Title of Document: DISINFECTION ABILITY OF BACTERIOPHAGES AGAINST LISTERIA MONOCYTOGENES BIOFILMS

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Abstract

Pathogenic foodborne bacteria, particularly species belonging to *Listeria* and *Salmonella*, pose a growing threat to public health because of their ability to form and/or grow within biofilms on various environments, specifically food processing facility. Within a biofilm, bacteria develop increased resistance to common disinfectants, making surface sterilization a challenge for businesses involved in food processing. In order to determine the viability of bacteriophages as an antibiotic alternative, this experiment attempted to explore the bacteriophage growth process as well as bacteriophage efficacy against *Listeria monocyogenes* as compared to *Salmonella enterica* serovar Typhimurium. A511 bacteriophage was grown and tested on *L. monocytogenes* 1/2a using previously studied P22 bacteriophage and *S. enterica* as a control case. While this experiment was unable to establish a defined efficacy of A511 against *L. monocytogenes*, repeatable results with *Salmonella* show promising potential for phage therapies.

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By

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INTRODUCTION

Increasing rates of bacterial infection by antibiotic resistant bacteria are a growing threat to public health. In 2013, the U.S. Center for Disease Control and Prevention (CDC) reported that two million people were infected by antibiotic-resistant bacteria and 23,000 people died from their infection in the U.S. alone (CDC, 2013). Overuse of antibiotics, particularly in the meat and dairy industry, is thought to cause many pathological bacterial species to develop resistance to multiple classes of antibiotics. (Landers, Cohen, Wittum & Larson, 2012) In the US, 80% of all antibiotics sold are administered in animal agriculture, 70% of which are relevant to human health (Martin, Thottathil, & Newman, 2015).

The adverse effects of bacteria not only impact medicine, but also agriculture, where strains of *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Listeria monocytogenes* affect both animals and humans, resulting in billion-dollar losses (Schroeder, 2012). Outbreaks of infection have amplified public concern. Cases of listeriosis caused by ice cream products contaminated with *L. monocytogenes* forced massive product recalls by Blue Bell Creameries in 2015 (U.S. Food and Drug Administration [FDA], 2015). From 2010 to 2016, there were 39 foodborne outbreaks of *L. monocytogenes*, resulting in 379 hospitalizations and 84 deaths in the U.S alone (CDC, 2018). Mortality rates are especially high in immunocompromised individuals, the elderly, children, and pregnant women (Colagiorgi, Di Ciccio, Zanardi, Ghidini, & Ianieri, 2016). As highlighted by U.S President Barack Obama in the Executive Order No. 13,676 (2014), entitled "Combating antibiotic-resistant bacteria," the significant implications

for public health and the global economy warrant a larger allocation of the federal budget towards research for antibiotic alternatives.

A promising solution to controlling antibacterial resistance is the implementation of phage therapy, which involves the use of bacteriophages to kill specific bacteria with minimal adverse effects. The inception of phage therapy is noted by Maura & Debarbieux (2011), who explain that the discovery of bacteriophages in 1915 by Frederick W. Twort quickly gave rise to various forms of phage therapy which became prevalent in the 1920s and 1930s. However, the development of antibiotics in the 1940s diminished consumer use of the phages. Phages then became models of experimentation that furthered the field of molecular biology (Maura & Debarbieux, 2011).

Bacteriophage therapy became popularized as an alternative to antibiotics in countries such as the former Soviet Union and England and is still being used successfully today (Kutter et al., 2010). The high regulatory hurdles of medical application mean that with only more higher-level clinical trials will phage therapy be accepted in the U.S. medical community (Matsuzaki et al., 2003).

Phage therapy has seen rapid advancement in the food industry, where applications towards pathogen prevention and treatment have been extensively researched (Maura & Debarbieux, 2011). These food safety measures include the inhibition of bacterial colonization in domesticated livestock, disinfection of inanimate surfaces, and post-harvest administration to food surfaces (Sulakvelidze, 2013). Products such as Listex™, ListShield™, Ecophage™, and Agriphage™ have manifested from this research. However, a lacking area in phage therapy research is the treatment of biofilms.

L. monocytogenes prevalence in the food industry is attributed to its ability to form biofilms on surfaces in cold temperatures via production of extracellular polymeric substances (EPS) (Di Bonaventura et al., 2008; Blackman & Frank, 1996). The EPS is comprised of sugars, proteins, and DNA that function in aggregation, adhesion, and protection of the inner bacteria (Flemming & Wingender, 2010). Antibiotics are less effective at killing *L. monocytogenes* in biofilms than when bacteria are free-floating, primarily due to the EPS physically blocking antibiotic treatment from coming into contact with cells, as well as the formation of dormant persister cells within the biofilm colony (Carpentier & Cerf, 2011; Wu, Yu, & Flint, 2017). In other studies, bacteriophages were also found to infect the metabolically inactive persister cells and secrete polysaccharide depolymerases (e.g. alginate lyases, hyaluronidases) that disrupt the biofilm matrix (Harper et al., 2014). However, the specific depolymerases and other enzymes responsible for EPS disruption in *L. monocytogenes* phage have yet to be identified. Research pertaining to biofilms may broaden the range of biosanitation uses for phages and further the field of antibiotic alternatives.

A number of bacteriophages have been isolated and proven to reduce bacterial populations of *L. monocytogenes* (Lee, 2017). A study from 2009 showed that a lytic strain of bacteriophage called A511 can infect 95% of all strains of *L. monocytogenes* (Guenther, Huwyler, Richard, & Loessner, 2009). Additionally, several examples of disinfectants composed of phage have been marketed and produced successfully. These products combine multiple phages into a cocktail to broaden the disinfecting scope (Sulakvelidze, 2013). In the context of food industry applications, application of *Salmonella* infecting

bacteriophages led to greater reduction of *Salmonella enterica* colonies at 4°C (the typical temperature of factories and processing facilities) than at 18°C (Galarce, Bravo, Robeson, & Borie, 2014).

Biofilm formation

Biofilm formation can be summarized in three basic stages: initial attachment, maturation, and dispersion (Monroe, 2007; O'Toole, Kaplan, & Kolter, 2000). These phases are illustrated in ***Diagram 1*** and then described below.

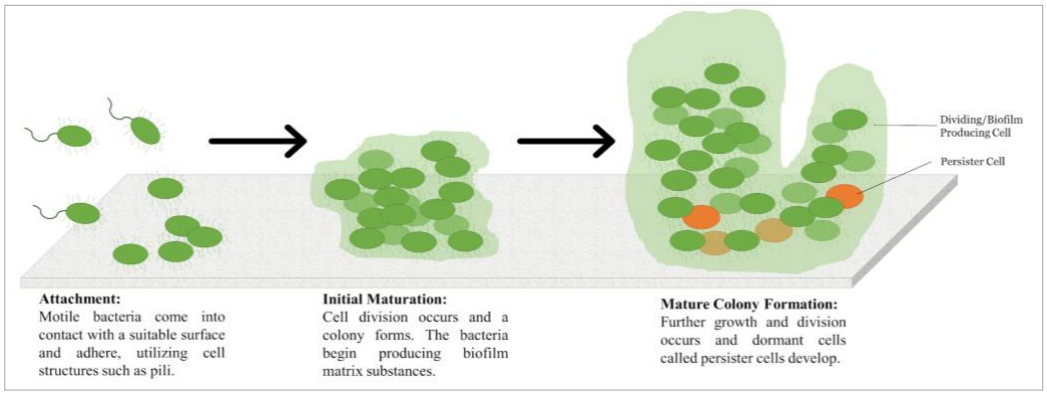

Figure 1: Biofilm Formation Phases. *An illustration of the attachment and maturation phases of biofilm formation. Diagram is based on information obtained from Monroe (2007) and adapted from a figure from Harper et al., (2014).*

Attachment Phases*:* Initial attachment involves the use of van der Waals and hydrophobic interactions between the cell and the attachment surface, aided by cell appendages such as pili, represented as thin external hairs in **Figure 1**. As surface roughness increases, colonization by bacteria increases due to the increased surface area and available interaction (Donlan,

2002). There have been experiments conducted testing the biofilm-forming ability of bacteria on smooth surfaces, unfortunately achieving this is difficult because only a few bacteria need to adhere in order for a successful biofilm to form and grow (Monroe, 2007). Once adhered, cells alter their gene expression to allow them to survive in the oxygen depleted environment of the biofilm, upregulating genes that will favor fermentation over aerobic respiration (Donlan, 2002).

Maturation Phases: As the biofilm grows, the bacteria will produce the EPS, which consist of an array of materials such as carbohydrates, polypeptides, metals, DNA and lipids in varying relative amounts dependent on the environment and native bacteria of the biofilm. Overall, carbohydrates are the most abundant, and typically DNA and lipids are present in only more trace amounts. As a biofilm matures, both the mass of EPS and the ratio of EPS to bacteria increase, suggesting that EPS production occurs at a faster rate relative to bacterial replication (Jiao et al., 2010). Once fully grown, EPS can make up 50%-90% of biofilm mass. EPS is also amphipathic and extremely insoluble, making it even more difficult to remove or penetrate with disinfectants. Additionally, while bacteria are isolated and growing inside a biofilm, they are in an ideal environment to exchange plasmids containing DNA that could confer resistance to certain antibiotics, increasing the portion of a bacterial population that is resistant. The later phases of maturation are also when persister cells begin to develop, as bacteria enter a dormant state in which they resist uptake of antibiotics (Donlan, 2002).

Dispersion: The dispersion stage (not illustrated in **Figure 1)** of biofilm development allows bacteria to proliferate and spread. Dispersal can

occur when bacteria in the biofilm break away, often due to nutrient changes in the environment. Since the biofilm is able to survive and re-adhere when pieces are broken off, incomplete attempts to remove it or any other stress on the surface can aid in its dispersal (Donlan, 2002). Bacteria in their dispersal phase have gene expression distinct from both bacteria in biofilms and free-floating bacteria. This altered gene expression may aid in a cell's ability to break away from the host biofilm and has been found to increase their virulence against macrophages (Chua et al., 2014).

Bacteriophage Structure and Replication

Bacteriophage are typically comprised of a nucleic acid - such as double-stranded DNA - and proteins that interact together to form the characteristic complex structure of the bacteriophage (Cann, 2001). The vast majority of bacteriophages possess the head-tail morphology, as detailed in **Figure 2**.

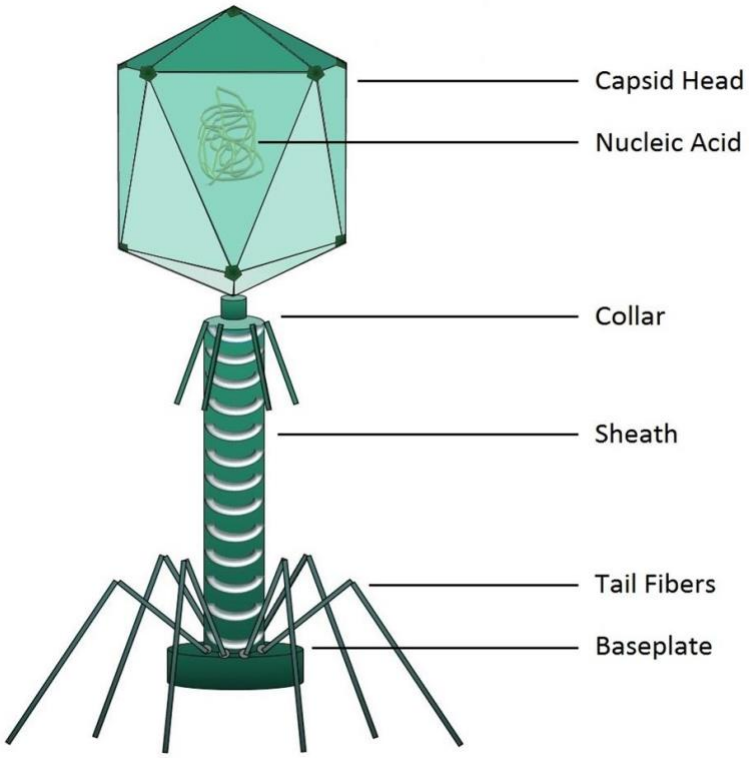

Figure 2: Morphology of Phage T4. *T4 is part of the Myoviradae family of viruses, characterized by their lack of a lipid bilayer enveloping the protein capsid of the head. Figure adapted from Kostyuchenko et al., (2003).*

Bacteriophages are highly specific to the strain of bacteria which they infect and are therefore generally non-toxic to humans or the normal bacterial gut environment of humans. Studies have shown that bacteriophages can be incredibly specific, such that that a strain which infects *E. coli* cells *in vitro* will not infect wild-type *E. coli* in the gut of mouse or even human test subjects. Additionally, many types of bacteriophage already inhabit the gut, and do no harm their host organism. (Bruttin & Brüssow, 2005; Chibani-Chennoufi et al., 2004).

When bacteriophages like A511 ATCC PTA-4608 detect and bind to the outer membrane of a bacterial cell, the tail portion undergoes a

conformational change that results in retraction of the base plate and tail. This retraction causes the sheath to pierce the membrane of the bacterial cell, creating a pathway through which the nucleic acid (dsDNA in the case of A511) can be inserted, traveling from the capsid head, through the sheath and into the bacterial cytosol (Orlova, 2009).

Once the bacteriophage nucleic acid is inserted into the cell, the cell can enter the lysogenic (integrative) or lytic (replicative) phases, as shown in **Figure 3**. In the lysogenic phase, the previously inserted bacteriophage DNA becomes a part of the bacterial genome. The integration of DNA into the bacterial chromosome suppresses phage reproduction. Primarily lysogenic phages contain host-controlled modification systems and restriction systems that have a negative effect on the quality and quantity of DNA translated into the bacterium. Studies conducted testing the lysogenic qualities of phages by using *Bacillus subtilis* show that the bacteriophages were successful in bacterial transduction*; however, the bacteria were unable to maintain high levels of transfection*. The study concluded that although bacteriophages have the capability to parasitize a host, the lysogenic phages either integrate their DNA into the host's genetic material or remain in the plasmid for an extended period of time (Yasbin, Wilson, & Young, 1973). Although this may be useful in certain situations, the paused reproduction state, also known as the temperate phase, renders this cycle less useful for application for disinfection. However, it is possible for lysogenic phages to revert back to the lytic phase through the removal of nucleic acid from the bacteria's chromosome or the addition of other reagents such as ultraviolet light (Campbell, 2003).

For disinfectant-related applications, the lytic phase is more desired due to its ability to lyse bacteria. During this reproductive phase, bacteriophage parts accumulate in the host cell's cytoplasm and eventually form into complete progeny phage. To release these progeny phage from the cytoplasm, enzymes called lysins must deteriorate peptidoglycan* in the cell wall of the bacterial host. The phage lysins require an additional protein, holin, to guide lysins through the bacterial membrane. Holin is a small membrane protein that provides the lysins with access to the cell wall, allowing the lysins to disrupt peptidoglycan bonds. These enzymes create large patches in the cell wall, which cause the bacteria to burst and release the phages into the environment (Fischetti, 2008; Wang, Smith, & Young, 2000; van Heijenoort, 2001).

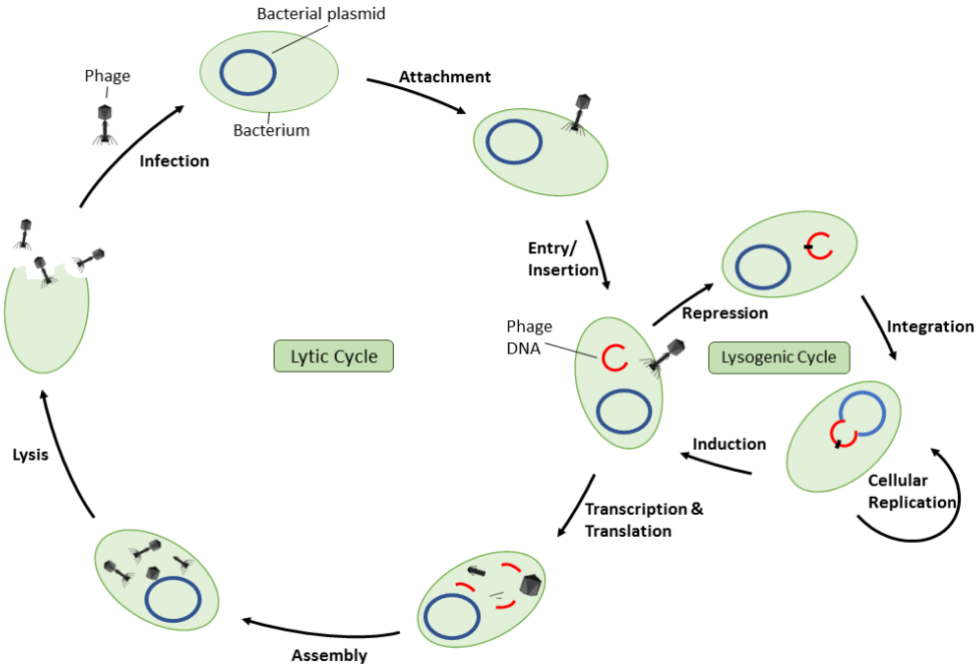

Figure 3: Life Cycle of the Typical Phage. *A bacteriophage will attach to the outer wall of the bacterial cell. During DNA insertion, a protein shell is*

left behind. From there, phages can enter the lytic or lysogenic phase to continue their life cycle. Diagram adapted from Campbell, A. (2003). The future of bacteriophage biology. Nature Reviews Genetics, 4(6), 471-477.

Bacteriophages possess a high specificity to the bacteria they infect, with their proteins acting similarly to that of the lock-and-key with bacterial surface proteins. When in an environment with no complementary bacteria, bacteriophages are inactive. However, in the presence of compatible bacteria, the phage's proteins automatically bind to the bacteria. In prior studies phages have significantly reduced or completely eliminated *Listeria* on the surface of cheese and had no negative effects when orally given to mice. Further mixtures with varying concentrations of phages resulted in varying degrees of effectiveness (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005). In the case of *E. coli*, the phage used was applied in various environments with differing conditions. For example, the phage was applied in cool or warm locations, in varying concentrations, and with different contact times (Hudson et al., 2013).

The use of bacteriophages in environments with varying temperatures may result in different results as well. A 2004 study showed that the greatest reduction of *Salmonella* on salmon was found in the cooler condition of 4 degrees Celsius, rather than at a warmer 18 degrees Celsius (Galarce, Bravo, Robeson, & Borie, 2014). Moreover, two phages were tested on *Salmonella* and *Campylobacter* grown on both roast and raw beef. Bacteriophages and their hosts were set in conditions with varying temperature, times, amounts of phages, as well as amounts of CO_2 (Bigwood, Hudson, Billington, Carey-

Smith, & Heinemann, 2008). The study suggests the greatest eradication occurred when the bacterial density was high and with contact time of 24 hours. For *Campylobacter* bacteria, the largest reductions in cell colonies were found in the case of a high host cell density on both raw and cooked beef. In both cases, inactivation of the pathogenic bacteria occurred as time increased. Reflection upon previously published literature leads to questions of what specific concoction of phages, as well as what conditions and settings, will maximize elimination of the most bacteria (Bigwood et al., 2008).

Targeted Environments for Phage Therapy

Foodborne diseases cost the U.S. over 5 billion dollars in medical costs and lost production per year (Fey, Mills, Coffey, Mcauliffe, & Ross, 2009). The potential lost profit is a large motivator for companies to take action against bacterial infection, and due to the increase of bacterial resistance, there is also pressure to search for alternatives to antibiotics. In addition to high costs and lost profits, foodborne illnesses pose a huge risk to public health. Over 2,000 hospitalizations and 500 deaths are caused per year by listeriosis, the disease caused by *Listeria monocytogenes* which is a common foodborne bacterial pathogen present in food processing (Fey et al., 2009). Bacteriophage A511 have previously been shown to have significant potential for addressing breakouts of *L. monocytogenes* (Guenther, Huwyler, Richard, & Loessner, 2009). Other types of bacteria such as *Salmonella* occur in an immense range of food products including leafy greens, tomatoes, and poultry products (Fey et al., 2009). Sectors where bacterial infection and the threats to

human health are a prevailing issue will be listed in ***Table 1*** and then examined in greater detail below.

Table 1: Common Biofilm Forming Bacteria, Symptoms, and Sources[#]

Bacteria	Symptoms	Primary Source(s)
Salmonella enterica	Salmonellosis: diarrhea, nausea and vomiting, fever	Meats, produce
Escherichia coli **O157:H7**	Stomach pain, nausea and vomiting, bloody diarrhea	Bovine meats, some produce
Listeria monocytogenes	Listeriosis: Fever, severe headache, nausea and vomiting	"Ready to Eat" frozen or refrigerated foods
Campylobacter jejuni	Campylobacteriosis: Diarrhea, muscle pain, fever, headache	Undercooked poultry products, pasteurized milk, vegetables
Cronobacter sakazakii	High fever, stiffness, or seizures as a result of developed meningitis	Powdered infant formula
Staphylococcus aureus	Nausea and vomiting, stomach pain, diarrhea	Meat, poultry and dairy products
Shigella	Shigellosis: Bloody diarrhea, stomach pain, fever, nausea and vomiting	Contaminated water, poultry products, vegetables, dairy
Mycobacterium avium **subspecies** *paratuberculosis*	Causative agent of Crohn's disease	Dairy, mostly milk
Clostridium perfringens	Fatigue, bloating and stomach pain, diarrhea	Poultry products

[#]A summary of the various different types of bacteria that are capable of forming or growing within biofilms, the symptoms infections by these bacteria can cause, and the common sources. Table information obtained from Endersen, O'Mahony, Hill, Ross, McAuliffe, & Coffey (2014).

Meat packing industry. The meat packing industry is highly susceptible to foodborne illnesses. Certain types of bacteria can emit an EPS. The EPS allows bacteria to adhere to each other and therefore collect on various surfaces in the form of biofilm, providing protection from certain environmental factors (Vert et al., 2012). One of the dangers of biofilms is that bacteria that reside within the biofilm have different properties than those

of free-floating bacteria; the protected environment of the film increases their resistance to common detergents and antibiotics.

Another important factor to consider is the colder temperature at which meat packing plants operate in order to preserve their products. Phage testing on cheeses have investigated the effects of temperature on phage disinfectants. Bacteriophage P100, which targets *Listeria*, was shown to be severely weakened under refrigeration compared to 10 degrees Celsius (Silva, Figueiredo, Miranda, & Almeida, 2013).

Hospitals. Hospitals suffer from medical instrument and surface contamination from bacterial biofilms. Biofilm contamination of polymeric substances, such as intravenous tubing and catheters, often go unnoticed and may lead to infection of the patient. Many potentially infections bacteria - including MRSA, vancomycin resistant *enterococcus*, and *Pseudomonas* species - were the most commonly found biofilm creating species (Vickery et al., 2014). Bacteriophages, however, are able to break down biofilms and infect the bacteria inside (Harper et al., 2014). Subsequent phage treatment to equipment also significantly reduced biofilm regrowth (Ryan, Gorman, Donnelly, & Gilmore, 2011).

Applications of Phages

Bacteriophages are a potential solution to the recent consumer demand for natural and safe antimicrobials as opposed to chemical preservatives in the agro-food industry. Use of certain phages in animal and plant food production, processing, and handling can prevent the spread of bacterial diseases and ultimately promote a safer environment. Phages can be utilized at 4 different stages of the food production process: phage therapy, biocontrol,

biosanitation, and biopreservation. Phage therapy in animals before slaughter or during animal growth could help reduce pathogens and possible cross-contamination with feces. Another agro-food application for phages would be biocontrol, which is applying phages directly on the surface of food such as milk, meats, or fresh produce (Sillankorva, Oliveira, & Azeredo, 2012). Phages are reported to lyse hosts at temperatures as low as 1°C, so they could also be used as a food biopreservation agent to prevent growth of bacteria on refrigerated foods (Greer, 1988). Once the food is returned to room temperature, the phages are more effective in limiting bacterial growth (Bigwood et al., 2008). The most feasible application for this investigation was determined to be biosanitation of the biofilm that forms on the surface of equipment used in the food industry.

Bacteriophage Delivery Methods

Aerosol. Research by Keyang et al. (2012) suggested that in order to disperse phages so that incidence with bacteria is increased, an aerosol distribution method is an option. The team explored which conditions best offer tuberculosis-specific phage D29 ideal generation within an aerosolized phage solution. Using a nebulizer and a closed chamber the efficacy of multiple spray liquids, sampling medium, storage temperature, different humidities, as well as the best method of sampling were all studied. The group determined, using the solution with the highest resultant concentration of culturable phages, that the aerosol solution functions best in a relatively low (<25%) humidity. Use of deionized water offered vastly more culturable D29 particles that were aerosolized than with other liquids. Irrespective of

temperature, both used sample media (SM buffer and nutrient broth) offered near identical results, and of the two sampling methods, a biosampler and the AGI-30, neither performed significantly better than the other (Keyang et al., 2012). Aerosolization of phages opens a wide field of applications, but still has yet to see marketable usage.

Spray. Leverentz, Conway, and Janisiewicz (2004) investigated the effectiveness of phages at combatting honeydew melons that were inoculated* with *L. monocytogenes*. Their approach included introducing the phages to the melon via a spray applicator, to increase the dispersion of phages along the fruit surface. The group studied how the timing of application of the phages affected the resulting culture of bacteria, as well as the concentration of phage applied. They found that for best results (undetectable bacterial population after 7 days) a concentration of 10^8plaque forming units/mL (PFU/mL) applied less than one hour after inoculation is necessary (Leverentz et al., 2004). The fact that the phage solution is applicable and still effective against *listeria* when sprayed on a fruit surface opens a wide field for all phage products to work within. There are a handful of existing products similar in nature to the scope of this research.

Product Examples

Several examples of phage disinfectants have been marketed and produced successfully. These products show that a phage disinfectant is possible to use against many strains of bacteria. However, these products fail to use a bacteriophage cocktail targeting all prominent infectious subspecies of

L. monocytogenes and combine them in some way with current antibiotics or disinfectants to rescue* them.

Listex™. The FDA-approved Listex P100™ contains a single lytic phage P100 and can be used to reduce biofilm matrices caused by *L. monocytogenes* in food processing environments. It was found that P100 is active against a wide range of *L. monocytogenes* in biofilm conditions as it significantly reduced cell populations when applied to stainless steel surfaces for a 24 hour period. However, different strains of *L. monocytogenes* had statistically different optical densities when comparing the control and phage treatment results. A cocktail* with mixtures of different phages might provide a more effective real-world disinfectant because in an uncontrolled, real-world setting different strains of bacteria and the possibility of phage resistance could cause issues (Soni and Nannapaneni, 2010). Also, the ratio of bacteriophages to host cells is important as it is shown that higher concentrations of bacteriophages, also known as multiplicity of infection (MOI), are more effective at controlling *L. monocytogenes*. The most effective concentration of P100 found was 7-log PFU/ml (MOI of 5.13) because it produced the greatest phage reductions in the least amount of time (Montañez-Izquierdo, Salas-Vázquez, Rodríguez-Jerez, 2011). The age of the biofilm is also important, as a one week old biofilm was measured as having a phage reduction of 2-log colony forming units/cm^2 (CFU/cm^2) less than a 2 day old biofilm (Soni & Nannapaneni, 2010).

ListShield™. ListShield™ is a phage cocktail* that contains more than 6 different types of lytic phages and has shown to be effective against 170 different types of *L. monocytogenes* (Nannapeneni & Soni, 2015). It is a

concentrated, aqueous, phage preparation that is stored at 2-6°C and then diluted with clean water when ready for use (Intralytix, n.d.). It is Environmental Protection Agency (EPA)-approved for use on surfaces in food facilities and is FDA-approved for meat, poultry, and fish products as a surface treatment (Nannapaneni & Soni, 2015). However, it is mandated by the FDA that ListShield™ cannot be used as a 'stand-alone' protocol but as part of the overall sanitization of a surface. Also, it is necessary to wait five minutes after application before using any other chemical product to ensure that the chemical sanitizers do not inactivate the phages (Sulakvelidze, 2013).

EcoShield™. EcoShield™ is a marketed bacteriophage cocktail* composed of three *E. coli* specific strains (Carter et al., 2012). Researchers tested the safety of the product, the significance of *E. coli* reduction in lettuce and beef under usual storage conditions and protect against recontamination. When EcoShield™ tested on artificially contaminated beef steaks, *E. coli* cells were infected by phages within the first 5 minutes and protection against the initial bacterial load was maintained over a period of 7 days of refrigeration. However, there was no significant protection against the recontamination with *E. coli.* Subsequent treatment of the meat with EcoShield™ yielded no bacterial resistance to the phage cocktail*. EcoShield™'s dependency on concentration or dilution was examined through contaminated lettuce leaves, which had EcoShield™ applied to them and then the leaves were treated with water to dilute the phages present. Although the moisture slightly reduced efficacy of the treatment, the dilution initially decreased bacterial load. Chemical analysis found that there were very low levels of non-phage ingredients, meaning the contribution of EcoShield™ to an individual's diet

would be negligible, and thus potentially viable for an aftermarket decontaminant (Carter et al., 2012).

Agriphage™. Agriphage™ is an experimentally licensed phage cocktail* product that is aimed at combatting bacterial disease among commercially grown plants. Its efficacy in doing so was studied by Obradovic et al. (2005) in connection with other products with similar goals although different methods. Functioning alone, Agriphage™ did nothing to slow or reduce bacterial infection of tomato leaves studied, however when used in conjunction with other antibacterials, it provided the best method of combatting infection (Obradovic et al., 2005). A different multiyear study was also conducted where sprays of Agriphage™ on greenhouse tomato plants were investigated for their efficacy in combating bacterial cankers. Agriphage™ was not only effective in combating and reducing the canker, it also outperformed other standard methods in doing so (Ingram & Lu, 2009). This is interesting to note as it is a potentially viable area for phages to fit in within the larger context of antibacterials in the agriculture industry.

As bacterial antibiotic-resistance continues to grow, research has shown that bacteriophages are an effective alternative to the classical wide-spectrum antibiotic. Bacteriophages may be manipulated to account for bacterial resistance. As such, the concern for bacterial resistance development, in comparison to antibiotics, is of a lower degree. With gene sequencing, it is possible to observe and extinguish resistance on the genetic level, whereas with antibiotics, it is nearly impossible to make such minute changes. Through the use of a phage cocktail*, it is possible to target most of the major, actively pathogenic strains of bacteria, such as *Listeria*, while not affecting the

ecosystem of other bacteria in the area. Furthermore, phages are effective against biofilms, while most classical cleaning methods fail to penetrate the biofilm. Bacteriophages, however, have the ability to not only infiltrate the biofilm but also lyse the bacteria within (Harper et al., 2014). A growing issue in the meatpacking industry, as well as related fields, is the biofilm-forming bacteria, but a phage cocktail* will be an effective countermeasure. A potential issue with the use of bacteriophages is if the virus enters the lysogenic cycle upon entering a bacterium, as opposed to the lytic cycle. In the first case, the incorporation of virus DNA into bacterial DNA could create a wholly new strain of pathogenic DNA and as such it is imperative that the phage cocktail* always results in 100% lytic phage. Through deliberate experimentation, such a product can be achieved, which will have significant implications for bacterial sanitation in the food production industry and beyond.

METHODOLOGY

1. Growth of Bacterial Stock

1.1 Strains and Culture Conditions

Frozen stocks of *L. monocytogenes* strain 1/2a originally purchased from American Type Culture Collection (ATCC BAA679), Manassas, VA, USA were cultured in Brain Heart Infusion (BHI) broth and maintained over time by culturing on BHI agar plates and periodically sampling single colonies for re-culturing on plates or in broth. Culture plates were stored in the 4°C before use. Stock cultures of *Salmonella enterica* serovar Typhimurium originally purchased from ATCC (ATCC14028) were maintained by the same method but in Trypticase Soy Broth (TSB) or Luria Broth (LB). Broths were prepared by adding the labeled weight amounts of powdered broth to corresponding amount of distilled water, sterilizing in an autoclave for 20 minutes, and storing at 4°C for up to two weeks. Plates were prepared similarly but by adding 1.5% bacto agar.

1.2 L. monocytogenes 1/2a Standard Curve for Average CFU

A single *L. monocytogenes* colony was lifted from an agar plate and swished in warmed BHI media in a 15 mL tube. The tube was shaken at 37°C for 24 hours, centrifuged for 8 min at 3000g, and resuspended in new media to an OD of 0.1 with an absorbance wavelength of 600 nm. The 0.1 OD solution of bacteria was serially diluted down to 10^{-7} of the original. Aliquots of 25µL of each dilution starting at 10^{-2} OD down to 10^{-7} OD were spread in triplicate onto a plate using a flattened pipette. Plates were incubated at 37°C for 24 hours and colonies were counted. This experiment was repeated twice, colony

count values were averaged, and CFU/mL values were plotted against Log (OD). Bacterial solutions with OD's as high as 0.5 and as low as 10^{-9} were also plated but resulted in colony over and under growth respectively.

2. Growth of Phage Stock and Modifications

2.1 Bacteriophage A511 Propagation

Phage A511 was obtained from ATCC in a lyophilized form, reconstituted using distilled water, and then stored at -20°C. A colony of *L. monocytogenes* was added to 10 mL of BHI broth and incubated, shaking, overnight. A small amount of A511 phage was scraped from the top of the freezer vial (without thawing) and swished into the culture of *L. monocytogenes*. The phage and bacterial mix were incubated for 24 hours, shaking. The tube was then removed, centrifuged at 3000 rpm for 10min, and the phage containing supernatant carefully pipetted into freezer tubes and stored at -20°C, reserving one tube for the plaque assay.

2.2 Modifications to A511 Growth Method

After several tests that yielded no plaques, different growth methods were attempted. To address contamination issues with early stocks, a filtration step with a 0.22 µm filter was added to remove any remaining bacterial cells not separated out by centrifugation. A larger volume of original ATCC phage stock, either 250 or 500 µL, was added to begin the culture, instead of just a scraping of frozen stock. To concentrate bacterial cells before treatment of phage, and to prevent further growth, the 25 mL, 24-hour culture of *L. monocytogenes* was spun down and resuspended in 10 mL of peptone water to

which the A511 was also added. Before centrifugation or filtering, 0.5 mL chloroform was added, mixed, and then allowed to evaporate off in an attempt to increase phage concentration by lysing any bacteria that contained phage but had not lysed already. After the first few trials, the above changes to the original procedure were kept for all subsequent experiments.

Different incubation times were varied throughout the growth procedure. A method of 'feeding' the stock was performed in which a 24-hour *L. monocytogenes* liquid culture was treated with A511, allowed to incubate overnight, and then 'fed' by using a loop to transfer three colonies of *L. monocytogenes* to the tube and incubating for 4 more hours. The bacterial culture was incubated for 48 hours before A511 was added from frozen stock, and then the phage and bacterial mixture was incubated, monitoring for two days for change in apparent cloudiness. In a separate experiment, time trials were performed in which a 24-hour liquid culture of *L. monocytogenes* were treated with A511 and then incubated for 24-hours, 'fed' with three additional colonies of *L. monocytogenes*, and then three aliquots were taken at one, two, and four hours. These aliquots were filtered and chloroformed, and then tested for titer.

The *L. monocytogenes* stock that had been used was checked under a microscope to verify that it appeared to be a bacillus type as it should be, and that there were no other contaminants in the stock. In addition, a new vial of *L. monocytogenes* was retrieved from the -80°C freezer and used for subsequent experiments.

New A511 phage was ordered from ATCC and then grown using standard 24-hour incubation times but using the peptone water concentration,

filtration, and chloroform methods described above. This growth trial was tested in a plaque assay experiment along with a control of pure, undiluted ATCC stock.

3. Plaque Assay and Modifications

3.1 Semi-Soft Agar Phage Titer Plaque Assay

Phage titer procedures were modified from standardized Amrita Laboratory online resources for *E. coli* B. Semi-soft BHI agar was prepared ahead of time by adding 0.7% bacto-agar to a standard preparation of BHI broth, autoclaving, and then storing at 4°C. All prepared media were used within two weeks. A volume of 100 μL of *L. monocytogenes* overnight culture suspension was spread onto each of six BHI agar plates. The semi-soft BHI agar was heated on a hot plate until fully melted and then cooled in a water bath down to 45°C. Once cooled, 5 mL of semi-solid was added into each of five tubes. A serial dilution of A511 phage stock of unknown concentration was performed down to 10^{-9} and 100 μL of each of the 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} phage dilution mixtures were mixed into the prepared tubes of 5 mL of semi-solid agar. Working quickly to prevent solidification of the semi-solid agar, the tubes were poured onto the prepared plates of *L. monocytogenes*. Once the agar had solidified, these plates were incubated upside down for 24 hours at 37°C.

3.2 Modifications to Semi-Soft Agar Plaque Assay

Due to initial struggles with the texture of the semi-solid agar being an irregular thickness and consistency when poured onto the plate, new mixtures

were made with 0.5% and 1% agar as well. One attempt was made to use the agar by cooling it straight from the autoclave, instead of storing in the fridge and re-melting. In later procedures, the 100 μL of bacteria was added straight to the mix of semi-solid and phage, instead of plating beforehand. In addition, semi-solid was heated with a microwave instead of a hot plate to achieve more complete and rapid melting.

Plaque assays were performed in which plates were left at room temperature instead of in the incubator. This slows the growth of bacteria and was an attempt to view plaques that may have formed before overgrowth. However, no difference in plaque formation was observed and the risk for contamination was higher, so plates were placed in the incubator for subsequent experiments.

When no clear plaques were observed even with successful spreading of the semi-solid agar, changes in the initial bacterial treatment were made as well. The initial bacterial suspension was diluted to 0.1 OD and further serial dilutions were made in the suspension until 0.0001 OD. These dilutions were mixed into the semi-solid with ratios of phage varying from undiluted to 10^{-4} of the original unknown concentration. For example, one experiment with 16 plates was performed with different combinations of 0.1, 10^{-2}, 10^{-3}, and 10^{-4} OD liquid bacterial culture mixed with prepared phage dilutions of 1:0, 1:10, 1:1,000, and 1:100,000 in media. Trials with different combinations were performed, some as low as 10^{-10} OD dilutions of the bacterial solution. These trials were also performed before and after different methods of growing A511 phage were tried.

3.3 Bacterial 'Lawn' Plaque Assay

In later experiments, an alternative method to semi-solid agar was adopted to test for PFU/mL. A single colony of *L. monocytogenes* is retrieved from a culture plate and spread thoroughly onto a BHI agar plate, making sure to cover the whole area of the plate several times. In triplicate, 10 µL aliquots of the A511 phage stock batch to be tested are dropped onto one half of the plate. The plates are incubated, right-side up, overnight at 37°C. If overgrowth was a concern, plates were checked at 8 and 12-hour time points or incubated at room temperature instead. While less accurate, only providing information about the minimum number of phage at a particular dilution where plaques appear, this method removes the possibility of improperly melted and rapidly solidifying semi-solid agar and is much quicker to perform, allowing for more efficient testing of the many different methods of A511 growth.

4. Salmonella *Growth, Phage Stock, and Biofilm Assay*

4.1 Growth of P22 Phage

A 50 mL volume of TSB broth was inoculated with a culture of *Salmonella* and incubated for overnight at 37°C. The bacteria were spun down and re-suspended in 30 mL of peptone water. An aliquot of 750 µL of P22 phage was added and the mixture was incubated overnight at 37°C. The stock was centrifuged at 3000 x g for 20 minutes and the supernatant was filtered with a 0.22 µm filter (VWR, USA). A few 1 mL aliquots were stored at -20°C, and the rest was kept for use at 4°C. The PFU/mL of this stock was tested using the bacterial 'lawn' plaque assay.

4.2 Biofilm Attachment Ability Assay

A liquid culture of *Salmonella* was incubated overnight in TSB broth and then diluted to 0.1 OD, or about 10^6 CFU/mL. Prepared 100µL aliquots of bacteria at 10^6, 10^5, and 10^4 CFU/mL were added to a 96-well plate in triplicate. Each of these was treated with 100µL of undiluted P22 phage stock, about 10^6 PFU/mL. This effectively tested three different multiplicity of infection (MOI) values, where $MOI = log\ (\frac{PFU}{CFU})$, and so MOI's tested were 0, 1, and 2. Controls of 100 µL 10^6 CFU/mL bacteria with 100 µL peptone water, and 100 µL TSB broth with 100 µL peptone water were used. After 24 hours of incubation, the cell suspensions in each of the wells of the 96-well plate were transferred to a new plate, being sure not to disturb the adhered cell layer. Absorbance readings at 570 nm for these cell suspensions, along with the controls, were obtained.

4.3 Enumeration of attached cells

The adhered cells from the 96-well plate were rinsed gently with 100 µL of peptone water to aspirate off loosely adhered cells. They were then mixed vigorously with another 100 µL of peptone water and diluted down to 10^{-3} and 10^{-4} of their original concentration with peptone water. Twenty-five microliter aliquots of each were spread in triplicate onto TSB agar plates divided into thirds. The plates were incubated for 24 hours and then colonies were counted within each third. This experiment was repeated three times, diluting to 10^{-5}, 10^{-6}, and 10^{-7} in order to make colony growth more defined and countable.

RESULTS

Prior to beginning phage-bacterial cell interaction test, standard curves for *Listeria monocytogenes* ½a were calculated. This growth curve was used to pick the volume of bacterial suspension needed for meeting the required CFU of multiplicity of infection (MOI) against bacteriophage. A standard curve for *L. monocytogenes* ½a was successfully developed as shown in **Figure 4.**

L. monocytogenes *Standard Curve*

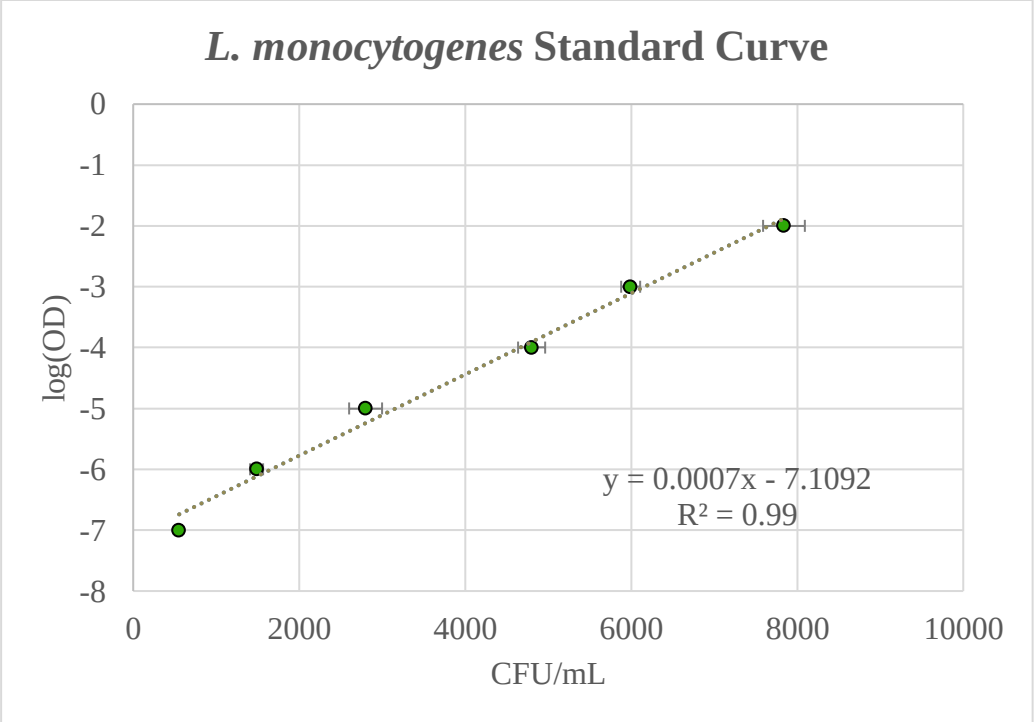

Figure 4: L. monocytogenes Standard Curve. *Standard curve showing resulting CFU/mL of bacterial solutions of various OD's (inferred from serial dilution). Results are representative of a triplicate experiment. Error bars represent standard error of the mean.*

As seen by the y-axis, ODs greater than 10^{-1} and less than 10^{-8} were unable to produce a measurable absorbance reading and resulting colony

count. This provided a method of controlling CFU/PFU for biofilm attachment ability and enumeration experiments.

Low L. monocytogenes *Concentration Tests*

It was found that the A511 phage was unable to produce plaques when applied using a semi-soft agar. To determine whether or not there was low or negligible activity of the phage stock, titer experiments were performed using high phage concentrations and low bacterial concentrations. A combination of 10^{-1} phage concentration and 10^{-10} bacteria concentration was tested, and there were no visible plaques as seen in **Figure 5**. At these extreme concentration values, at which the phage concentration is at its upper bound and the bacterial concentration is at its lower bound, there are still no visible plaques seen on the agar plate.

Figure 5: Ascertaining Phage Stock Titer. *Titer experiment with semi-solid 1% agar, bacterial concentration of 10^{-10} OD and A511 phage concentration of 10^{-1} of the unknown stock concentration. No visible plaques at such low bacterial concentrations could potentially indicate low phage activity.*

Irregularity of Semi-Soft Agar

The first iteration of titer experiments utilized a semi-soft agar in order to allow the bacteria and phage to interact with each other. Different concentrations of semi-soft agar were utilized to determine the optimal agar concentration for spreading. First, a 1% semi-soft media was used which can be seen in **Figure 6A & B**. Following this, a 0.7% semi-soft media was tested as well which can be seen in **Figure 6C**. Both concentrations resulted in inconsistencies in spreading as seen by the uneven surfaces produced by the agar on the plates.

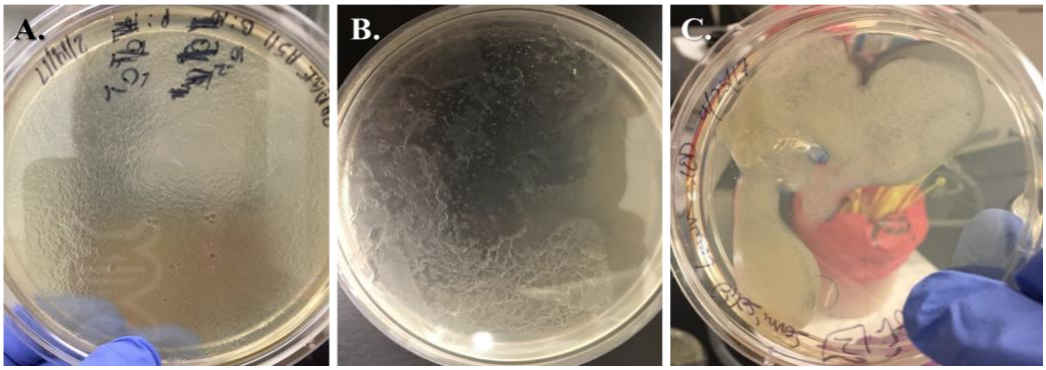

Figure 6: Variability of Semi-soft Agar. *Titer experiments showing variability of the agar spreading. Image A used 1% agar semi-soft media mixed with bacteria at 10^{-2} OD and phage at 10^{-1} of the stock. Image B used 1% agar semi-soft media mixed with bacteria at 10^{-3} OD and phage at 10^{-4} of the stock. Image C used 0.7% agar. In each case agar was melted with a microwave until completely liquid and then cooled to 50°C.*

L. monocytogenes *Lawn Plaque Assay Initial Results*

Following the series of semi-soft agar experiments performed, grass experiments were utilized in order to determine methods of improving the phage stock testing process. The phage stock was directly applied to a grass lawn of bacteria. This initially resulted in what was thought to be promising results as seen in **Figure 7A,** but it was soon realized that the plaque-like structures on the plate were actually just disturbances in the bacterial layer from the initial dropping of phage onto it**.** Due to the inconclusive results from the initial grass experiments, further testing was performed with newly grown phage at various phage and bacterial concentrations. After multiple iterations, it was determined that there were no plaques visible, even at high phage concentrations as seen in **Figure 7B.**

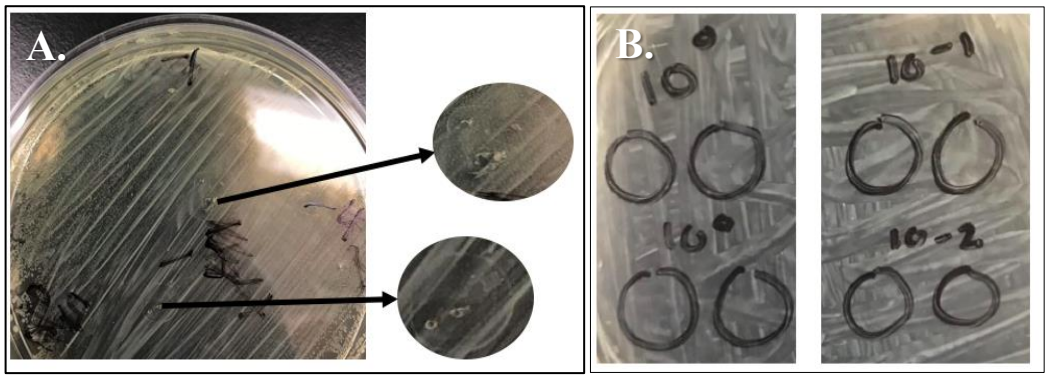

Figure 7: Lawn Plaque Assay. *Image A shows a lawn experiment resulting in bacterial disturbances, but no plaques. Initially, the results were interpreted to be plaques. Image B shows a lawn experiment using a new trial of grown A511 at undiluted (10^0), 10^{-1}, and 10^{-2} dilutions of the trial stock. However, no plaques are visible.*

To further confirm that plaques were not simply obscured by bacterial overgrowth, various temperatures for bacterial growth were used to slow the growth rate. As seen in **Figure 8,** a room temperature environment did not help improve the presence of plaques, despite the decrease in bacterial growth.

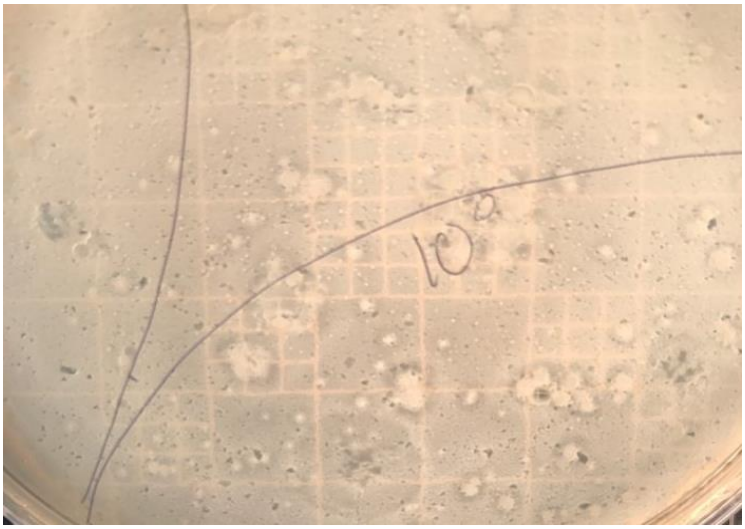

Figure 8: Lawn Plaque Assay at Room Temperature. *Lawn experiment using Listeria at room temperature to slow bacterial growth rate, in order to determine whether plaques were formed but then overgrown. Resulting plate is cloudy and unreadable, indicating no plaques.*

In order to verify that the stock of *L. monocytogenes* that was being used was not contaminated at some point, the bacterial stock from the freezer was Gram-stained and placed under a microscope. **Figure 9** shows that the morphology of the cells appears to be consistent with the expected coccobacilli of *L. monocytogenes.* This showed that the stock used for previous experiments were likely not affected by a contaminated bacterial source. Despite this confirmation, future experiments were performed with a newly thawed *L. monocytogenes* 1/2a stock from the laboratory storage.

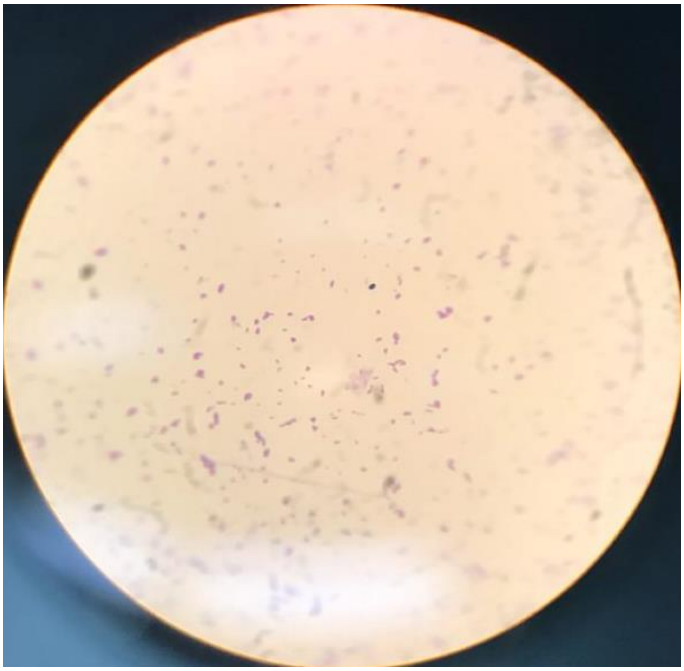

Figure 9: Gram-stain of *L. monocytogenes*. *Contamination check via staining to confirm presence of Listeria at 1000x magnification. Cells appear to be coccobacilli and Gram-stain purple, consistent with Listeria monocytogenes.*

Using newly ordered ATCC A511, several more grass experiments to determine titer of phage grown with that new stock were attempted. As seen in **Figure 10A & B,** no plaques appeared to be present despite the newly purchased phage.

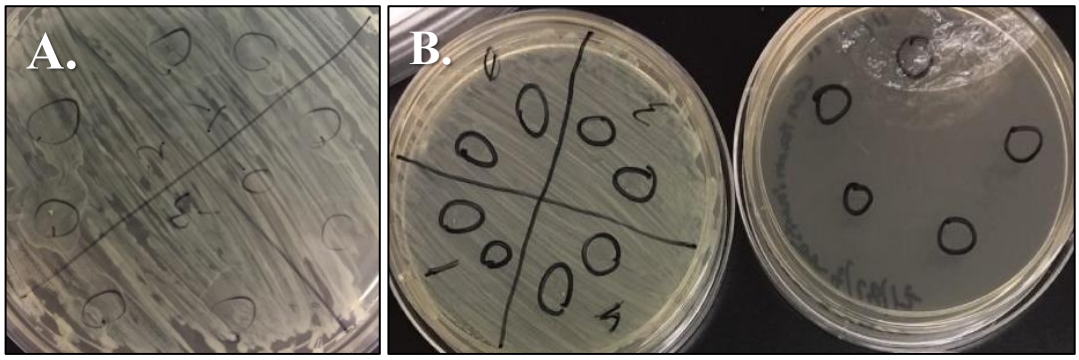

Figure 10: Lawn Plaque Assay Using New ATCC A511. *Image A shows a lawn experiment using new ATCC stock from a second order. Stock was filtered and chloroformed, but no plaques were visible. Image B shows two plates, a test and a control. The left shows a lawn experiment using fresh phage and bacterial stocks with phage stock concentrations from 10^{0} to 10^{-4}. No plaques present. The right shows a control plate with the bacteriophage stock plated in absence of bacteria. The lack of growth on this control plate shows that our bacteriophage stock was not contaminated with bacteria.*

A511 phage straight from the small volume obtained from ATCC was plated on a grass experiment. **Figure 11** shows that while plaques formed, they were only present up to the 10^{-2} dilution of the original ATCC A511 stock. This means that the purchased stock had a concentration of about 10^{4} PFU/mL, which is relatively low.

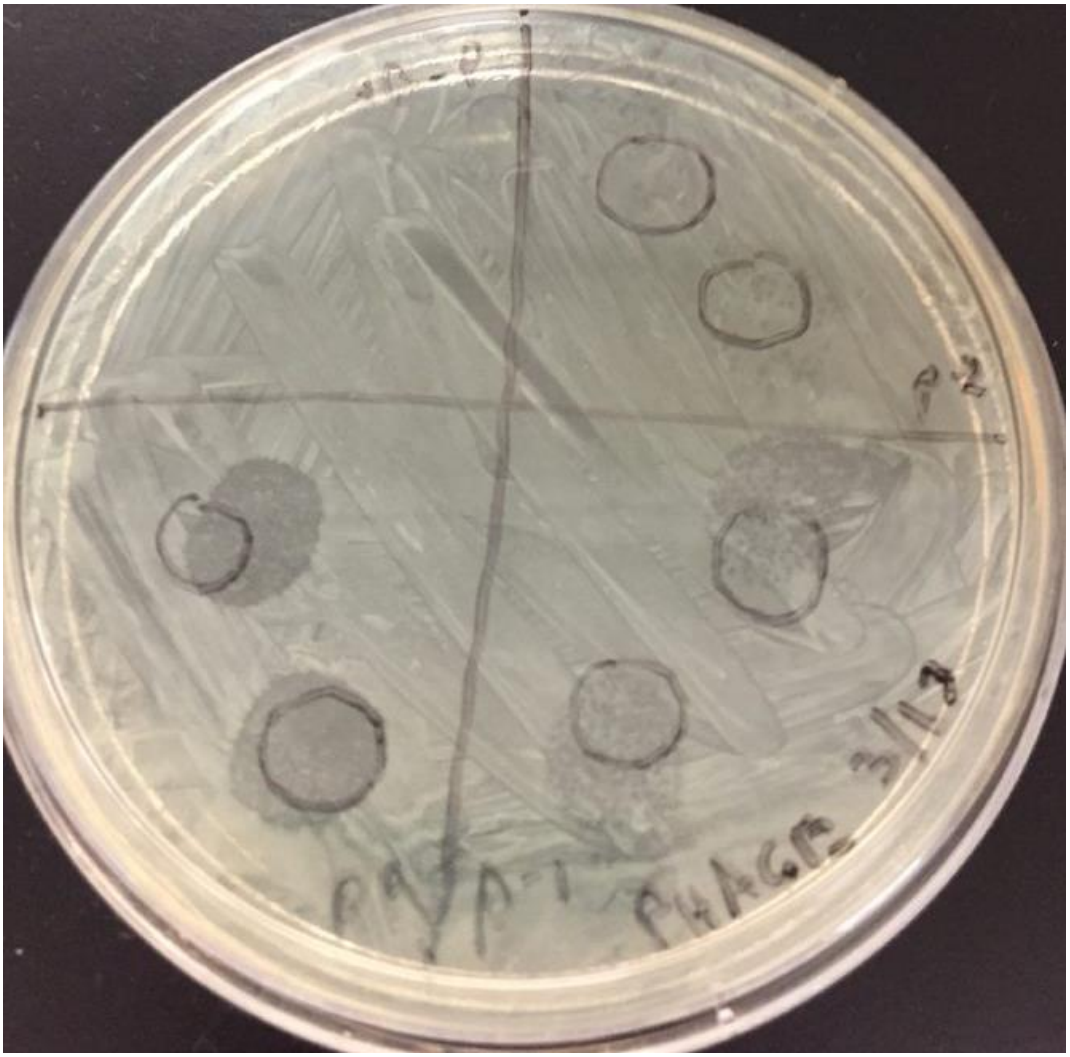

Figure 11: Lawn Plaque Assay Using Undiluted ATCC A511. *Direct application of pure, reconstituted ATCC A511 phage stock. Top left quadrant: control quadrant. Top right: phage concentration of 10^{-2}. Bottom right: phage concentration of 10^{-1}. Bottom left: phage concentration of 10^{0}(undiluted pure stock). Very few plaques are present in the 10^{-2} quadrant, indicating low phage titer.*

Because prior attempts to grow A511 from this ATCC stock had failed to show plaques, a parallel experiment was performed using *Salmonella* and P22 phage so the methods of phage growth could be verified. **Figure 12** Shows that while the A511 and P22 were grown following the same methods,

the A511 sample failed to show plaques on *L. monocytogenes* while P22 showed plaques on *Salmonella* even at very low dilutions (P22 dilutions of 10^{-5} and 10^{0} are shown in **Figure 12**). This lead to the conclusion that the methods and materials currently available to the team were insufficient to successfully grow A511 phage, and marked the pivot to *Salmonella* tests as a control case.

L. monocytogenes *vs.* S. enterica *serovar Typhimurium:*

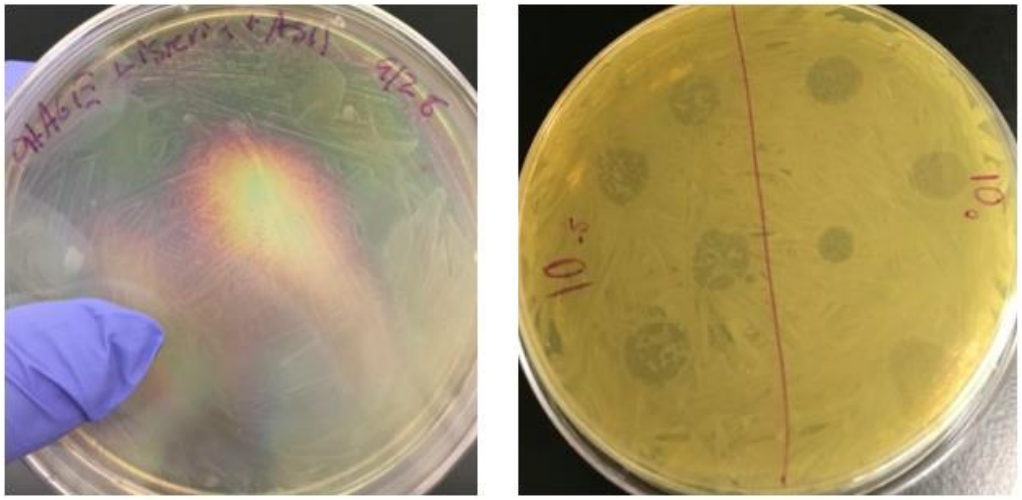

Figure 12: Parallel Lawn Plaque Assays of A511 and P22. *Parallel testing of grass experiments using Listeria (left) and Salmonella (right). Same techniques used for both stocks, only Salmonella showed plaques.*

Figure 13: Plaque Formation of P22 Application on *S. typhimurium*. *Salmonella grass experiment using a P22 10^{-5} phage stock dilution. Presence of plaques at this concentration indicate an MOI of 10^{7} PFU/CFU.*

For testing on *Salmonella* biofilm, the working stock of P22 was determined to have a titer of 10^{7} PFU/mL, as shown in **Figure 13**, a cropped and magnified version of **Figure 12**. This was tested on biofilms in varying MOIs but results were limited due to time constraints.

DISCUSSION

Prior to beginning phage experimentation, standard curves for both *S.* Typhimurium and *L. monocytogenes ½a* were calculated. These growth curves were necessary for later calculations of multiplicity of infection (MOI). A standard curve for *L. monocytogenes ½a* was successfully developed as shown in **Figure 4.** As seen by the y-axis, ODs greater than 10^{-1} and less than 10^{-8} were unable to produce a measurable absorbance reading and resulting colony count. This provided a method of controlling CFU/PFU for biofilm attachment ability and enumeration experiments.

Bacteriophage titer measurement was attempted via semi-soft agar plaque assay (3.1) At first, this method seemed to limit total bacterial growth; however, because there were no clear plaques on these plates, it was inconclusive whether or not this was caused by the phage activity or some other issue with the procedure. After further testing, it became clear that bacterial overgrowth occurred, so variations in the original method were made to reduce starting bacterial concentration in the plaque assay (3.2)

When bacterial overgrowth occurs, the phage tend to infect cells less successfully due to the threshold MOI for lysogeny not being reached (Abedon, 2016). To decrease bacterial concentration on these plates, bacterial solutions were diluted to lower ODs which correspond to lower concentration of bacteria per mL in accordance with the standard curve. This was to ensure that the CFU:PFU ratio was more skewed in favor of the phage, and ensure that the minimum lysogenic MOI was reached. However, even with varied initial plating concentrations, consistent plaque formation was not observed even at extremely low bacterial dilutions (**Figure 5**). Because of other

difficulties with performance of the semi-soft agar, it was inferred that there may have been an issue with semi-soft agar composition or melting method (3.1) In many experiments, the semi-soft agar solidified rapidly after melting such that it would fail to spread evenly on the plates or appear lumpy after spreading. This would prevent an even distribution of bacteria and phage across the whole plate, making any resulting plaque count un-observable or unreliable (**Figure 6 A-C**). It was also thought that very viscous agar reduces the rate of diffusion of phage across the plate, which inhibits its ability to infect the target bacteria. At first, instead of plating bacteria and then pouring the phage/semi-soft agar mixture on top, both bacteria and phage were added to the semi-soft agar and poured onto a clean plate to attempt a more even mixing. When no plaques were observed and agar spreading problems persisted, different agar preparation methods were attempted.

Modifications to the semi-solid agar composition proved unreliable, with lower agar percentages failing to solidify properly. Different reheating methods such as high temperature microwave melting followed by cooling in a hot water bath led to contamination issues from the water bath (3.2). It is also possible that initially autoclaving the semi-soft agar led to inconsistencies within the mixtures themselves, rendering any re-melting or remixing ineffective. Even when these modifications in methods resulted in an even spread of agar across the plate and extremely low initial bacterial concentrations were plated, plaque formation was not observed. Therefore, a new method of a ‘lawn’ plaque assay was adopted, allowing for the elimination of semi-soft agar as an experimental variable (3.3). While this method is less accurate as it does not allow for a controlled CFU/mL to be

plated in each experiment, it provides a minimum PFU/mL measurement. If plaques are visible at a particular phage dilution, then there must be at least one phage within the plated volume, and the PFU/mL can be estimated.

Result of the 'grass' method were initially hopeful, as plates showed what appeared to be plaques (**Figure 7A & B**). However, results were varied and unrepeatable. It may have been that when the phage solution was dropped onto the plate, the bacterial layer was disturbed, resulting in an observation of displaced bacteria rather than lytic activity. After several trials, it was concluded that the phage stock used for these trials may simply be too low concentration to produce plaques. A set of new trials to increase concentration of the phage stock began.

Initially phage stocks were contaminated, so a filtration step was added to the procedure. In an attempt to make bacterial cells more susceptible to infection, they were grown first in a normal BHI broth and then concentrated into a smaller volume of A511-treated nutrient-less peptone water that would essentially "starve" the bacteria and prevent further growth. After these initial changes to the growth procedure still failed to produce plaques in a 'lawn' experiment, methods to concentrate the stock were attempted. Phage stock was 'fed' with cultures of bacteria from a loop and then incubated and filtered again. To slow growth of bacteria, some trials were left at room temperature, to ensure that bacterial overgrowth was not covering up existing plaques, but these trials showed no plaques (**Figure 8*)***.

To further ensure there was no contamination within the *L. monocytogenes* culture, the cells were observed under a microscope and no evidence of contamination was found (**Figure 9**). To be absolutely certain,

fresh stocks of *L. monocytogenes* were retrieved from the -80℃ freezer and a fresh A511 stock from ATCC was purchased and used in a new phage growth trial. Even with these efforts using fresh stocks, no plaques were observed (**Figure 10A & B**). Returning to the theory that the A511 phage was not aggressive enough to observe plaque formation, pure ATCC phage stock was used on bacterial cultures (**Figure 11**). The pure ATCC stock showed a titer of 10^2 PFU/mL, which is very low. This led to the tentative conclusion that the purchased pure A511 phage vial as a whole may have had a low titer. The ATCC acquired A511 phage was not only of an unknown concentration, but several years old. The phage sent to us by ATCC was preserved via lyophilization, a process which has been found to decrease the titer of *Staphylococcus aureus* phage ISP as a result of the destabilization of the phage components during the freezing process. Immediately after lyophilization, phage titer in sucrose solutions decreased by a factor of two and continued to decrease by as much as a factor of six after three years of storage. Higher concentrations of sucrose lessen the degradation to only a factor of two over that three-year time scale (Merabishvili et al., 2013). It is also possible that mistakes were made by our team in the initial inoculation of the phage, leading to a reduced titer. When treating the bacteria with phage, the overall treatment was likely not high enough to kill enough bacteria for phage propagation. This led to having a phage solution with a high amount of bacterial growth and a low or non-existent phage. These circumstances could in part explain difficulties with lack of phage growth and subsequently lack of observing phage growth on varying concentrations of *L. monocytogenes* cultures, even after significant alterations to phage growth procedures.

As a control for the procedures used with *L. monocytogenes* against A511 phage, *Salmonella* against P22 phage were tested. *Salmonella* has previously shown susceptibility to P22 phage (Ahn, Kim, Jung, & Biswas, 2013). As shown in **Figure 12**, an experiment in which exactly the same phage growth, purification, and plaque assay methods were used resulted in plaque formation for *Salmonella* treated with P22, but no plaque formation for *L. monocytogenes* treated with A511. These results are evidence that there were issues with our personal phage stock, which limited plaque formation.

Using the bacterial "lawn" assay, the P22 phage stock titer was found to be about 10^6 PFU/mL (**Figure 13**). This stock was stored and used for *S. enterica* biofilm experiments. Initial *S. enterica* biofilm tests showed that bacteria initially plated at 0.1 OD resulted in lower adhered bacterial concentrations after incubation with phage (0.519, 0.547, and 0.379 OD) than those initially plated at 0.01 OD (0.874, 0.705, and 0.954 OD). A similar pattern of overgrowth was apparent in the planktonic cell count as well. This may be due to an overgrowth of *S. enterica* at 0.1 OD, leading to a lack of nutrients left available over time and causing a higher amount of bacterial death at that concentration. It is possible that the P22 phage solution of 10^6 PFU/mL was not concentrated enough to overcome *S. enterica* growth at this MOI, though efforts could be made to re-grow this phage stock and concentrate it to 10^{10} PFU/mL, the concentration at which it showed activity previously (Ahn, Kim, Jung, & Biswas, 2013). Other studies have shown a reduction in *Salmonella* planktonic cell count of 10^5 CFU/mL after four hours of incubation with phage (Birendra, Kim & Kim, 2013). Due to time limitations, however, a concentrated stock of P22 was not tested here.

Furthermore, across nearly all of the experiments, a constant temperature of 37°C was used to grow the phage-applied bacteria plates. Despite an attempt to incubate the bacteria at room temperature, no other trials were conducted under different temperatures. One of the driving factors for phage efficacy involves determining the optimal temperature for phage receptors to adhere to the bacterial cells. An experiment testing the efficiency of two *Listeria* phages similar to P-100, LP-048 and LP-125, found that temperature is an extremely important parameter to control when applying phage. The study found that at 37°C, LP-048 exhibited severe decrease in adsorption efficacy and plaque formation in response to strains 1/2a, 1/2b, 4a, indicating a downregulation of phage receptor rhamnose. Additionally, LP-048 was shown to produce plaques at 37°C on a mutated strain lacking N-acetylglucosamine, suggesting that competition between N-acetylglucosamine and rhamnose for glycosylation sites occurs during infection (Tokman, Kent, Wiedmann, & Denes, 2016). As such, the temperature that we incubated our plates at seem to be detrimental to phage infection and multiplication. Successful A511 and P100 testing at 6°C and 20°C on ready-to-eat foods corroborate the implication that 37°C is not an ideal temperature for growing or examining *Listeria* phage (Guenther, Huwyler, Richard, & Loessner, 2009).

At higher temperatures, the ability of A511 to infect bacteria begins to significantly decline. A study testing *Listeria* phage A511 on *Listeria ivanovii* 3009 discovered that this phage was the most sensitive to environmental changes with respect to other commonly used phages such as *Salmonella* phage Felix O1 and *E. Coli* phage T7. At 4°C, there was no detectable activation of the phage kinetics for A511. Additionally, at 37°C it was found

that A511 activation was slowed down and had lost 2 log PFU after 21 days. At 60°C, the sensitivity of the A511 increased even higher, losing 4 log PFU in 5 minutes (Kim, 2007). Therefore, temperature is a key factor that can be further varied in future experiments and is likely a large contributor to the lack of activity witnessed across the data collected.

Current Research

The use of bacteriophage against bacterial biofilms is a promising research area. During the three years of research for this project, thousands of research articles were published describing the positive aspects of using bacteriophage as disinfectants. Despite our work being inconclusive, other scientists have performed similar experiments and reported significant success. One group tested two *Salmonella* phages against *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis biofilms on rubber, stainless steel, and lettuce (Sadekuzzaman, Mizan, Yang, Kim & Ha, 2018). They carried out a method similar to the team's, including finding ways to concentrate phage stocks. On stainless steel, the phage treatment reduced biofilm cells by approximately 10^3 CFU/cm^2. On the rubber surface, the phage treatment reduced biofilm cells by more than 10^2 CFU/cm^2. Trials on lettuce were tested at varying temperatures, however, the bacterial load was significantly reduced for each temperature. To assess phage treatment in a controlled smooth surface, an MBEC assay was performed and showed that the phage treatment reduced biofilm cells up to $10^{3.6}$ CFU/peg (Sadekuzzaman, Yang, Mizan, Kim & Ha, 2018).

A secondary reason bacteriophages are thought to be effective against biofilms, beyond their penetration of the protective matrix, is their ability to

infect persister cells. Persister cells are inactive bacterial cells embedded in biofilms with relatively no metabolic activity, and regain normal function if damage occurs to the biofilm. These persister cells are a major facet of biofilm tenacity. Bacteriophages have the ability to infect these dormant cells, and when the cell activates, the bacteriophage lyses the cell. A study by Harper et al. in 2014 confirmed this phenomenon in *Pseudomonas aeruginosa* biofilms, noting the ability of phages to kill biofilms in situations where antibiotics could not. A study found that 97% *S. enteritidis* biofilm reduction after only 4 hours of contact with their phage stock, with 100% of phage adsorption achieved after only 15 minutes (Tiwari, Kim, Kim).

Other studies corroborate phage killing of persister cells immediately upon metabolic activation. The same ability was recorded using the hipA7 strain and the λ-phage, showing 90% reduction of the colonies under microscope. Furthermore, the lytic activation of phages was shown to directly follow the resumption of persister cell growth functions. The researchers make the point that this is another adaptive ability of phage, as phage lysis of nutrient-deprived cells has also been studied. T4 phages can enter stasis if the host bacteria is starved of resources, reactivating only when nutrients are acquired in order to optimize phage production. The flexibility and adaptability of phages is the foundation of their effectiveness against biofilms (Pearl, Gabay, Kishony, Oppenheim & Balaban, 2008).

There are many other directions that phage therapy has taken. Some institutions have heavily researched phage endolysins - lytic enzymes used to lyse the host cell by enzymatically degrading the peptidoglycan in the bacterial cell wall (Schmelcher & Loessner, 2016). This method skips the

process of infecting bacteria with bacteriophage and moves directly to lysing the host cell. Some endolysins, such as PlyGRCS, have shown to be effective in directly lysing cells in biofilms (Linden et al., 2015.) However, it is most effective on Gram-positive bacteria, since the outer membrane of Gram-negative bacteria interfere with the activities of endolysins (Love, Bhandari, Dobson, & Billington, 2018). Furthermore, the endolytic phage derivatives were also shown to lyse bacteria in biofilms more quickly than topical phage application. One group of researchers measured the efficacy of a phage cocktail composed of bacteriophage K and 6 modified derivatives on *Staphylococcus aureus* found that the cocktail was able to inhibit biofilm growth after 48 hours, when applied immediately. Treatment with the same cocktail after 72 hours of biofilm growth, however, yielded an even larger drop in biomass, showing that a later application was more effective. A different group of researchers then used the phage lysin $CHAP_k$ on the same *Staphylococcus* bacteria, reporting that it only took 4 hours to eliminate the biofilm (Endersen et al., 2014). This is evidence that even more effective anti-biofilm products can be derived from phages, such as endolysins, though more research is needed directly comparing whole bacteriophages and endolysins.

All of these methods have applications in food safety, and even clinical care due to the specificity of both bacteriophage and their endolysins. The large body of work between these directions also emphasizes the relevance and promise of phage therapy as an antibacterial technique.

Future Directions

Due to the limited timespan and extensive scope of the project, any obstacles in research severely interfered with data collection. The main factors that hindered progress were time sensitivity of experiments, delays in receiving materials, and significant issues with phage growth.

Over a year of research was spent testing and altering experiments with *Listeria* phage A511 - growing and concentrating the phage stock, attempting to optimize the titer experiment, and troubleshooting bacteriophage infection of *Listeria* biofilms. The team invested a significant amount of time and money, and with the prior restrictions on phage access it was difficult to pivot to a different phage in a timely fashion. Investigation of the original lyophilized *Listeria* phage A511 received from ATCC revealed it to be more than 12 years old which may have reduced its activity below effective levels.

An additional several months to a year would make a significant impact on the amount of data collected. When the team decided to switch from testing *Listeria* to *Salmonella*, it was the fall semester of the final year. As mentioned previously, the *Salmonella* phage P22 was shown to be effective on *Salmonella*, thus providing the team with an alternative route which would allow for testing an active, concentrated phage stock as a control for the procedures used on *Listeria* and a proof-of-concept. Unfortunately, switching at this point in the timeline did not leave sufficient time for all of the initial project goals to be completed.

There are multiple different tests that the team planned on conducting. The first would be to alter the temperature at which the phage stock was incubated on the bacteria.This would allow more relevant data for the food-

processing industry, which keeps its work areas typically at near-freezing. Next would be to test the effectiveness of bacteriophage cocktails in conjunction with non-phage substances, like peracetic acid, to boost total bacterial eradication. Another interesting extension of the project that the team planned was to combine bacterial species in a biofilm, such as *Salmonella* and *Listeria,* and observe the effects of bacteriophage on this consortium. It is possible that multiple types of bacteria residing in the biofilm could afford more protection, reducing the effectiveness of a bacteriophage cocktail. Further research can also be done on the most effective application method of phages. There is evidence that aerosolized sprays can be effective, but there a wide range of options in product design to explore. While the team was not able to demonstrate efficacy of *Listeria* phage A511 against *L. monocytogenes 1/2a* within this time frame, the team was able to explore and report a host of different methods for phage growth, as well as reproduce promising data for P22 phage against *Salmonella enterica* serovar Typhimurium. The wealth of recent research and continued expansion of commercial phage disinfectant products show that bacteriophages are promising alternative to antibiotics.

Appendix: Glossary (denoted by "*")

Aliquot: A portion of a given substance.

Brain-heart infusion broth: A nutrient growth medium used for culturing bacteria which combines dextrose broth and brain tissue.

Burst size: Average number of phage particles liberated when an infected bacterium is lysed.

Extracellular polymeric substance (EPS): An extracellular matrix composed of a conglomeration of biopolymers that assist in biofilm formation.

Inoculate: To introduce one thing to another, generally cells to a medium or a treatment to cells, and generally a specific measurement.

Peptidoglycan: The structure that makes up the cytoplasmic membrane of nearly every eubacteria.

Persister Cell: A form of effectively dormant bacterial cell that grows within a biofilm and resists uptake of antibiotics but can give rise to whole new colonies of bacteria.

Phage cocktail: The mixture of selected phages as well as any other supporting agents in a solution.

Rescue: Restoration of the effectiveness of an antibacterial agent that bacteria have become immune to by addition of another agent.

Titer: Measurement of the concentration of a given solution.

Transfection: The purposeful introduction of nucleic acid material into cells.

Transduction: Bacteriophage infection to incorporate foreign DNA into a bacterial cell

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