comparison to untreated control. T4 was the next most effective phage treatment, with a reduction of 0.72 OD_{600} ($P \leq 0.0001$) in comparison to control at 24 h. While the T1 and rV5 treatments did not have any significant differences from the untreated control at 24 h, both were able to prevent significant growth of bacteria for 11 hours.

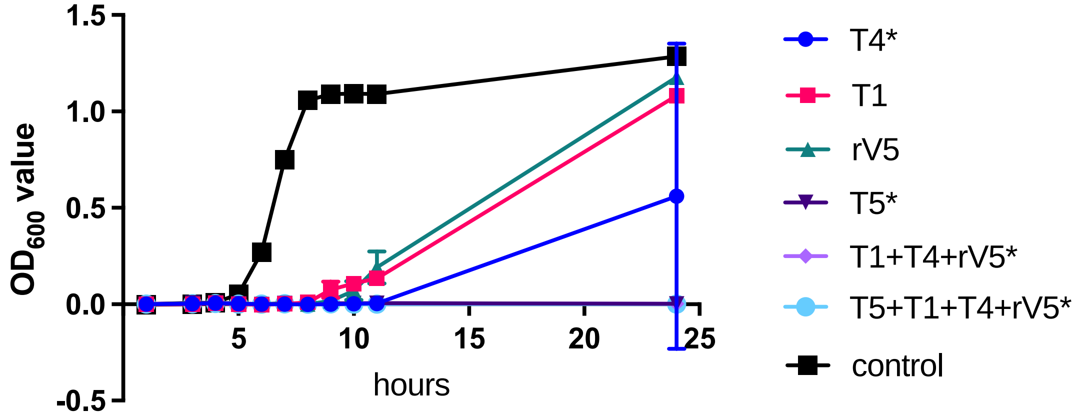


Figure 3.8: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 E318N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For E318N cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.9*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T1+T4+T5+rV5 and T1+T4+rV5 treatments, both resulting in a reduction of 1.0 OD_{600} (P = 0.0001) in comparison to untreated control. T1, T4, T5, and rV5 phage treatments did not result in a significant reduction in turbidity at 24 h in comparison to untreated control, making E318N the only strain to have only phage cocktails result in a significant reduction at 24 h in comparison to untreated control.

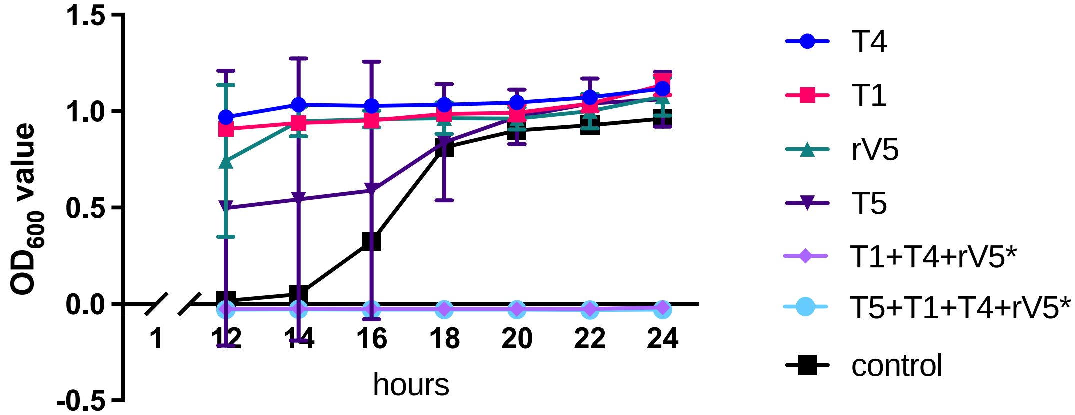


Figure 3.9: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 E318N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For C0281-31N cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.10*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T1+T4+T5+rV5 treatment resulting in a reduction of 1.5 OD_{600} ($P \leq 0.0001$), followed by T1+T4+rV5 resulting in a reduction of 1.2 OD_{600} ($P \leq 0.0001$) in comparison to untreated control. T1 was the next most effective phage treatment, with a reduction of 0.40 OD_{600} ($P = 0.0011$) at 24 h in comparison to untreated control. Treatment with T5 resulted in a reduction of 0.28 OD_{600} ($P = 0.0479$) at 24 h in comparison to control. T4 and rV5 did not result in a significant reduction in turbidity at 24 h in comparison to untreated control.

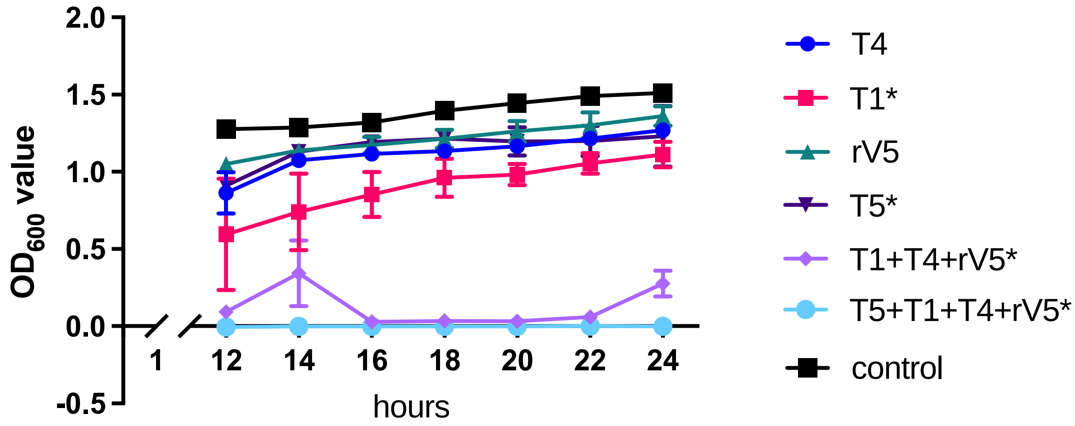


Figure 3.10: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 C0281-31N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For H4420N cultured for 24 hours, with measurements at 12, 14, 16, 18, 20, 22, and 24 hours (Figure *3.11*), the most effective phage treatments that resulted in the least amount of turbidity at 24 h were the T1+T4+T5+rV5 and T5 treatments, both resulting in a reduction of 1.3 OD_{600} ($P \leq 0.0001$) in comparison to untreated control. T1+T4+rV5 was the next most effective treatment, resulting in a reduction of 1.0 OD_{600} ($P = 0.0002$) at 24 h in comparison to untreated control, followed by treatment with T4, which resulted in a significant reduction in comparison to control with a 0.40 OD_{600} ($P = 0.0146$) reduction at 24 h. T1 and rV5 did not result in a significant reduction in turbidity at 24 h in comparison to untreated control.

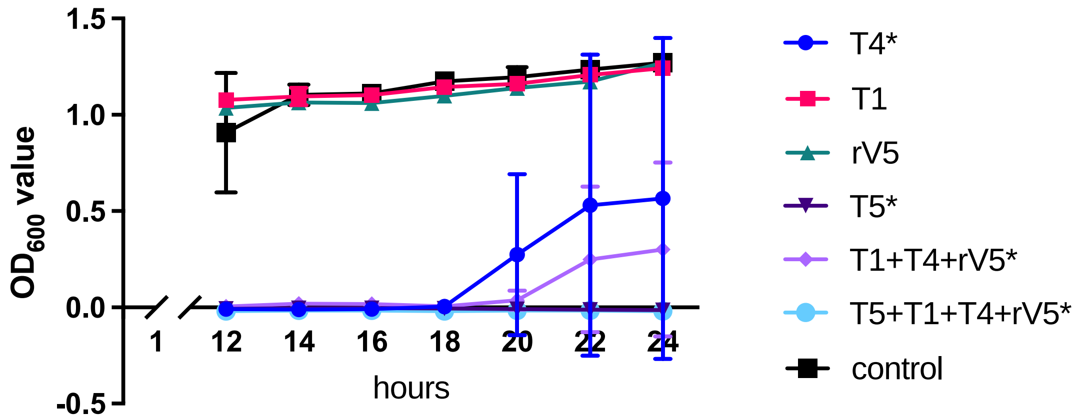


Figure 3.11: Preliminary exploration of O157 strains: Increase in optical density of cultures of phage-treated *E. coli* O157 H4420N.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

Phage treatment	EDL933		E32511		R508N		E318N		CO281-31N	H4420N
Trial	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 2	Trial 2
T4	+	-	+	+	++	-	+	-	-	+
T1	+	-	-	-	+	-	-	-	+	-
rV5	+	-	-	-	+	-	-	-	-	-
T5	++	++	++	++	++	++	++	-	+	++
T1+T4+rV5	+	++	+	++	++	++	++	++	+	++
T5+T1+T4+rV5	++	++	++	++	++	++	++	++	++	++

Table 3.4: Summary of the efficacy of phage treatments on six strains of STEC over 24 h of growth as measured by optical density

The efficacy is ranked as follows: -: no significant difference from control at 24 h; +: significantly different from control at 24 h; ++: no significant growth observed

3.3.3 Phage lysis kinetics

Due to titre variations in the phage stocks and bacterial concentration of ELD933, the MOIs varied, with the first trial having the highest average MOI of 7940±5549, the second trial having an average MOI of 4082±2852, and the third trial having an average MOI of 2242±433 (Table *3.5*).

Trial	Phage	MOI
Phage lysis kinetics trials 1	T1	5843.14
Phage lysis kinetics trials 1	T4	3267.97
Phage lysis kinetics trials 1	T5	3777.78
Phage lysis kinetics trials 1	rV5	18431.37
Phage lysis kinetics trials 1	T1+T4+rV5	8492.37
Phage lysis kinetics trials 1	T1+T4+T5+rV5	7830.07
Phage lysis kinetics trials 2	T1	3004.03
Phage lysis kinetics trials 2	T4	1680.11
Phage lysis kinetics trials 2	T5	1942.20
Phage lysis kinetics trials 2	rV5	9475.81
Phage lysis kinetics trials 2	T1+T4+rV5	4366.04
Phage lysis kinetics trials 2	T1+T4+T5+rV5	4025.54
Phage lysis kinetics trial 3	T1	1600.00
Phage lysis kinetics trial 3	T4	2900.00
Phage lysis kinetics trial 3	T5	2000.00
Phage lysis kinetics trial 3	rV5	2333.33
Phage lysis kinetics trial 3	T1+T4+rV5	2411.11
Phage lysis kinetics trial 3	T1+T4+T5+rV5	2208.33

Table 3.5: MOI of the three phage lysis trials

In the optical density analysis of the first trial (Figure *3.12*), the most effective phage treatment was T5, which resulted in a reduction of 1.3 OD_{600} ($P \leq 0.0001$) compared to the untreated control at 24 h. The combination treatment using T1+T4+T5+rV5 was less effective ($P \leq 0.0001$) in lysing EDL933 compared to the single T5 phage treatment. In the EDL933 culture treated with the combination T1+T4+T5+rV5, we observed a reduction of 1.0 OD_{600} ($P \leq$ 0.0001) in comparison to the untreated control at 24 h. The T1+T4+rV5 cocktail treatment of EDL933 resulted in significantly less growth (P = 0.0015) at 24 h than the individual component phage treatments of T1, T4, and rV5. The T1 treatment in particular only had a significant difference from the untreated control up until 7 h, and then was not significantly different from the control.

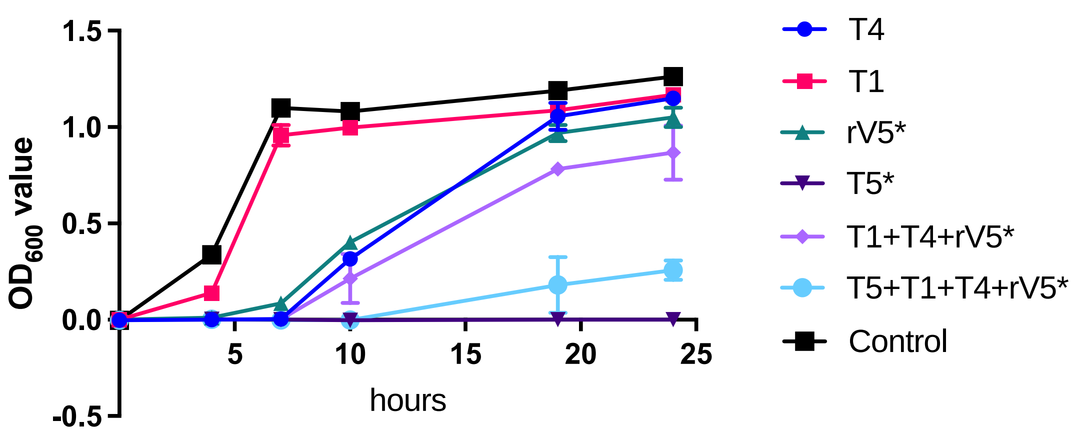


Figure 3.12: Phage lysis trial 1: Increase in optical density of cultures of phage-treated *E. coli* O157 EDL933.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

Serial dilutions on TSA plates to determine CFU/ml was also utilized to obtain a more accurate estimation of EDL933 concentration. In the first phage lysis trial (Figure *3.13*), the most effective phage treatment was T5 alone, resulting in a reduction in bacterial population of EDL933 of 7.2 $\log_{10}$ CFU/ml ($P \leq 0.0001$) at 7 h, a reduction of 6.1 $\log_{10}$ CFU/ml ($P \leq 0.0001$) at 19 h, and a reduction of log 4.9 CFU/ml (P = 0.0008) at the 24 h time point in comparison to the untreated control. The T1+T4+T5+rV5 cocktail treatment had a reduction at 7 and 19 h with a 6.1 $\log_{10}$ CFU/ml ($P \leq 0.0001$) and 5.0 $\log_{10}$ CFU/ml ($P = 0.0005$) in comparison to the untreated control, but did not have a significant reduction at 24 h, which was significantly different ($P = 0.0277$) from the T5 reduction at 24 h. The three-phage cocktail treatment T1+T4+rV5 only had a reduction of 4.7 $\log_{10}$ CFU/ml ($P = 0.0013$) in comparison to the control at 7 h , and was not significantly different from the control at 19 and 24 h. T4 had a 3.9 $\log_{10}$ CFU/ml ($P = 0.0098$) reduction compared to untreated control at 7 h, but no significant reduction at 19 and 24 h. rV5 had a 3.2 $\log_{10}$ CFU/ml ($P = 0.0440$) reduction compared to untreated control at 7 h, but no significant reduction at 19 and 24 h. T1 was the least effective at lysis, with no significant difference of bacterial growth from the untreated control at any time point.

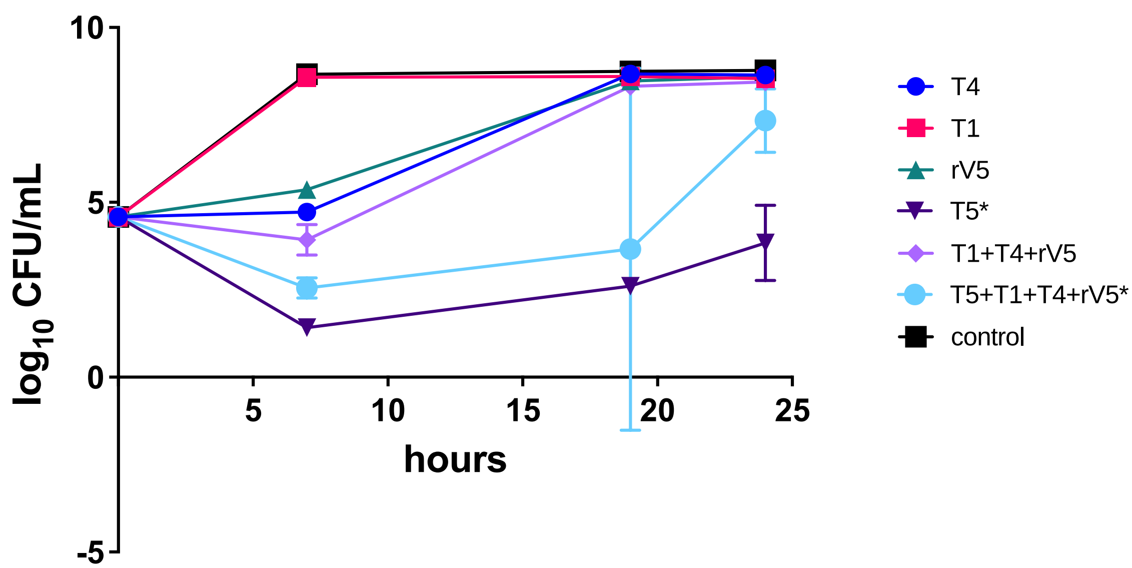


Figure 3.13: Phage lysis trial 1: Growth of phage-treated *E. coli* O157 EDL933 in mTSB for 24 h at 37°C at 170 RPM at average MOI of 7940.

The asterisks on the legend mark statistically significant difference in growth from control at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For the optical density analysis of the second phage lysis trial (Figure *3.14*), the most effective phage treatment was the combination treatment T1+T4+T5+rV5, with a reduction of 0.98 OD_{600} ($P \leq 0.0001$) compared to the untreated control at 24 h. The combination T1+T4+rV5 was the next most effective at lysing EDL933, with a reduction of 0.32 OD_{600} ($P \leq 0.0001$) compared to the untreated control at 24 h. T1 had a reduction of 0.27 OD_{600} ($P = 0.0262$ compared to the untreated control at 24 h. While the individual phage treatments T4, rV5, and T5 showed no difference compared to the untreated control treatment at 24 h, a significant suppression of bacterial growth occurred for the first 7 h for all three individual treatments.

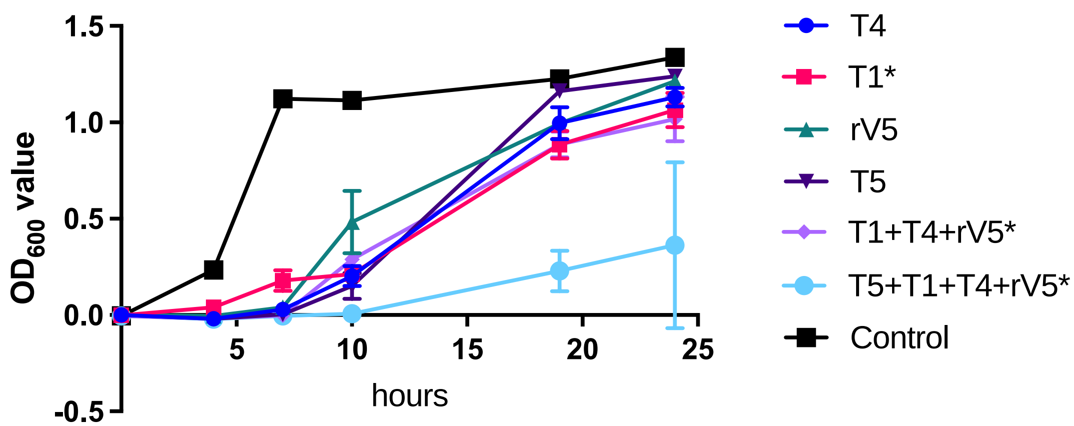


Figure 3.14: Phage lysis trial 2: Increase in optical density of cultures of phage-treated *E. coli* O157 EDL933.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For the CFU/ml measurements for the second trial (Figure *3.15*), the most effective phage treatment was T1+T4+T5+rV5, with a 6.8 $\log_{10}$ CFU/ml ($P \leq 0.0001$) reduction of EDL933 at 7 h, a 4.0 $\log_{10}$ CFU/ml ($P \leq 0.0001$) reduction at 19 h, and a 1.9 $\log_{10}$ CFU/ml ($P = 0.0018$) reduction at 24 h in comparison to the untreated control. The other phage treatments were able to considerably reduce bacterial growth by 3.2-5.1 $\log_{10}$ CFU/ml ($P \leq 0.0001$), but produced no reduction at 19 and 24 h.

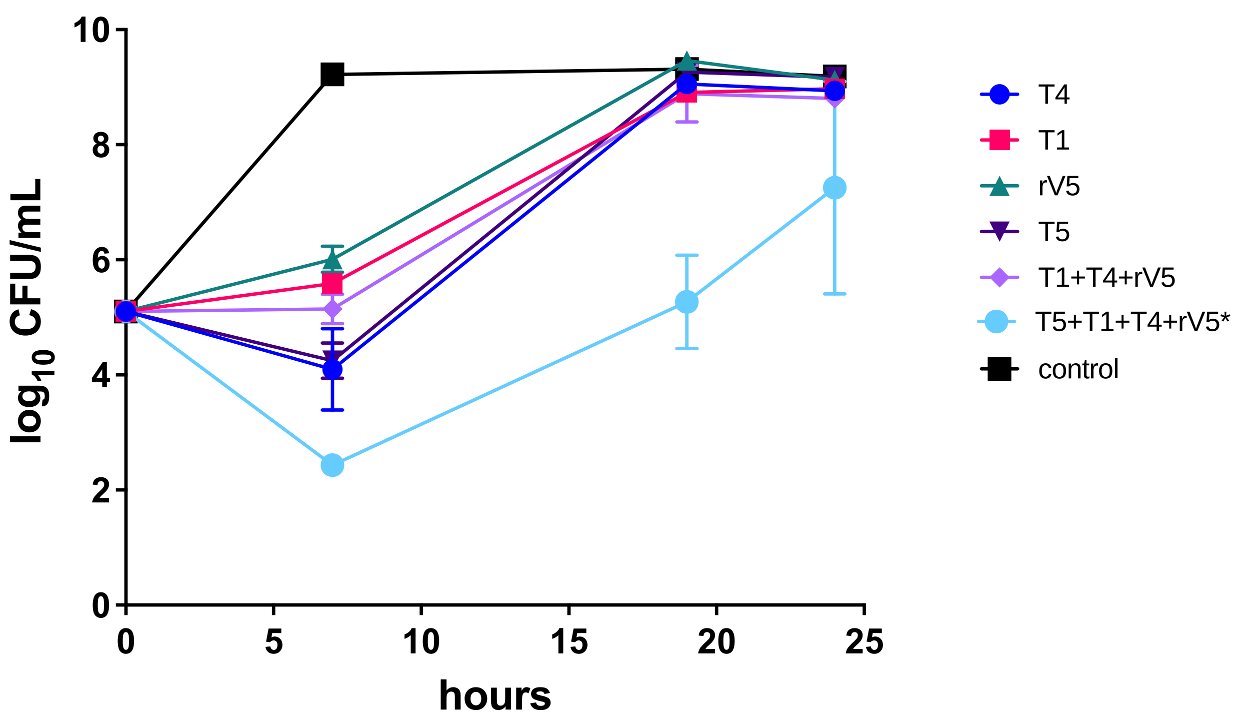


Figure 3.15: Phage lysis trial 2: Growth of phage-treated *E. coli* O157 EDL933 in mTSB for 24 h at 37°C at 170 RPM at average MOI of 4082.

The asterisks on the legend mark statistically significant difference in growth from control at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

For the optical density analysis of the third trial (Figure *3.16*), the most effective phage treatment at reducing turbidity was the combination treatment T1+T4+T5+rV5, with a reduction of 0.84 OD_{600} (P = 0.0033) compared to the untreated control at 24 h. The other phage treatments of T1+T4+rV5, T1, T4, rV5, and T5 had no significant differences from the untreated control at 24 h. However, T4, rV5, and T5 all supressed the growth of EDL933 for the first 7 hours.

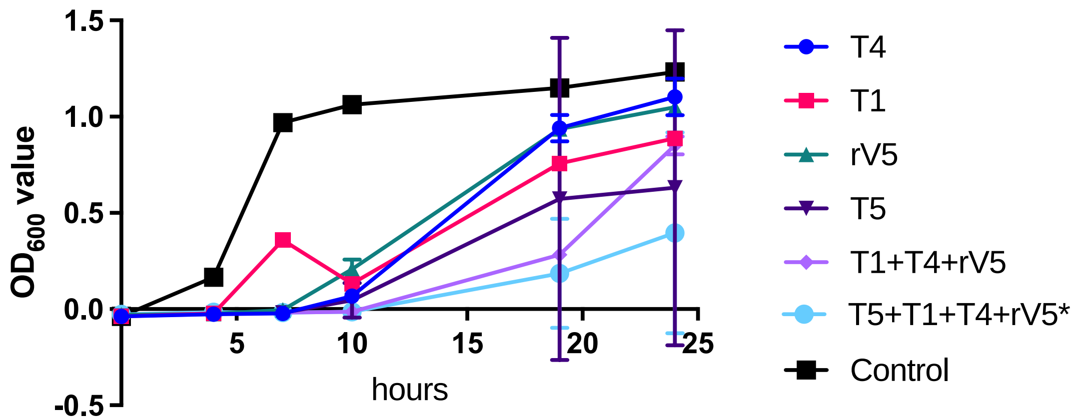


Figure 3.16: Phage lysis trial 3: Increase in optical density of cultures of phage-treated *E. coli* O157 EDL933.

The asterisks on the legend mark statistically significant difference in growth compared with the control culture at the 24 h time point. If no error bars are seen, the error was too small to appear on the graph.

In the CFU/ml measurements of the third trial (Figure *3.17*), the most effective treatment was T1+T4+T5+rV5, with a 5.9 $\log_{10}$ CFU/ml reduction at 7 h, and a 2.7 $\log_{10}$ CFU/ml reduction at 19 h in comparison to the untreated control, with no significant reduction at 24 h. The T1+T4+rV5, T5, T4, and T1 treatments were able to considerably reduce bacterial growth by 3.7-5.7 $\log_{10}$ CFU/ml (P = 0.0235) at 7 h, but produced no reduction at 19 and 24 h. The rV5 treatment did not have a significant reduction in comparison to the untreated control at any time point.

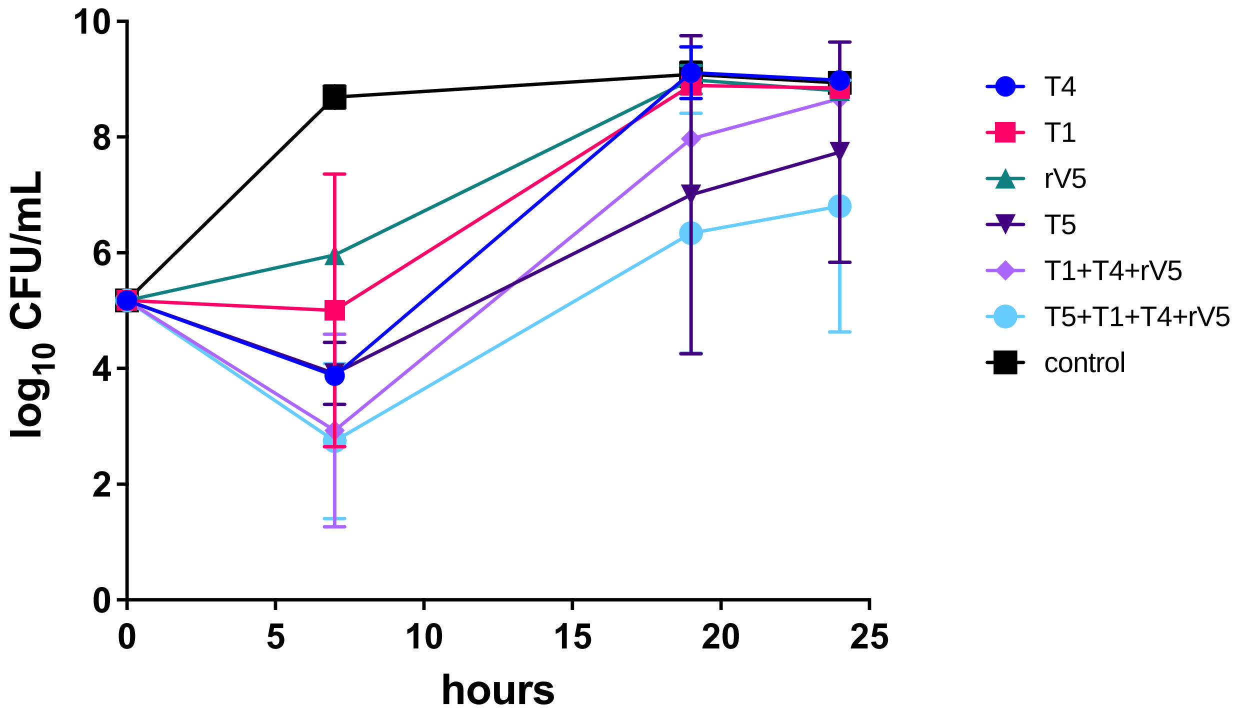


Figure 3.17: Phage lysis trial 3: Growth of phage-treated *E. coli* O157 EDL933 in mTSB for 24 h at 37°C at 170 RPM at average MOI of 2242.

The asterisks on the legend mark statistically significant difference in growth from control at the 24 h time point (no significant differences from control for any treatment at 24 h). If no error bars are seen, the error was too small to appear on the graph.

Across all three phage lysis trials, the time point where the phage treatments were most effective was 7 h, with 16 out of 18 phage treatments resulting in a significant reduction in EDL933 as compared to the untreated control. The 24 h time point only had significant reduction as compared to the control in 3 treatments, the T5 and T1+T4+T5+rV5 treatments in Trial 1, and the T1+T4+T5+rV5 treatment in Trial 2.

3.3.4 Effect of incubation time on phage concentration.

Counting of the phage present at different time points was only completed for Trial 1 (Figure *3.18*). The cocktails counts, as noted in the methods, were separated into three categories of small, medium, and large phages, with both rV5 and T4 in the small phage category. For individual phage treatment, T4, T1, rV5, and T5 did not have any significant increases or decreases in the concentration of phage present over the experimental period. The total number of phages in T1+T4+rV5 did not have any significant increases or decreases in concentration, and the small plaque phages (T4 and rV5) in T1+T4+rV5 did not have a significant change in concentration, but the large plaque phages (T1) differed significantly over the course of 24 h incubation. The titre of T1 dropped to 3.0 $\log_{10}$ PFU/mL at 7 h, was not detectable at 19 h and increased to 3.9 $\log_{10}$ PFU/ml PFU/mL at 24 h. The total number of phages in T1+T4+T5+rV5 did not have any significant differences in concentration at any of the time points, and the small plaque phages (T4 and rV5) in T1+T4+ T5+rV5 did not have a significant change in concentration, but the medium plaque phage (T5) had a significant increase of 5.3 $\log_{10}$ PFU/ml (P = 0.0040) between 19 and 24 h. The large plaque phage (T1) decreased in concentration gradually from 6.0 $\log_{10}$ PFU/ml at 7 h to 2.8 $\log_{10}$ PFU/ml at 19 h and was no longer detectable at 24 h.

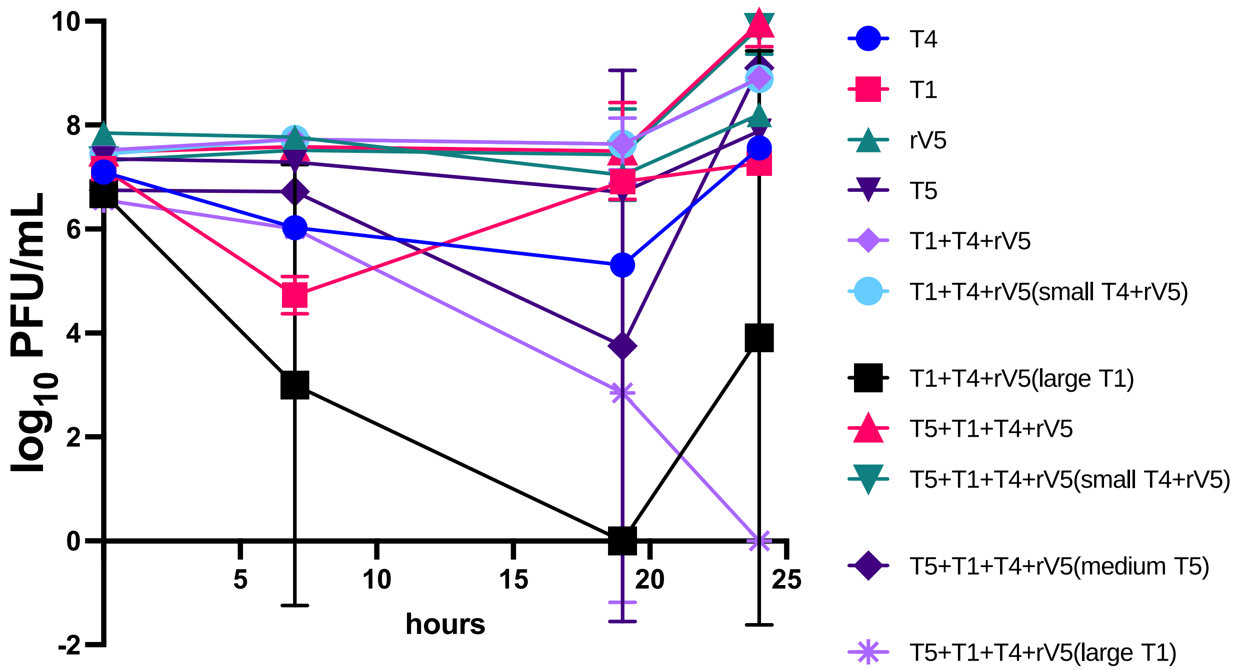
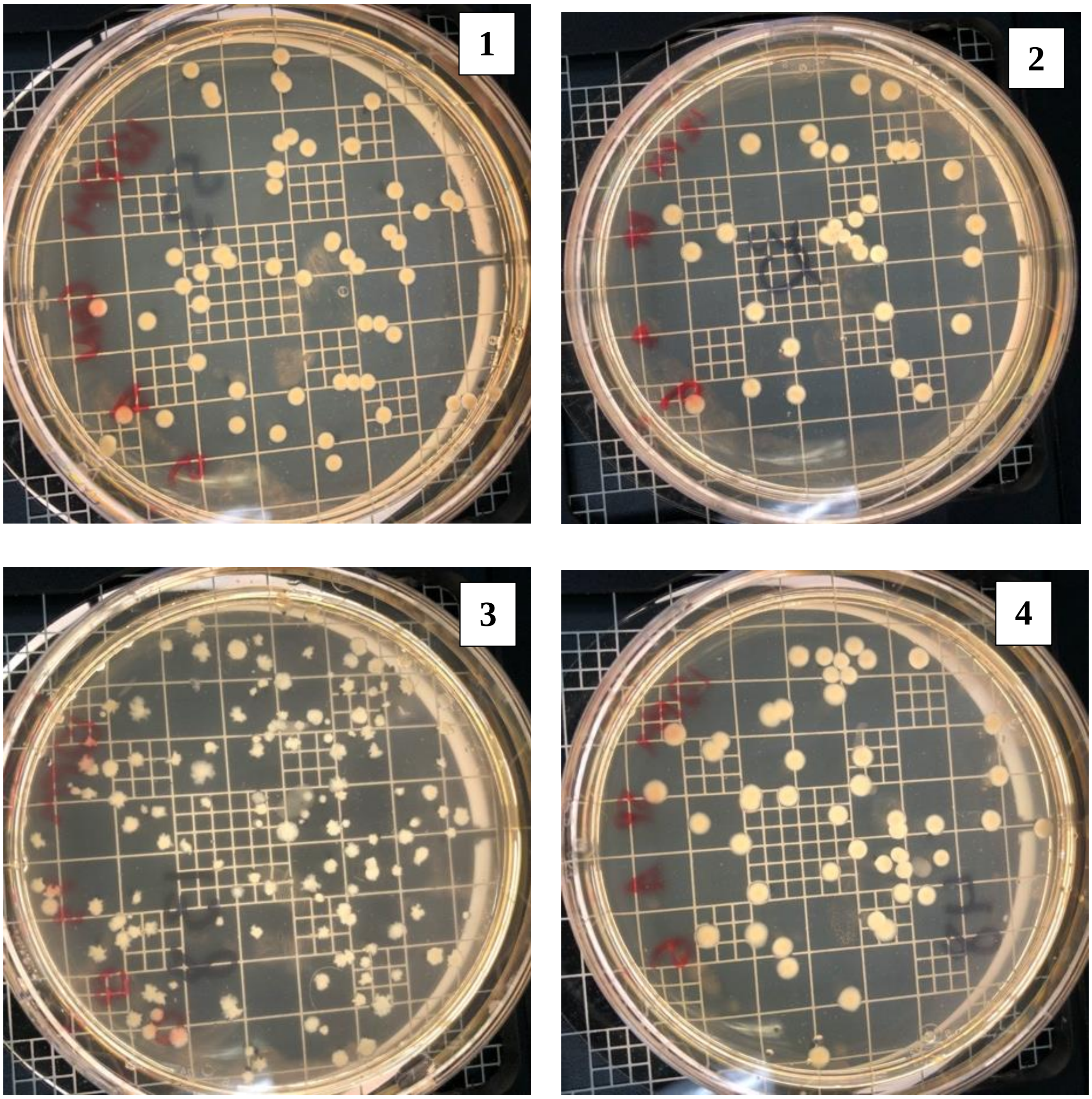


Figure 3.18: Phage lysis trial 1: Phage concentrations after growth in *E. coli* O157 EDL933.

3.3.5 Evaluation of potential development of anti-phage resistance of EDL933 after exposure to phage treatments

Certain colonies present on the plates from Trial 1 had atypical appearances (Figure *3.19*). The only treatment that resulted in different colony morphologies at all three time points (7, 19, and 24 hours) was T5-exposed EDL933, with a small and large colony at 7 and 19 h, and a small, medium, and large colony at 24 h. Additionally, the colonies on T5 had an atypical shape with irregular edges, unlike the smooth, round colonies of the control. T1+T4+rV5-exposed EDL933 and T1+T4+T5+rV5-exposed EDL933 had two distinct morphologies each at the 24 h time point, small and large. T1+T4+T5+rV5-exposed EDL933 also showed unusually large colonies in comparison to control. T1-exposed EDL933, T4-exposed EDL933, and rV5-exposed EDL933 did not have more than one distinct morphology.


1
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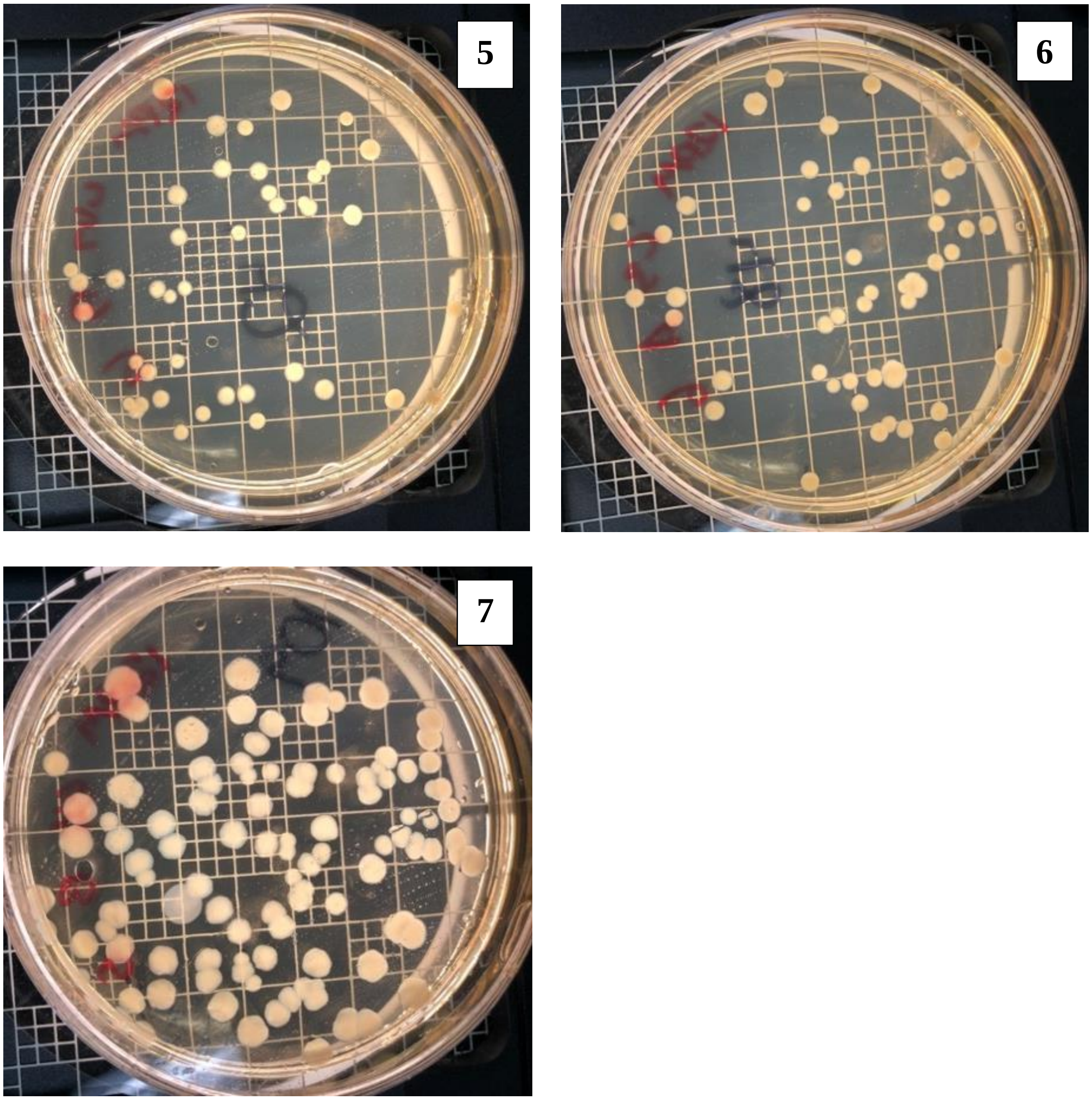

Figure 3.19: Colonies of EDL933 from Trial 1 at 24 h.

1 : Control, 2: T1, 3: T5, 4: rV5, 5: T4, 6: T1+T4+rV5, 7: T1+T4+T5+rV5

The susceptibility was measured by the MOI change in the exposed strain of EDL933 as compared to the parental strain when both were cultured with the same phage treatment (Table *3.6*). For T1, there was no change in the category of susceptibility between the control strain of EDL933 and the T1-exposed strain. For T4, there was a substantial decrease in susceptibility between the control and the T4-exposed strain, with the control MOI as 1.01×10^{-4} (extremely susceptible) and no lysis seen in the T4-exposed strain at any phage concentration. For rV5, neither the control or the rV5-exposed strain of EDL933 had any lysis. For T5, there was an increase in resistance for all of the mutants, requiring 10 – 1000 as many phages to result in complete lysis, but the mutants did remain susceptible to lysis by the phage treatments. For T1+T4+rV5-exposed EDL933, both the small and large phenotypic mutants showed a substantial decrease in susceptibility, with no lysis present at any tested concentration of phage treatment, whereas the control was extremely susceptible with an MOI of 2.27×10^{-4}. For T1+T4+T5+rV5-exposed EDL933, a similar pattern was seen, with the small and large phenotypic mutants showing no susceptibility to the T1+T4+T5+rV5 phage treatment, and the unexposed control showing extreme susceptibility with an MOI of 2.09×10^{-6}.

Post-treatment EDL933	Minimum MOI for complete lysis	Untreated control EDL933	MOI
T1	1.64×10^{-4}	T1	1.01×10^{-4}
T4	> 1	T4	1.26×10^{-5}
rV5	> 1	rV5	> 1
T5 7 SM	1.33×10^{-4}	T5	1.56×10^{-7}
T5 7 LG	1.20×10^{-4}	T5	1.56×10^{-7}
T5 19 SM	1.13×10^{-5}	T5	1.56×10^{-7}
T5 19 LG	7.40×10^{-6}	T5	1.56×10^{-7}
T5 24 SM	3.07×10^{-4}	T5	1.56×10^{-7}
T5 24 MD	1.02×10^{-4}	T5	1.56×10^{-7}
T5 24 LG	5.85×10^{-5}	T5	1.56×10^{-7}
T1+T4+rV5 SM	> 1	T1+T4+rV5	2.27×10^{-4}
T1+T4+rV5 LG	> 1	T1+T4+rV5	2.27×10^{-4}
T1+T4+T5+rV5 SM	> 1	T1+T4+T5+rV5	2.09×10^{-6}
T1+T4+T5+rV5 LG	> 1	T1+T4+T5+rV5	2.09×10^{-6}

Table 3.6: Susceptibility of phage exposed and unexposed (control) EDL933 challenged with the same phage treatment

Susceptibility is based on the multiplicity of infection (MOI: The lowest phage:bacteria ratio which results in complete bacterial clearance of a microplate well after 5 hours incubation in mTSB at 37°C. The phage-exposed strain notes the phage treatment that EDL933 has been exposed to and the plaque morphology, if it differs from control (SM: small plaque; MD: medium plaque; and LG: large plaque). The control strain column is EDL933 that has not previously been exposed to phages, cultured with the noted phage treatment.

Phage treatment	Trial 1	Trial 2	Trial 3
T4	-	-	-
T1	-	+	-
rV5	+	-	-
T5	++	-	-
T1+T4+rV5	+	+	-
T5+T1+T4+rV5	+	+	+

Table 3.7: Summary of the efficacy of phage treatments on three repeat trials of EDL933 over 24 h of growth, as measured by optical density.

The efficacy is ranked as follows: -: no significant difference from control at 24 h; +:

significantly different from control at 24 h; ++: no significant growth observed

3.4 Discussion

We used *in vitro* experiments in which different strains of *E. coli* O157 were infected with a single species of phage or cocktail of phages to assess if a combination of phages is more effective at reducing the concentration of bacteria and could decrease the potential for the emergence of mutants.

3.4.1 Preliminary exploration of *E. coli* O157 strains using optical density

Of the six strains of O157 examined, EDL933 was selected for further exploration. EDL933 is the laboratory reference strain of STEC O157:H7, initially isolated from ground beef associated with the so called 'hamburger outbreak' in Michigan, USA in 1982[114]. The genome of EDL933 has been fully sequenced [115], which facilitates the characterization of mutations associated with exposure to phages.

While not all evaluated O157 strains were equally susceptible to the same phages, we noted some similarities. Overall, T5 was the most effective phage species, which is consistent with previously published literature, where T5 was shown to be effective at lysing O157 [97,111]. On the other hand, T1 was the least effective phage species against the O157 strains, in contrast to previous findings, where earlier studies observed that T1 was effective at lysing O157[111]. In our experiments, there was not a single trial where T1 was the most effective at lysing *E. coli* O157, and furthermore, it was frequently the least effective against the six strains of *E. coli*. However, there was an interesting pattern seen with T1 in the first trial that used EDL933. The OD_{600} measurement increases from 6 to 8 hours, and then decreases again. Although this was not confirmed with determining phage counts at those time points, this increase and decrease may suggest active therapy, where phage amplification might take place and the newly generated phages were able to lyse EDL933[25].

Of the strains where the lysis experiments were repeated (EDL933, R508N, E32511, and E318N) there were differences between the 0-12 vs 12-24 OD_{600} measurements. The experiments had the same MOI between the first and second rounds, but a key difference was in the amount of mixing between the two trials. In the first round, where the cultures were grown for 24 h and measurements were taken from 0-12 hours, the cultures were vortexed every time the OD_{600} was measured. In the second round, the cultures were vortexed every time the OD_{600} measurements were taken, but the measurements did not start until the cultures had already been growing for 12 hours. While the cultures were being shaken at 170 RPM during growth, the additional mixing provided by the intermittent vortexing may have contributed to the change in lysis between the two trials. As phages rely on random encounters to interact with bacterial surface receptors, it is possible that an increase in mixing resulted in more bacterial-phage interactions, although this has not been seen in literature.

Some of the treatments within the trials, especially with measurements taken from 12-24 h, had large error bars due to variation in the two replicates. It is not known exactly what caused this variation between replicates, but it could have potentially been due to the volume of the cultures. As volume increases, the potential number of spontaneous mutations of the bacteria increases[116], and could potentially lead to changes between replicates.

The different susceptibilities demonstrate that there is a range of efficacy of phages even within the subgroup of O157. One of the main benefits of using phages against bacteria is the highly specific nature of phage infection[21]. This specific nature does lead to the need to accurately identify any target bacteria prior to the use of phage treatments, but there is research that indicates the host range of phages may not be as specific as previously thought, due to similar host receptor proteins[117–119].

3.4.2 Phage lysis kinetics

While the three trials had dissimilar MOIs, some conclusions can be drawn from the general interactions between the phage treatments and EDL933. The lysing efficacy of the cocktails in comparison to the individual phages varied. In the first phage lysis trial, the most effective treatment was T5, and in the second and third trials, the most effective treatment was T1+T4+T5+rV5. The three-phage cocktail T1+T4+rV5 was less or equally effective than T1+T4+T5+rV5 across the three trials, contradicting previous literature that suggests the three-phage combination will be more effective at lysis where Niu et al. suggested that T5 had an antagonistic effect on other phages when applied in a cocktail[97], but the comparison of T5 treatment alone to the two cocktails that were tested does not indicate an antagonistic effect. The experiment conducted by Niu et al. used 200 μl volumes, whereas our experiments used 10 mL volumes. The 50X increase in volume could have resulted in an increase in the number of phage-bacteria interactions, which could have potentially led to more mutations.

While there was no conclusive 'best' treatment over the three trials, the T5 treatment and the cocktail treatment containing T5 (T1+T4+T5+rV5) were the most effective. The T5 phage was very effective at lysis in these experiments, which aligns with previous work completed with T5[111].

In certain phage treatments (such as T1 in Trial 1), there was no difference in turbidity as compared to the untreated EDL933 control. As T1 has previously been shown to have lytic activity against *E. coli* O157, and was capable of lysis in the second and third phage trial, the reason behind the limited lysis observed in our experiments compared to other treatments seen in all three of the trials, especially at 24 h, is unknown.

The rV5 treatment also performed poorly, with no difference at any time point between the rV5 and the T1 treatment in the third and fourth trial. Niu et al. found rV5 to be less effective at lysis of O157 than T5 and T1 alone in previous work[97].

The changes between the three main trials could potentially be attributed to the differences in MOI across the three trials. The T5 treatment alone contained four times the amount of T5 phages as compared to the T1+T4+T5+rV5 treatment. As the first phage lysis trial had the highest average MOI, the amount of T5 phages present could have resulted in a different growth pattern in comparison to the second and third trial. Fischer et al. and Carey-Smith et al. postulated that the initial suppression of growth in bacterial cultures treated with phages could be due to only a phage susceptible population, which potentially already had some resistance to infection by phage[120,121]. Although the experiments of Fischer et al. were on a continuous culture, and our experiments used a clonal culture from a single strain of *E. coli*, it is possible that the process of preparing the cultures for the three phage lysis trials could have resulted in a subpopulation of bacteria that were not as susceptible to the phage treatments in comparison to the other lysis trials. The bacterial cultures were first grown in a broth culture, then plated, and a single colony was selected for another broth culture for each phage lysis trial. While the original *E. coli* source was the same, mutations in *E. coli* can occur quickly, with Foster et al. demonstrating a mutation rate of 1-2 × 10^{-3} mutations per generation per genome[122]. Additionally, as noted above, the large volume cultures could lead to an increased number of mutations[116].

Previous research by Kocharunchitt et al. suggested that a temporary, acquired, non-specific phage resistance phenomenon may arise in bacteria exposed to phages [123]. Kocharunchitt's work was in *Salmonella enterica*, another member of the same family as *E. coli*, *Enterobacteriaceae*. They found that after an initial suppression of growth of 2-3 hours, the lytic phages were unable to inhibit growth of *S. enterica*. This could be a possible explanation for the initial lag in growth measured following phage infection (such as with T4, rV5, and T1+T4+rV5 in Trial 1) which was followed by an increase in turbidity.

The phage counts from Trial 1 indicate that there were certain phages that outcompeted others. The counts of T1 over time were different when it was a component of the two cocktails compared to when it was the individual treatment. When the treatment was T1 alone, the concentration decreased at 7 h, but increased after that measurement until at 19 and 24 h it was at a comparable level to the initial concentration. In the three-phage cocktail T1+T4+rV5, T1 was significantly outcompeted by T4 and rV5, with no detectable T1 phage present at 19 h before increasing at 24 h. In the T1+T4+T5+rV5 phage cocktail treatment, T1 decreases at every time point until it was undetectable at 24 h. T1 produced the largest plaque size of the four individual phages, which suggests that it had a large burst size, which describes the number of progeny virus particles released from a single infected cell[91]. However, T1 performed poorly individually in the lysis trials, and had a lower amount of replication than the rV5+T4 combination. Considering that the T1 treatment of EDL933 resulted in the least amount of lysis compared to the untreated control, and, like the other three individual phages, T1 is a lytic phage that requires lysing of the host to release progeny viruses, T1 replication could have been hindered by the limited amount of lysis. T5 also showed a different replication pattern when present in the T1+T4+T5+rV5 cocktail than when alone. This might have been due to T1 being outcompeted by the other phages in the cocktails. T5 alone did not show major fluctuations in PFU/mL over the 24 h time period, but when present in the T1+T4+T5+rV5 cocktail, there was a significant decrease at 19 h, before increasing again at 24 h. This could have been T4 and rV5 outcompeting T5 in replication between 7 and 19 h, resulting in fewer T5 progenies. The low numbers of T5 could have also been from a high amount of T5 being inside the bacterial cells, and not available to count.

3.4.3 Evaluation of potential development of anti-phage resistance of EDL933 after exposure to phage treatments

The different morphological appearances of the colonies in the T5, T1+T4+rV5, and T1+T4+T5+rV5 treatments post-24 h exposure to the phages potentially suggests phenotypic change of EDL933, however, as the sequencing was not completed at this moment, we do not know the genetic basis of these changes. O'Flynn et al. previously published a study where changes in colony morphology, including the colony shape and size, was observed following exposure to phages of the *Myoviridae* and *Siphoviridae* families, remarkably these exposed strains of *E. coli* O157 were found to be insensitive to subsequent phage infection [90]. Although we did not examine the bacteria from these mutant colonies under a microscope, it could be of interest to evaluate any potential physical changes in the bacterial cells, in addition to sequencing.

The assay which could determine susceptibility changes in the phage exposed strains of EDL933 suggested that there was an increase in resistance to phages in EDL933. As the rV5 control did not have any lysis, we cannot draw any meaningful conclusions from the inclusion of rV5. As such, any change in phage resistance in the cocktail-treated cultures is assumed to be due to the other phages present, not rV5. Of the EDL933 that showed no morphological change after 24 h exposure to phage treatments, the T1 treatment showed no difference in the MOI required to achieve complete lysis. On the other hand, the T4 exposed EDL933 showed a marked increase in resistance to T4. In the EDL933 exposed to T5 alone, which had the most morphological variants, a higher MOI was required to fully lyse the bacteria regardless of their morphology. A substantial increase in resistance to cocktail phage treatments was observed in both of the cocktail-treated EDL933 cultures when compared to the non-phage exposed EDL933. EDL933 exposed to T1 and T5 individually showed less of an increase in phage resistance than all of the cocktail-treated phenotypic atypical colonies. This finding is of interest as one of the main reasons cocktail phage treatments are being investigated is to reduce the onset of anti-phage resistance mutations.

This finding contradicts previous research that suggests that cocktails are more effective at minimizing anti-phage mutations. Fischer et al. determined that while phage cocktail treatments were only marginally more effective at reducing bacterial densities of *Campylobacter jejuni*, the cocktail treatment resulted in a lower level of phage resistance[125]. Korf et al. also found that phage resistance mutations arose when pathogenic *E. coli* was cultured with a single species of phage, but not with phage cocktails [126].

A previous publication examining T1, T4, and T5 phage treatments demonstrated that even though mutations arose in *E. coli* O157 on a beef matrix during phage treatment, no significant decrease in sensitivity to subsequent phage exposures was observed[111]. In our experiments, all the *E. coli* O157 that were exposed to phage cocktails had a reduction in phage sensitivity.

Another study found that a similar occurancef anti-phage *E. coli* O157 mutants resulted from both single and cocktail treatments with *Myoviridae* and *Siphoviridae* phages, and those anti-phage mutations became sensitive to phage infection again within 50 bacterial generations when the pressure of the phage presence was removed[90].

EDL933 that was treated with T5 resulted in the most morphological changes to the colonies, especially from the samples that were taken at 24 h. As T5 was highly effective at lysis, it is possible that only bacterial cells that developed visible mutations were able to escape infection and lysis by T5.

3.4.4 Limitations

The differences in MOI and the efficacy of lysis between the three trials makes it difficult to compare the three trials. More specific repeats may allow more confidence in comparison.

The use of a spectrophotometer to track the turbidity of bacterial cultures, while quick and inexpensive, is an inherently imprecise method of tracking growth. As the spectrophotometer only reads the amount of 600 nm light absorbed when shone through a culture, it cannot distinguish between live and dead cells, or other contaminating cells.

There were limitations to the method utilized to count the phage cocktail plaques. The dilutions used to count the plaques had to be calculated on the basis of the highest titre phage present in the cocktail, which often meant that a lower titre phage would not be at a high enough concentration to be countable. Furthermore, the two phages with the smallest plaques (T4 and rV5) were not distinguishable from one another, resulting in a count that included both of the smaller plaques as one. A qPCR to track replication of specific phages might be a more precise method of phage titration, which could be developed with sequencing data from the phages.

The microplate virulence assay has limitations in evaluation, as the visual analysis of the culture turbidity offers less accuracy than the use of a spectrophotometer to evaluate turbidity.

No genetic sequencing was performed over the course of the experiments, which reduces the ability to confirm any genetic mutations in either the phages or the *E. coli* treated with the phages.

This study could have also benefitted from repeats of some experiments due to some variability in replicates.

Chapter 4

4 General discussion

4.1 Implications

4.1.1 Methane mitigation

A common issue with microbial studies centering around growth of certain microbes is difficulty in culturing, especially highly fastidious microbes. *Methanobrevibacter spp.* has a long list of requirements for growth, including anaerobic conditions and a number of components to the media. In addition, the methods utilized for tracking growth were not ideal, with no overlap between the methane testing and the optical density measuring. A more refined protocol adapted to work in a local lab may allow more consistent growth of the methanogens, and determining a more precise method, or multiple methods, to measure the growth of the cultures could result in being able to track more consistent growth. It would also be of interest to determine if the lytic viris found within the genome of *M. ruminantium* is capable of being an effective tool for controlling the population of methanogens.

While the original goals of this project were not accomplished, there still lies a great deal of potential for the utilization of viruses in the control of specific microorganisms, including *M. ruminantium*. Utilizing existing viral controls for ecological issues holds promise, and the potential to use not only whole virus particles but also specific viral enzymes demonstrates the flexibility of such an approach.

Although the complexity of the rumen makes manipulation of the microbiome difficult, increasing knowledge of the microbial communities within and their interactions with each other is promising for methane mitigation[127].

4.1.2 Control of *E. coli* O157 through phage therapy

These experiments add to the existing theory that phage cocktails are not just the sum of their parts. Predicting phage treatment efficacy is complex, and more experimentation into phage efficacy is required to further evaluate the use of phages to control bacteria. Further examination of individual phages versus cocktails of those phages may reveal the best course of action for control or treatment of pathogenic bacteria.

There are benefits and drawbacks to use of cocktail and single phage treatments[96]. Single phage treatments may be more difficult to customize to specific applications, while cocktail phage treatments may have a higher cost to maintain a diverse bank of phages, although there is potential to target a wider array of hosts.

Although the resistance assays suggested that resistance mutations quickly emerged in phage-treated bacteria, the development of anti-phage mutations do not necessarily indicate that the particular phage treatment is not of value. Developing resistance to phage infection and lysis can have fitness trade-offs which may lead to decreased resistance to antibiotics, reduction in virulence, or establishment and colonization defects[128–131]. Such fitness costs can leave virulent bacteria more open to pressures from antibiotic therapy and the immune system, as suggested by

Mangalea and Duerkop[132]. Oechslin notes that fitness costs for phage-resistant bacteria often lie in virulence factors, such as slower growth rates, increased susceptibility to antibiotics, and higher LD50 (lethal dose to kill 50% of the population), especially for bacteria that colonize the gastrointestinal system[133].

4.2 Future directions

4.2.1 Methane mitigation

There are several potential improvements to the growth conditions of archaea that could be made in the future.

If multiple methods of measuring growth of the methanogens could be used in concert, the reliability of the growth measurements might increase compared with spectrophotometer results. While these methods of tracking growth have some specificity and accuracy advantages over using OD_{600}, using a spectrophotometer may remain the least expensive and quickest option. If measuring methane production as an indicator of growth is available, using both methane and turbidity increases in the culture may be a very effective method to monitor growth.

To isolate the proposed archaeal virus, we intended to adapt the protocol of Weidenbach et al[32] for the isolation of lytic viruses from *Methanosarcina spp*. which would involve several steps of filtration and centrifugation to isolate the predicted virus from rumen fluid. Additionally, we planned the induction of the archaea virus which is detectable in the genome of *M. ruminantium* by stressing the archaea. Induction of bacteriophages have been completed using a variety of methods, including chemical induction with Mitomycin C[134], chemical stress factors[135], and UV radiation[136], and such methods may be effective for inducing viral lysogeny in methanogens.

If methanogenesis could be reduced or eliminated from the rumen, there are a number of experiments that would be of interest, such as analysing the energy requirements of methanogenesis, which is significant, and can account for 2-12% of the gross energy intake that the ruminant consumes[137]. Examining potential weight gain after reduction of methanogenesis would be an interesting avenue to explore.

4.2.2 Control of *E. coli* O157 through phage therapy

Further exploration is needed to examine the nature of phage-phage interactions within cocktails, and the implications for potential phage therapies.

The exposed bacteria should also be sequenced to determine if any genetic changes occurred after a 24 h exposure to the different phages. As bacterial resistance to phages can develop in as little as 19 h[133] or even sooner[122], it is possible that resistance developed during the experiment, or possibly was present in the strain of EDL933 prior to the onset of the experiments. The experimental phages are common in commercial feedlots[110], the original source of *E. coli* O157, and it is possible that resistance was already present.

In comparing a change in susceptibility, it would be of interest to specifically examine any genetic differences between the EDL933 exposed to the individual versus the cocktail of phages, compared to unexposed EDL933 strain.

The atypical colony formation of some of the phage-exposed EDL933 cultures, suggests an alteration in the receptor usage of phages. A number of surface antigens act as receptors, and are responsible for the shape of the colony, such as O antigens and type 1 fimbriae[138,139]. It is not completely understood how these proteins influence colony morphology, but the deletion or mutation of these proteins results in an altered colony formation[138]. A mutation in a surface antigen of a bacterial cell that allows a reduced rate of entry by phage may be beneficial for survival which could have implications on colony formation and the growth of the bacteria.

The use of high doses of phages, hence, high MOIs, aids to limit the emergence of anti-phage mutants[140]. A threshold may exist where the benefits of a very high MOI may taper off, and it would be of interest to examine this in future experimentation.

Considering the fitness costs of resistance[132], it would be of interest to examine how phage-resistant *E. coli* strains compete with other bacteria in a multi bacterial system.

Previous studies have proposed that certain phage treatments are comparable to an antibiotic treatment[118], especially when a strain of bacteria is resistant to one or the other.

4.3 Conclusions

As antimicrobial resistance increases in both humans and livestock, the need for alternative treatments will increase as well. There is great potential for the use of phage therapy to combat both environmental and infectious disease challenges that are facing us today and in the future, but the optimal application strategy of phages remains to be determined.

While we were not able to reach to point of utilizing a virus against methanogenic archaea, the possibility remains of viruses being utilized for control of microbes other than pathogenic bacteria.

The research completed in this thesis suggests that interactions between phages in cocktails are promising area of research, especially as interest in phage therapy grows. While the use of phage cocktails holds a great deal of potential, we have yet to fully understand the interactions between phages when used in combination with each other. Gaining understanding of ideal, specific applications of viruses and phages for complex issues in both human and veterinary medicine can allow for more effective treatments in the future. While phage therapy may never fully replace antibiotics, an approach that utilizes a combination of phages, archaeal viruses, antibiotics, and other technologies may be a promising way forward.

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5 Appendices

5.1 DSMZ 141 media

Microorganisms

DSMZ

141. METHANOGENIUM MEDIUM (H_2/CO_2)

KCl	0.34	g
$MgCl_2 \times 6\ H_2O$	4.00	g
$MgSO_4 \times 7\ H_2O$	3.45	g
NH_4Cl	0.25	g
$CaCl_2 \times 2\ H_2O$	0.14	g
K_2HPO_4	0.14	g
NaCl	18.00	g
Trace element solution (see below)	10.00	ml
$Fe(NH_4)_2(SO_4)_2 \times 6\ H_2O$ solution (0.1% w/v)	2.00	ml
Na-acetate	1.00	g
Yeast extract (OXOID)	2.00	g
Trypticase peptone (BD BBL)	2.00	g
Na-resazurin solution (0.1% w/v)	0.50	ml
$NaHCO_3$	5.00	g
Vitamin solution (see below)	10.00	ml
L-Cysteine-HCl x H_2O	0.50	g
$Na_2S \times 9\ H_2O$	0.50	g
Distilled water	1000.00	ml

Dissolve ingredients (except bicarbonate, vitamins, cysteine and sulfide), sparge medium with 80% H_2 and 20% CO_2 gas mixture for 30 – 45 min to make it anoxic. Add and dissolve bicarbonate and adjust pH to 7.0, then dispense medium under 80% H_2 and 20% CO_2 gas atmosphere into anoxic Hungate-type tubes or serum vials to 30% of their volume and autoclave. After sterilization add cysteine and sulfide from sterile anoxic stock solutions autoclaved under 100% N_2 gas. Vitamins are prepared under 100% N_2 gas atmosphere and sterilized by filtration. Adjust pH of complete medium to 6.8 – 7.0, if necessary. For incubation use sterile 80% H_2 and 20% CO_2 gas mixture at two atmospheres of pressure.
Note: If the medium is being used without overpressure then adjust pH with a small amount of sterile anoxic 1 N HCl, if necessary.

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Modified Wolin's mineral solution:

Nitrilotriacetic acid	1.50	g
$MgSO_4 \times 7\ H_2O$	3.00	g
$MnSO_4 \times H_2O$	0.50	g
NaCl	1.00	g
$FeSO_4 \times 7\ H_2O$	0.10	g
$CoSO_4 \times 7\ H_2O$	0.18	g
$CaCl_2 \times 2\ H_2O$	0.10	g
$ZnSO_4 \times 7\ H_2O$	0.18	g
$CuSO_4 \times 5\ H_2O$	0.01	g
$KAl(SO_4)_2 \times 12\ H_2O$	0.02	g
H_3BO_3	0.01	g
$Na_2MoO_4 \times 2\ H_2O$	0.01	g
$NiCl_2 \times 6\ H_2O$	0.03	g
$Na_2SeO_3 \times 5\ H_2O$	0.30	mg
$Na_2WO_4 \times 2\ H_2O$	0.40	mg
Distilled water	1000.00	ml

First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals. Adjust final to pH 7.0 with KOH.

Wolin's vitamin solution:

Biotin	2.00	mg
Folic acid	2.00	mg
Pyridoxine-HCl	10.00	mg
Thiamine-HCl	5.00	mg
Riboflavin	5.00	mg
Nicotinic acid	5.00	mg
Ca-D-pantothenate	5.00	mg
Vitamin B_{12}	0.10	mg
p-Aminobenzoic acid	5.00	mg
(±)-α-Lipoic acid	5.00	mg
Distilled water	1000.00	ml

For DSM 1498 and DSM 22353 adjust pH to 6.5.

For DSM 2373 increase the amount of trypticase to 6.00 g/l.

For DSM 4254 add a filter-sterilized, anoxic solution of L-histidine to a final concentration of 80.00 mg/l.

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5.2 Jay's media volatile fatty acid mixture

Ingredient	**Amount**
Valeric acid	25.0g
Isovaleric acid	25.0g
2-methylbutyric acid	25.0g
Isobutyric acid	25.0g
Distilled water	1000ml
Concentrated NaOH	To adjust pH to 7.5

5.3 Rumen Medium 2 (RM02)

<u>**RM02 base**</u>

distilled H_2O	950	ml	
KH_2PO_4	1.4	g	10 mM (MW = 136.09)
$(NH_4)_2SO_4$	0.6	g	5 mM (MW = 114.01)
KCl	1.5	g	20 mM (MW = 74.55)
trace element solution SL10	1	ml	
selenite/tungstate solution	1	ml	
0.1% (w/v) resazurin solution	4	drops	

Mix and boil to drive off dissolved gases. Cool in an ice bath while bubbling with 100% CO_2.

Once cool, add:

$NaHCO_3$	4.2	g	50 mM (MW = 84.01)
L-cysteine·HCl·H_2O	0.5	g	3 mM (MW = 175.64)

Dispense 9.5 mL of medium into Hungate or Balch tubes while gassing the tubes with 100% CO_2, seal and cap the tubes appropriately. Alternatively, dispense into other suitable culture vessels while gassing the vessels with 100% CO_2. Autoclave for 20 min at 15 psi at 121°C. Store in the dark for 24 h before using.

Before use, add:

vitamin 10 mix	0.1 ml per tube	
Mg/Ca mix	0.1 ml per tube	4mM $CaCl_2$, 4 mM$MgCl_2$

plus substrates, yeast extract, etc., as required

or clarified rumen fluid	0.5 mL per tube

(containing substrates, yeast extract, vitamins, etc)

Preparation of rumen fluid

Make sure rumen fluid has been centrifuged twice for 20 min at 10, 000 *x g*.

Preparing base clarified rumen fluid

Kenters et al. (2011)

1. Thaw rumen fluid stock and centrifuge at 10,000 *x g* at 4°C for 20 minutes.
2. Bubble with N_2 (100%) for 20 minutes and autoclave under N_2 at 121°C for 20 minutes (to inactivate viruses, etc.). This autoclaving step is best carried out in serum vials closed with a butyl rubber stopper and crimp-sealed. The anoxic conditions are to limit the formation of unwanted oxidation products. Allow to cool.

SAFETY NOTE: serum vials are not made of Pyrex or Duran glass, and so will crack and possibly shatter if cooled suddenly.

3. With stirring, add the following per 100 mL of rumen fluid:

$MgCl_2 \cdot 6H_2O$	1.63 g	0.08 M (MW = 203.30)
$CaCl_2 \cdot 2H_2O$	1.18 g	0.08 M (MW = 147.02)

A heavy precipitate will form. This precipitate is removed by centrifuging at 10,000 *x g* at 4°C for 20 minutes. The supernatant is the clarified rumen fluid.

No substrate-rumen fluid-vitamin mix (NoSubRFV)

Kenters et al. (2011)

1. Add the following per 100 mL of clarified rumen fluid:

yeast extract	2 g	2% (wt/vol)

2. Mix and bubble with N_2 (100%) gas for 15 minutes. Transfer to a N_2-flushed sterile serum vial through a 0.22 µm pore size filter using a syringe and needle (Note 1).
3. Per 100 mL of preparation, add using syringe and needle:

Vitamin 10 concentrate (Note 2)	2 mL

Add 0.5 mL of NoSubRFV to 9.5 mL of medium. This will result in final concentrations of 5% (vol/vol) rumen fluid, 4 mM each of Ca^{2+} and Mg^{2+}, and 1 g of yeast extract per litre.

Note 1: To sterilise the empty serum vials, put 100 µl distilled H_2O in the vial, gas with 100% N_2 for 20 min, and cap with a rubber butyl stopper and aluminium seal and autoclave.

Note 2: This is NOT the vitamin 10 mix, but the concentrate from which the vitamin 10 mix is prepared.

Vitamin 10 concentrate

Kenters et al. (2011)

distilled H_2O	1000	ml	
4-aminobenzoate	40	mg	
d-(+)-biotin	10	mg	(store biotin at 4°C)
nicotinic acid	100	mg	
hemicalcium D-(+)-pantothenate	50	mg	(store pantothenate at 4°C)
pyridoxamine hydrochloride	150	mg	(store pyridoxamine at 4°C)
thiamine chloride hydrochloride	100	mg	(store thiamine at 4°C)
cyanocobalamin	50	mg	(store cyanocobalamin at 4°C)
D,L-6,8-thioctic acid	30	mg	(store thioctic acid at 4°C)
riboflavin	30	mg	(store riboflavin at 4°C)
folic acid	10	mg	(store folic acid at 4°C)

Vitamin 10 concentrate is sterilized by filtration into sterile N_2-filled (100%) serum through sterile 0.22 µm pore-size polyethersulfone (PES) filters. (Note 1) The vials are wrapped in aluminium foil to protect against light, and stored at 4°C.

Note 1: To sterilise the empty serum vials, put 100 µl distilled H_2O in the vial, gas with 100% N_2 for 20 min, and cap with a rubber butyl stopper and aluminium seal and autoclave.

Vitamin 10 mix (working stock)

Add 1 mL of vitamin 10 concentrate to 9 mL sterile de-gassed H_2O.

The vitamin 10 mix is added at 0.1 mL per 10 mL of medium to give concentrations used by Janssen et al.

To prepare the sterile de-gassed H_2O, boil distilled H_2O, and add 9 mL aliquots to Hungate tubes while gassing the tubes with 100% N_2. Autoclave.

Alternatively: add 1 mL of vitamin 10 concentrate to 9 mL of sterile medium e.g. BY medium, to use this as a stock for adding to other tubes at 0.1 mL of this working stock per 10 mL of culture medium.

Mg/Ca mix

distilled H_2O	80	ml	
$MgCl_2 \cdot 6H_2O$	6.5	g	(MW = 203.30)
$CaCl_2 \cdot 2H_2O$	4.7	g	(MW = 147.02)

make up to 100 mL with distilled H_2O

Mix and boil. Cool in an ice bath while bubbling with CO_2. Once cool, dispense into Hungate tubes while gassing the tubes with CO_2, 10 mL Ca/Mg mix per tube. Autoclave.

This does not need to be added if clarified rumen fluid is added, which already contains Mg and Ca.

Selenite/tungstate solution

The selenite/tungstate solution (Tschech and Pfenning, 1984) is autoclaved aerobically, in 50 mL aliquots in 100 mL screw-capped bottles.

distilled H_2O	1.0	L
NaOH	0.5	g
$Na_2SeO_3 \cdot 5H_2O$	3	mg
$Na_2WO_4 \cdot 2H_2O$	4	mg

Trace element solution SL10

The components of the trace element solutions SL10 (Widdel et al., 1983) are added and dissolved in the order listed.

25% HCl	10	ml
$FeCl_2 \cdot 4H_2O$	1.5	g
distilled H_2O	1.0	L
$CoCl_2 \cdot 6H_2O$	190	mg
$MnCl_2 \cdot 4H_2O$	100	mg
$ZnCl_2$	70	mg
H_3BO_3	6	mg
$Na_2MoO_4 \cdot 2H_2O$	36	mg
$NiCl_2 \cdot 6H_2O$	24	mg
$CuCl_2 \cdot 2H_2O$	2	mg

The trace element solution is autoclaved aerobically, in 25 mL aliquots in 50 mL screw-capped bottles. Large stocks do not need to be sterilised for storage.

0.1% Resazurin solution

Reazurin (note)	0.1	g
distilled water	100	ml

Dissolve resazurin in distilled water.

Note: Weigh resazurin carefully to ensure powder does not spill as resazurin is a dye. Place paper towels around the balance to catch any powder particles.

References

Kenters N, Henderson G, Jeyanathan J, Kittelmann S, Janssen PH (2011) Isolation of previously-uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. Journal of Microbiological Methods 84: 52-60.

Tschech A, Pfennig N (1984) Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. Archives of Microbiology 137: 163-167

Widdel F, Kohring GW, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Archives of Microbiology 134: 286-294

5.4 *Methanobrevibacter spp.* preservation solutions

DMSO solution (50%)

To make 100 mL:

Salt Solution A	17	ml	
Salt Solution 2B	17	ml	
Distilled water	16	ml	
DMSO	50	ml	
$NaHCO_3$		0.5	g
Resazurin (0.1% solution)	1	drop	
L-cysteine-HCl	50	mg	

Add all the components, except L-cysteine-HCl, to an Erlenmeyer flask. Mix thoroughly and bring to the boil in the microwave.

Immediately bubble with O_2-free 100% CO_2. Cool in an ice bath, while still bubbling with O_2-free CO_2.

When the solution is cold, add the L-cysteine-HCl.

Dispense into CO_2 filled Hungate tubes; 8.0 mL per tube.

<u>Autoclave for 20 minutes at 115°C at 15 psi</u>.

Add 1 mL to the culture (~ 9 mL) to be frozen at a final concentration of 5%.

Salt Solution A

NaCl	15	g

KH_2PO_4	7.5 g
$(NH_4)_2SO_4$	3.75 g
$CaCl_2.2H_2O$	1.98 g
$MgSO_4.7H_2O$	3.0 g

Make up to 2.5 litres with distilled water.

Salt Solution 2B

$K_2HPO_4.3H_2O$	19.65 g
or	
K_2HPO_4	15 g

Make up to 2.5 litres with distilled water.

5.5 SOP-901: Phage research stock preparation of pathogenic *E. coli* infecting phage

1. Purpose

1.1. This protocol outlines the procedures used to propagate Pathogenic *E. coli* infecting bacteriophage for master, working and laboratory stocks.

1.1.1. NOTE: Master stocks should only be accessed once per year for the purpose of generating working stock. Working stock must be prepared from the master stock.

2. Equipment and Materials

2.1. Equipment

2.1.1. Incubator

2.1.2. Shaker

2.1.3. Biological Safety Cabinet (BSC)

2.1.4. Pipettes and tips

2.1.5. Centrifuge

2.1.6. Optical Spectrophotometer

2.2.Media and Reagents

2.2.1. Tryptic Soy Broth (TSB) ($\leq$ 1 week old)

2.2.2. Tryptic Soy Broth with 10mM $MgSO_4$ (mTSB)

2.2.3. $MgSO_4 \cdot 7H_2O$

2.3. Supplies

2.3.1. 0.2µm syringe filters and 3mL syringes

2.3.2. 0.2µm 50mL, and 500mL bottle top filters.

2.3.3. 50mL falcon tubes

2.3.4. 2mL microcentrifuge tubes

2.3.5. Pathogenic *E. coli* (EC) strain(s) host for propagation (BRLC Must be ≤ 3 days old, access the BRWS vial only once)

2.3.6. Bacteriophage purified Eluate 3, master stock or working stock.

2.3.7. Glass vials for stock storage

2.3.8. Test tubes

2.3.9. Disposable inoculation loops

2.4. Miscellaneous

2.4.1. Gloves

2.4.2. 70% Ethanol

2.4.3. 10% Bleach

2.4.4. Test tube and microtube racks

3. Procedure

3.1. Day 1:

3.1.1. Preparation of the EC host(s): Inoculate a singular colony from an EC BRLC plate (≤ 3 days old) into 10mL TSB (Use ≤ 1 week old TSB). Vortex well and incubate for approximately 2-2.5 hours in a rotary shaker at 37ºC and 170rpm until moderately turbid (OD_{600} = 0.5-0.6, ~10^8 CFU/ml).

3.1.1.1. Alternatively, incubate 18-20hours at 37°C without shaking, add 1mL of this overnight culture to 9mL TSB and incubate this new culture at 37ºC and 170rpm for 45-60 minutes (OD_{600} = 0.5-0.6, ~10^8 CFU/ml).

3.1.2. Once the log-culture is ready, vortex and **immediately** transfer 1mL of the culture to 9mL cold PBS. Place this PBS culture tube in ice and place the ice bucket to the 4ºC refrigerator.

3.1.2.1. As time allows, continue serial dilutions of this PBS/culture mixture, creating 10^{-5} and 10^{-6} dilution tubes.

3.1.2.2. Plate 100 µl of the 10^{-5} and 10^{-6} dilutions onto TSA for EC enumeration. Plate in duplicate and add the plates to a 37ºC incubator for 16-18 hours.

3.1.2.3. Record the number of colonies averaged between two plates and calculate the initial culture's CFU/ml using the following equation:

3.1.2.3.1. Initial CFU/ml = $\#colonies_{average}$ x 10 x reciprocal dilution of EC.

3.2. Day 2:

3.2.1. **If preparing working stock or laboratory stock, follow directions starting at 3.2.4.**

3.2.2. Purification of phage Eluate 3: In the BSC, vortex thoroughly and transfer 100 µl of eluate 3 into 900 µl of lambda diluent in a sterile microtube. Centrifuge at 11,000 x g for 10 minutes. Use a 3mL syringe and a 0.2µm syringe filter to filter the resulting supernatant into a new sterile microtube.

3.2.3. **For low titre E3:** Transfer 3 E3 tubes (~2.7mL) into three 1.5mL sterile microtubes. Centrifuge at 11,000 x g for 10 minutes. Use a 3mL syringe and a 0.2µm syringe filter to filter the resulting supernatant into a sterile 5mL falcon tube.

3.2.4. Add TSB with 10mM $MgSO_4$ (mTSB) to a sterile culture tube/bottle. The volume of mTSB added should be 20mL for Master/Working stock (in a 50mL falcon tube), or 300mL for Laboratory stock (in a 1L media bottle).

3.2.4.1. Transfer 500 µl of this media into a sterile 2mL microtube (for total volumes below 2mL) or a 5mL falcon tube (for volumes above 2mL).

3.2.5. Infect the mid-log phase EC culture prepared in 3.1 with phage at a low MOI of approximately 0.1. **Additionally, consider preparing MOIs of 0.01 and 0.001 for uncharacterized phage.**

3.2.5.1. Add the appropriate volumes/dilutions of EC culture and purified Eluate 3 to the 500 µl of mTSB set aside in a microtube/falcon tube. **Vortex thoroughly** and incubate this tube for 15 minutes at 37ºC and 130rpm.

3.2.5.2. For PRLS, use ~9mL of *E. coli* to guarantee proper growth of the bacteria over the course of the experiment.

3.2.5.2.1. **Note: The appropriate volumes and dilutions will depend on E3 titre estimated by prior plaque assays. To determine what should be added, follow the**

values and calculations in the "Phage Research Stock Experiment Forms" **excel spreadsheet.**

3.2.5.2.2. MOI is critical for the production of a high titre phage stock and needs to be pre-tested and optimized. Highly virulent phage should be used at an MOI $\leq$ 0.1.

3.2.6. After the 15-minute incubation, vortex and transfer the EC/phage mixture to the bottle/container containing the mTSB. Transfers should be conducted by pouring the culture into the container and using a pipette to transfer any remaining liquid/residue.

3.2.7. Incubate at 37ºC and 190rpm for 4-6 hours until the first round of lysis has finished. Record experimental observations at 0.5-1 hour intervals using a Turbidity Worksheet.

3.2.8. Note: Lysis onset is marked by a distinct decrease in the turbidity of the culture caused by bacterial lysis. Bacterial debris and precipitate should be visible, while the silkiness and Schlieren patterns of an unlyzed culture should not be.

3.2.9. Once lysis has been confirmed, centrifuge the culture at 4800 x g for 30 minutes (20mL volumes) or 45 minutes (300mL volumes) (Beckmann swing rotor) at 4ºC. For 300mL PRLS, centrifuge 2 x 150mL volumes in centrifuge bottles. Transfer the supernatants into new containers and sterilize these using a 0.2µm bottle-top filter.

3.2.9.1. IF preparing more than 2 PRLS, centrifugation will have to be done more than once. In this case, transfer cultures awaiting centrifugation into ice to slow any growth.

3.2.10. NOTE: If phage stocks must be removed from the lab, EVERYTHING must be sterilized using 10% bleach to avoid contaminating the environment.

3.2.10.1. Aliquoting and storage of filtered phage:

3.2.10.2. Dispensing of phage should be done in the BSC.

3.2.10.3. Cover the BSC with absorbent pads in case of phage spillage.

3.2.10.4. Using the bottle top filter, filter ~half of the bottle's max volume of the supernatant obtained in 3.2.7.

3.2.10.5. Dispense 3mL aliquots of this filtrate into screwtop vials.

3.2.10.5.1. For PRLS, store 300mL volumes in the filtration unit used.

3.2.10.6. Seal the lid with parafilm and label the vial as follows:

3.2.10.6.1. PRMS (or PRWS/PRLS for working or laboratory stocks) – Sample ID – Host for propagation – Lot# - Vial# - Production Date – Phage Titre (determined by a later overlay plaque assay).

3.2.10.6.2. Example: PRMS-11(ECL20885)-001-Vial01-June 17, 2019 – 4.3×10^9 PFU/mL.

3.2.11. Autoclave all waste, glassware and centrifuge bottles, making sure that the caps of the centrifuge bottles are fully removed before autoclaving.

3.2.12. Complete the "Phage Research Laboratory Preparation Form".

3.3. Day 3:

3.3.1. Enumerate the phage stock prepared following SOP-006 for Double Agar Overlay Assay and place the remaining stock back into the 4ºC refrigerator. Make 10^{-5}, 10^{-6}, 10^{-7} dilutions of the phage for this assay.

3.3.2. Master and Working phage stocks should be stored at 4ºC in 3mL aliquots in glass screw top vials. Lab stocks can be stored at higher volumes for less than 3 months at 4ºC.

3.3.3. Update the "Phage Research Stock Preparation Form" with all applicable information.

4. Reagents

4.1. TSB

Substance	**Amount per litre**
TSB powder	30 g
Distilled water	1000.0 mL

1. Mix to dissolve
2. Autoclave at 121°C for 20 min.
3. Store at 4°C for up to 3 month.

4.2. $MgSO_4$

Substance	**Amount per litre**
$MgSO_4 \cdot 7H_2O$	246.47 g
Distilled water	1000.0 mL

1. Mix to dissolve
2. Filtrate with 0.2 µm SFCA serum filter

5. References

5.1. **“**Phage Research Stock Preparation-May2019” provided by Dr. Dongyan Niu.

5.2. Sambrook, J. and D. W. Russell. 2001. Molecular cloning: a laboratory manual-3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

5.6 Enumeration of pathogenic *E. coli* infecting bacteriophage: double agar overlay plaque assay

1. PURPOSE

1.1. This protocol outlines the procedure used to determine titre of Pathogenic *E. coli* -infecting bacteriophages (phages).

2. EQUIPMENT AND MATERIALS

2.1. Lambda diluent

2.2. Modified Nutrient Agar (MNA**,** Dalynn, PN88-11041)

2.3. Tryptic Soy Broth (TSB, EMD 1.05459.0500)

2.4. $MgSO_4 \cdot 7H_2O$

2.5. UltraPure Agarose (Invitrogen # 15510-027)

2.6. Pathogenic *E. coli* (EC): host for enumeration

2.7. 0.22 µm sterile syringe filters (Pall life science, VWR, CA28143-400)

2.8. Disposable bottle-top filter with sterile bottle (Nalgene Filtration 0.2 µm SFCA membrane filter, VWR, 28199-677(250ml);28198-505(500ml); 289199-698(1000ml))

2.9. 3 mL sterile syringes (Luer-Lok Tip, 309585)

2.10. Multi-channel pipettes and tips

2.11. 0.5 mL Assay block (Costar, 3956)

2.12. 96-well silicone plate seal (Fisher, 03-396-50)

2.13. 2 mL microcentrifuge tubes (DiaMed, AD2000-N)

2.14. 5ml polyproylene tubes (Falcon 12 x 75 mm)

2.15. Reservoir (VWR, 82026-352)

2.16. 5-ml tips

3. PROCEDURE

3.1. **Preparation of host for titration**: Inoculate 10 mL TSB with one isolated colony OF EC from a freshly streaked plate. Incubate 3-5 h at 37°C, 170 rpm, until moderate turbid (10^7~10^8 CFU/ml). Alternately, incubate 10-ml TSB with one isolated colony of EC overnight at 37°C and transfer 1 mL of the overnight culture to 9 mL TSB and incubate for 1~1.5 hour until moderate turbid (10^7~10^8 CFU/ml, OD_{600} = 0.5 to 0.6) at 37 C, 170 rpm.

3.1.1. Once the culture is ready, vortex and **immediately** transfer 1mL of the culture to 9mL PBS. Place this PBS culture tube in ice and add the ice bucket to the 4ºC refrigerator.

3.1.1.1. As time allows, continue serial dilutions of this PBS/culture mixture, creating 10^{-5} and 10^{-6} dilution tubes.

3.1.1.2. Plate 100 µl of the 10^{-5} and 10^{-6} dilutions onto TSA for EC enumeration. Plate in duplicate and add the plates to a 37ºC incubator for 16-18 hours.

3.1.1.3. Record the number of colonies averaged between two plates and calculate the initial culture's CFU/ml using the following equation:

3.1.1.3.1. Initial CFU/ml = #colonies$_{average}$ x 10 x reciprocal dilution of EC.

3.2. **Preparation of MNA plates:** Take out MNA plates from 4°C to room temperature 1-2 days ahead to dry. **NOTE**: The state of the "underlay medium" is very important; it should not to be too dry or too moist, since both of these factors will negatively affect plaque development. There should be several wrinkles shown on the surface of the MNA plates dried appropriately. If no wrinkles, dry MNA plates uncovered in an operating biological safety cabinet for 5-10 minutes just before plating.

3.3. **Preparation of diluent:** For each sample to be titred, pipette 450 µl of Lambda diluent into an assay block. Include a column of 450 µl of Lambda diluent for the negative control A (to which only EC host strain will be added) and a column of 450 µl of Lambda diluent for a blank.

3.4. **Preparation of sample:** Centrifuge 1.8 mL of a 1:10 dilution (lambda diluent) of feces at 11,000 *g* for 10 min in order to sediment the fecal material. Filter the supernatant, which will contain the phages, through a 0.22 um syringe filter. The resulting filtrate represents the initial aliquot for titration. **For determining titre of previously prepared phage lysates**, no centrifugation or filtration of the sample is required.

3.5. **Serial dilutions:** Add 50 µl of the filtrate or 50 µl of the phage preparation to the first well containing the 450 µl of Lambda diluent. Prepare serial dilutions using a column of eight wells for each sample (10^{-1} through 10^{-8}). Mix each dilution by pipetting up and down three times and ensure to use fresh pipette tip for each transfer, continue making 10-fold dilution.

3.6. **Plating dilutions**: To two 5 mL polypropylene tubes, add 100 µl of the selected dilution of phages and the host culture (prepared in 3.1). For the Blank Control, add 200 µl of Lambda diluent; For the Negative Control A, transfer 100 of

Lambda diluent and 100 µl of the host culture (prepared in 3.1); For the Negative Control B, only transfer 200 µl of the host culture (prepared in 3.1). Add 3 mL of molten 0.6% UltraPure agarose with 10 mM $MgSO_4$ to each tube, invert 2X to mix, and quickly pour onto pre-hardened MNA plates. Swirl gently to cover the plate and evenly distribute the EC-agarose mixture. Duplicate tube is required for the dilution. Using a fresh pipette tip for each dilution tube, repeat the above procedure to pour the overlays for the remaining phage dilutions. After plating the dilutions, directly add 3-ml Agar of a tube without any addition onto a plate for controlling Agar quality. **NOTE**: 1) pipette molten agarose in a 56°C using 5-ml tips; 2) After addition of *E. coli* culture into the phages dilutions, these phage-*E. coli* mixtures should not be allowed to stand at room temperature more than 10 min before plating.

3.7. **Incubation**: Allow the overlays to harden for 10~20 min, and incubate the plates inverted in a single layer at 37°C for 18-24 h.

3.8. **Record plaques of plates** when **Blank** – no colony (NC), **Negative control A and B**, no plaques (NP); Agar only – no colony(NC). Count the plaques without halo on plates with 30~300 plaques or the plaques with halo on plates with 15~150 plaques, averaging the count from duplicate plates.

3.9. **Calculation:** Determine the titre of the original phage preparation by using the following calculation = average number of plaques on plates × 10 × reciprocal of dilution (PFU/ml).

3.10. Use a 5% hypochlorite solution to disinfect work area upon completion.

4. REFERENCES

4.1. Sambrook, J. and Russell, D.W. 2001. Plating Bacteriophage λ. In: Molecular Cloning: A laboratory manual 3rd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York. pp 2.25-2.31.

4.2. Kropinski M. A., Mazzocco A., Waddell T.E., Lingohr E., Johnson R.P. 2009. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol. 501, 69-79.

5. MEDIA and REAGENT Preparations

5.1 MODIFIED NUTRIENT AGAR (MNA)

Substance	Amount per litre	
Nutrient broth	20.0	g
NaCl	8.5	g
Agar #1	10.0	g
$CaCl_2$ (8.325 mg/L)	8.3	mg
$FeCl_3$ (1.15 mg/L)	1.1	mg
$MgSO_4 \cdot 7H_20$	0.5	g
Deionized water	1000.0	mL
30% Glucose (*add after autoclaving*)	10.0	mL

1. Mix to dissolve
2. Autoclave at 121°C for 20 min.

3. Cool to 50 □ C

4. Add glucose and dispense.

Quality Control:

1. Randomly select 2 plates from the batch. Incubate overnight at 37°C. If growth occurs, discard batch.

2. Randomly select 2 plates from the batch. Plate phage and EC strain to get a bacterial lawn containing plaques. Incubate overnight at 37°C. If no plaques or lawn of bacteria is present, discard batch.

5.2 LAMBDA DILUENT

Substance	**Amount per litre**
1 M Tris Cl (pH 7.5)	10.0 mL
$MgSO_4 \cdot 7H_20$	2.0 g
Deionized water	990.0 mL

1. In a volumetric flask combine Tris-Cl (see recipe below), $MgSO_4$ and a portion of distilled water.

2. Swirl flask until $MgSO_4$ is completely dissolved.

3. Add distilled water to 1.0 L.

4. Dispense into appropriate aliquots.

5. Autoclave at 121°C for 20 min.

6. Store at room temperature for up to 6 month.

Tris-Cl (1M)

Dissolve 121.1 g of Tris base in 800 mL of water. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 mL
7.5	60 mL
8.0	42 mL

NOTE:

1. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1L with water. Dispense into aliquots and sterilize by autoclaving.
2. If the 1M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solution is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, 0.05 M solution has pH values of 9.5, 8.9 and 8.6 at 5°C, 25°C and 37°C, respectively.
3. Store at room temperature for up to 6 month.

5.3 TSB

Substance	**Amount per litre**
TSB powder	30 g
Distilled water	1000.0 mL

4. Mix to dissolve
5. Autoclave at 121°C for 20 min.
6. Store at 4°C for up to 3 month.

5.4 Overlay or Top agar

Substance	**Amount per litre**
UltraPure Agarose	6 g
$MgSO_4 \cdot 7H_2O$	2.46 g
Distilled water	1000.0 mL

1. Mix and heat to complete dissolve the above chemicals.
2. Autoclave at 121°C for 20 min.

3. Store at 4°C for up to 3 month.
4. For use, heat the tubes containing the overlay medium to melt the agar and hold at 50°C in a water bath (**NOTE:** It really is important that the agar be brought to a full boil, or there are likely to be crystalline areas remaining that make the plates very hard to interpret**.**

5.7 SOP-002: Preparation and management of bacterial research working stocks

1. PURPOSE

1.1. To outline the procedures used to prepare, manage, and quality assure Bacterial Research Working Stocks**.**

1.1.1. Bacterial Research Master Stocks, Bacterial Research Working Stocks, and Bacterial Research Laboratory Cultures comprise a three-tiered system for cultures used to conduct relevant research.

2. REFERENCES:

2.1. FRM-04 – Bacterial Research Working Stock Inventory Form

2.2. FRM-05– Bacterial Research Working Stock Preparation Form

2.3. FRM-06 – Bacterial Research Stock Inventory Electronic Form

3. RESPONSIBILITY:

3.1. The supervisor/designate will ensure that personnel using these procedures are trained appropriately.

3.2. Personnel using this procedure must ensure they follow it appropriately.

4. DEFINITIONS/ABBREVIATIONS:

4.1. BRMS - Bacterial Research Master Stock, the bank of bacteria used to prepare the Bacterial Research Working Stock. This may include cultures contained in the *Escherichia coli* Reference Culture Collections of Niu’s Lab.

4.2. BRWS - Bacterial Research Working Stock, the bank of bacteria used to prepare a Bacterial Research Laboratory Culture of a strain of bacteria.

4.3. BRLC - Bacterial Research Laboratory Culture, the culture of bacteria used to propagate phage and conduct research experiments.

5. EQUIPMENT AND MATERIALS

5.1. Equipment

5.1.1. Rotary Shaker

5.1.2. Pipettors and sterile pipettes

5.1.3. Biological Safety Cabinet (BSC)

5.2. Media, Reagents & Ingredients

5.2.1. Tryptic Soy Broth

5.2.1.1.Note: Use sterile TSB less than 2 weeks old

5.2.2. Tryptic Soy Agar

5.2.3. Sterilized 100% glycerol (200 µl/vial)

5.3. Supplies

5.3.1. Sterile screw cap vials

5.3.2. Sterile culture vessels

5.3.3. Sterile culture loops

5.3.4. Sterile polystyrene reservoirs

5.4. Miscellaneous Supplies

5.4.1. Gloves

5.4.2. 70% ethanol

5.4.3. Test tube & microcentrifuge tube racks, various sizes

5.4.4. Weighing paper & spatula

5.4.5. Biohazard bags

6. PROCEDURE FOR THE PREPARATION OF BACTERIAL RESEARCH WORKING STOCKS:

6.1. Prepare an agar plate streaked with the BRMS.

6.1.1. Remove vial(s) from freezer inventory and transfer to the laboratory.

6.1.2. Using a disposable inoculation loop, dip into the vial, remove a scraping of inoculum (frozen slush) and streak onto a TSA plate. Incubate the plate at 37ºC ± 2ºC for 15−18 hours.

6.2. Record use of the BRMS in the Research Master Stock Inventory FRM-01, if applicable. Destroy the vial of BRMS by autoclaving.

6.3. Prepare a liquid culture of the strain to make the BRWS.

6.3.1. Inoculate TSB with a single colony of the strain from section 7.1. Use a volume of broth appropriate to the size of the BRWS desired. Incubate the culture at 37ºC ± 2ºC with shaking (150~190 rpm) until mid-log phase of growth (OD_{600} = 0.5~0.6).

6.3.2. Alternately, incubate 10 mL TSB with a single colony of the strain and incubate at 37°C ±2°C with shaking (150 RPM) for 18-24 hours. Then inoculate 1 mL of overnight culture into 9 mL TSB and incubate the culture at 37ºC ± 2ºC with shaking (150~190 rpm) until mid-log phase of growth (OD_{600} = 0.5~0.6).

6.3.2.1. For large volumes, inoculate 5 mL of the overnight culture into 50 mL of TSB and incubate at 37ºC ± 2ºC with shaking (150~190 rpm) until mid-log phase of growth (OD_{600} = 0.5~0.6), approximately 2.5 hrs.

6.4. Dispense 1 mL of the log-phase culture into sequentially numbered storage vials containing sterilized 200 µl of 100% glycerol and labelled with the lot number of the BRWS (**NOTE: Must be done within BSC).**

6.4.1. Note: For large volumes add 11 mL of sterile glycerol to the 2.5 hr culture and swirl thoroughly to mix. Pour the culture/glycerol mix into a sterile polystyrene reservoir and dispense 1.2 mL into labelled sterile storage vials.

6.4.2. Assign a lot number that includes a) BRWS identifier, b) the identity of the strain, and c) a sequential unique identifying number (e.g. BRWS-ATCC35352-001).

6.4.3. Assign a sequential unique vial number to each vial (e.g. Vial 001).

6.4.4. Label Date of Production

Example of BRWS label: BRWS-R508-001-Vial 01 (08-Nov-11)

6.4.5. Prepare as many BRWS as necessary to use for research purposes.

6.4.5.1. Typically make 40-50 vials, for storage reasons do not prepare more than 81 (the number of vials one cardboard freezer box can hold).

6.5. Distribute vials to freezer boxes labelled with the lot number of the BRWS and its storage location.

6.6. Contact the technician responsible for maintaining the -80°C freezer regarding the best location for the new working stock; transfer the freezer boxes containing the working stock to the -80°C freezer.

6.6.1. Provide the technician with necessary information regarding the research stock to update the isolate library database (FRM-06 – Bacterial Research Stock Inventory Electronic Form).

6.7. Autoclave any waste culture/materials, and thoroughly disinfect all pipettes, supplies, and work surfaces with 70% ethanol.

6.8. Complete the Bacterial Research Working Stock Inventory Form (FRM-04) and Bacterial Research Working Stock Preparation Form (FRM-05) and add them to the Bacterial Research Stock Inventory Binder.

5.8 Serial dilutions for bacterial enumeration and phage titration

Phage(s)	7h-Dilutions	19h-Dilutions	24h-Dilutions
T4	10^{0}, 10^{-1}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T4	10^{0}, 10^{-1}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T1	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T1	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
rV5	10^{-1}, 10^{-2} ,10^{-3}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
rV5	10^{-1}, 10^{-2} ,10^{-3}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T5	10^{0}, 10^{-1}	10^{0}, 10^{-1}	10^{0}, 10^{-1}
T5	10^{0}, 10^{-1}	10^{0}, 10^{-1}	10^{0}, 10^{-1}
T1+T4+rV5	10^{0}, 10^{-1}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T1+T4+rV5	10^{0}, 10^{-1}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
T1+T4+rV5+T5	10^{0}, 10^{-1}	10^{-5}, 10^{-6}	10^{-5}, 10^{-6}
T1+T4+rV5+T5	10^{0}, 10^{-1}	10^{-3}, 10^{-4}	10^{-3}, 10^{-4}
Control	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}
Control	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}	10^{-6}, 10^{-7}

Table 5.1: Serial dilutions for phage lysis experiment bacterial enumeration

Phage(s)	7h-Dilutions	19h-Dilutions	24h-Dilutions
T4	10^{-3}, 10^{-4}	10^{-5}, 10^{-6} ,10^{-7}	10^{-5}, 10^{-6} ,10^{-7}
T4	10^{-3}, 10^{-4}	10^{-5}, 10^{-6} ,10^{-7}	10^{-5}, 10^{-6} ,10^{-7}
T1	10^{-5}, 10^{-6}	10^{-5}, 10^{-6} ,10^{-7}	10^{-3}, 10^{-4} ,10^{-5}
T1	10^{-5}, 10^{-6}	10^{-5}, 10^{-6} ,10^{-7}	10^{-3}, 10^{-4} ,10^{-5}
rV5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
rV5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
T5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
T5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
T1+T4+rV5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-6}, 10^{-7} ,10^{-8}
T1+T4+rV5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
T1+T4+rV5+T5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-5}, 10^{-6} ,10^{-7}
T1+T4+rV5+T5	10^{-5}, 10^{-6}	10^{-6}, 10^{-7} ,10^{-8}	10^{-6}, 10^{-7} ,10^{-8}

Table 5.2: Serial dilutions for phage lysis experiment phage enumeration

5.9 SOP-903: Pathogenic *E. coli* microplate phage virulence assay

1. PURPOSE

1.1. This protocol outlines the procedure used to determine the host range and multiplicity of infection (MOI) of pathogenic *E. coli* bacteriophages (phages).

2. EQUIPMENT AND MATERIALS

2.1. Media/Reagents

a. Tryptic Soy Broth (TSB)

b. Phosphate Buffered Saline (PBS)

c. $MgSO_4 \cdot 7H_2O$

d. Tryptic Soy Agar (TSA)

2.2. Organisms

a. Pathogenic *E. coli* strains

b. High titre phage research laboratory stocks

2.3. Supplies

a. Multi-channel pipettes and tips

b. Sterile, clear, 96-well flat bottom polystyrene microplates with lids.

c. Reservoirs

d. Spreaders

e. Test tubes

f. Inoculation loops

2.4. Equipment

a. Biosafety Cabinet (BSC)

b. Rotary Shaker

c. Incubator

3. **PROCEDURE**

3.1. **Day 1:** Prepare a Bacterial Research Laboratory Culture (BRLC) of the standard and tested EC strains using the streak-out method onto TSA. Incubate plates at 37°C for 16-18 hours. Remove to the 4°C fridge once grown. BRLC used for the experiment should be ≤ 7 days old.

3.2. **Day 2:** Pick a single colony from the produced BRLC plate(s) and transfer it to 10mL TSB (≤ 1 month old). Vortex thoroughly and incubate these overnight cultures at 37°C for 18-20 hours.

3.3. **Day 3:** Virulence Assay Preparation: Using a reservoir and multichannel pipette, add 180 µl of 10mM $MgSO_4$ TSB (mTSB) to columns 1-12 of a sterile 96-well microplate. Columns 1-9 will be used for test strains, column 10 will be a negative control with EC but no phage, and column 11 will be used for a blank (mTSB alone).

- **a.** **NOTE: 3 EC strains can be tested in triplicate per plate.**
- **b.** Using a multichannel pipette and reservoir, add 20 µl of a high titre phage research laboratory stock (PRLS) (~10^9 PFU/mL) to wells 1-9 of the plate's first row (Row A).
- **c.** Using a multi-channel pipette, prepare a series of 20 µl 10-fold phage dilutions down the microplate, making sure to mix the wells by aspirating/ejecting 7 times. **Change pipette tips after each dilution.** Remove 20 µl from the final row (Row H).

3.4. Preparing the EC test strains:

a. Vortex and transfer 1mL of the overnight EC culture(s) prepared in 3.2 into 9mL TSB. **NOTE: STRAINS WILL BEGIN GROWING IMMEDIATELY.** To minimize growth, use **cold** 9mL TSB, place the dilution in ice and transfer the ice container to the 4°C fridge after transfer.

b. Use separate reservoirs for different EC strains. Transfer 2-3mL of a diluted EC culture into a reservoir using a 1mL pipette. Using a multichannel pipette, pipette 20 µl of this culture into the first 3 columns of the previously prepared microplate. Repeat this with each EC culture to be tested, (changing tips after each addition) pipetting into columns 1/2/3, 4/5/6, and 7/8/9, for each strain. Repeat this for column 10 using a 10^{-1} broth dilution of a standard host strain for the negative control.

c. Once done, cover the microplate and incubate for 5 hours at 37°C.

3.5. Examine the wells and record their turbidity.

a. Blank control should show no growth or evidence of bacterial presence (debris etc.). This shows there was no media contamination and is used to score other wells for "complete lysis".

b. Negative control wells should show bacterial growth, indicating no phage contamination and showing the expected results if there is no lysis occurring.

c. Positive control wells will display a range of turbidities from none (complete lysis) through intermediate (partial lysis) to fully turbid (no lysis).

3.6. Score the wells as follows:

a. Positive (+): complete lysis, similar to the blank (no obvious turbidity)

b. Partial (+/-): partial lysis (less turbidity than control, but not completely clear)

c. Negative (-): no lysis, very turbid, similar to control without phage.

d. Note the highest dilution (lowest amount) of the phage that results in complete clearing (lysis) of the culture.

3.7. EC test strain enumeration:

3.8. Using a new microplate, add 180 µl of PBS to the first 6 rows (A-F) of wells. Repeat this in each column based on how many strains are to be enumerated. Transfer 20 µl of the previously prepared 10^{-1} broth dilutions to the top wells of the microplate.

3.9. Using a multichannel pipette, prepare 10-fold serial dilutions of each *E. coli* strain to be enumerated by transferring 20 µl of the first row to the second row, continuing down the plate until a 10^{-6} dilution is made. Make sure to mix each row by aspirating/ejection 7 times, and change tips between dilutions.

3.10. Plate 50 µl of the 10^{-5} and 10^{-6} dilutions (Rows E and F) onto TSA plates In duplicate. Incubate for 18-20 hours and use these to determine the colony count of the 10^{-1} broth cultures.

3.11. CFU/ml of 10^{-1} Broth Culture = Colonies Counted$_{\text{Average}}$*20*$10^{-\text{dilution used}}$.

3.12. Use 10% bleach solution to soak the reservoirs and microplates used for at least 2 hours. Disinfect the work area and all equipment (that can be safely cleaned) with 10% bleach. Afterwards, wipe equipment with 70% ethanol to remove the bleach. Dispose of all waste in the proper areas.

3.13. Determining the MOI of the phage against the test EC:

a. Based on the phage research laboratory stock's titre, calculate the number of phages (PFU) in each microplate row dilution, and from EC enumeration the number of *E. coli* cells (CFU) in each well.

b. Using these values, determine the MOI of the highest phage dilution that results in the complete lysis of its host.

c. MOI = (Phage Stock Titre (PFU/mL) * Highest Phage Dilution)/Titre of the 10^{-1} broth culture.

3.14. Record observations and values in the file "EC MOI datasheet template".

7. REFERENCES

7.1. Adapted from: Microplate Phage Virulence Assay SOP: Provided by Dr. Dongyan Niu.

7.2. Johnson, R. P. Guelph Laboratory, Health Canada, Ontario, Canada. Personal Communication. 2008 MAY.

8. MEDIA and REAGENT Preparations

5.1 TSB

Substance	Amount per litre
TSB powder	30 g
Distilled water	1000.0 mL

7. Mix to dissolve
8. Autoclave at 121°C for 20 min.
9. Dispense aliquots to make 9-ml of 10-ml TSB tubes.
10. Bag them and store at 4°C for up to 2 month.

5.2 1M $MgSO_4$

Substance	Amount per litre
$MgSO_4 \cdot 7H_2O$	246.47 g
Distilled water	1000.0 mL

3. Mix to dissolve
4. Filtrate with 0.2 µm SFCA serum filter

5.3 TSB with 10mM $MgSO_4$

Substance	Amount per litre
$MgSO_4$ (1M)	10 mL
Sterile TSB	1000.0 mL

1. Transfer MgSO4 (1M) to sterile TSB
2. Store at room temperature for up to 2 months.

5.4 TSA preparation (commercially available, Dalynn PT80) or prepare according an introduction of the TSA powder product.

5.5 PBS (pH 7.2)

Substance	Amount per litre
NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

1. Adjust pH to 7.2.
2. Adjust volume to 1L with additional distilled H2O.
3. Autoclave at 121C for 20 min.
4. Store at room temperature for up to 6 months.