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Efficacy of Listeria phage in reducing Listeria monocytogenes under both experimental and food processing conditions

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EFFICACY OF *LISTERIA* PHAGE IN REDUCING *LISTERIA MONOCYTOGENES* UNDER BOTH EXPERIMENTAL AND FOOD PROCESSING CONDITIONS

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In Dedication to My Parents

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ABSTRACT

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Title: Efficacy of *Listeria* Phage in Reducing *Listeria Monocytogenes* under Both Experimental and Food Processing Conditions
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Listeria monocytogenes (*L. monocytogenes*) is a foodborne pathogen able to flourish in different food processing environments. Bacteriophages are viruses that target bacteria and may be effective in controlling bacterial contamination in different environments. In 2006, the US Food and Drug Administration approved a six-*Listeria* phage product for use as a food additive in controlling *Listeria* contamination in ready-to-eat meat and poultry products. In this study, we evaluated the efficacy of bacteriophages in controlling *Listeria monocytogenes* growth on non-food contact surfaces under different conditions.

Stainless steel coupons or sterilized bags were inoculated with *Listeria monocytogenes* (LM94 or LM-GFP) and a cocktail of six *Listeria* bacteriophages under conditions that could limit or enhance phage treatment efficacy: pre-treatment of coupons with phages, protection of phages in oil, presence of competing organic matter, and presence of competing bacteria. In each case, inoculated coupons or bags were incubated for 18 h at 21°C and *Listeria monocytogenes* concentrations were compared between phage-treated and untreated samples. Phage impact on *L. monocytogenes* growth was measured by ANOVA through comparing viable bacteria cell counts between phage treated and untreated group with two sample t-tests and WMW tests based on data distributions. Differences were considered statistically significant at $P < 0.05$.

Phage treatment significantly reduced *Listeria monocytogenes* on stainless steel coupons co-inoculated with bacteria and bacteriophages ($P < 0.05$). Phage treatment remained effective in the

presence of soil, fat, and competing (non-*Listeria*) bacteria ($P < 0.05$). Phage treatment was not effective, however, in reducing bacterial concentrations in the presence of blood, as well as when used as a preventative, i.e., phages applied to coupons 4 h prior to *Listeria* challenge. Protecting phages in oil prior to application did not significantly improve efficacy of the treatment.

These data indicate that *Listeria* phages may be effective in reducing *Listeria monocytogenes* contamination of non-food contact surfaces in food processing environments. Under some conditions, however, phages may need some form of protection to ensure their efficacy (e.g., to prevent desiccation and inactivation).

CHAPTER 1. LITERATURE REVIEW

1.1 *Listeria Monocytogenes*

1.1.1 Prevalence of *Listeria monocytogenes*

The genus *Listeria* is currently comprised of 17 bacterial species, including: *Listeria aquatica, Listeria booriae, Listeria cornellensis, Listeria fleischmannii, Listeria floridensis, Listeria grandensis, Listeria grayi, Listeria innocua, Listeria ivanovii, Listeria marthii, Listeria monocytogenes, Listeria newyorkensis, Listeria riparia, Listeria rocourtiae, Listeria seeligeri, Listeria weihenstephanensis, Listeria welshimeri*. Among the 17 species, *L. ivanovii* and *L. monocytogenes* are identified as pathogens. While *L. ivanovii* causes diseases in ruminants, *L. monocytogenes* is associated with illness in both humans and other species. *L. monocytogenes* is among the top three foodborne pathogens causing fatalities in humans (*Scallan et al., 2011*). In the US, *L. monocytogenes* causes an estimated 1500 illnesses with 200 deaths annually (*Scallan et al., 2011*). Although the absolute number of infections is comparatively low, the mortality rate of clinical *L. monocytogenes* infections is as high as 20% (*Lee et al., 2012*). *L. monocytogenes* infection is more prevalent in the young, elderly, immunocompromised, and pregnant women. In pregnant women, *L. monocytogenes* is associated with a variety of illnesses including meningoencephalitis, septicemia, and abortions.

The probability of contracting listeriosis (the disease associated with *Listeria* infections) from a food source is low when *L. monocytogenes* loads in food are below 100 CFU/g, and increases with increasing infectious dose (*Thevenot et al., 2006*). Due to the severity of listeriosis, the US has zero tolerance for *L. monocytogenes* contamination in prepared foods, which include both ready-to-eat and fully cooked foods that are reheated before consumption. Europe and Canada each have a tolerance of 100 CFU/25 g in ready-to-eat foods. Food products failing to meet these

standards lead to recalls, which are estimated to cost $0.16 to $0.3 million per recall in the US (*Katan et al., 2004*). Together with productivity losses, hospitalization, and other costs, the annual cost of listeriosis in the US is estimated to approach $2.6 billion (*Hoffmann et al., 2012*).

1.1.2 *Listeria* biology

L. monocytogenes is a gram-positive, rod-shaped, facultative anaerobe. There are a total 13 *L. monocytogenes* serotypes according to O-antigenic patterns, of which serotypes 1/2a, 1/2b, and 4b are most often associated with foodborne disease outbreaks (*Lee et al., 2012*). While the optimal temperature for *L. monocytogenes* growth is between 30 to 37°C, *L. monocytogenes* can grow from 1 to 45°C (*Farber et al., 1991*). In addition to extreme temperature, *L. monocytogenes* can withstand a wide pH range of 4.0 to 9.6 (*Lado et al., 2007*). Because of its ability to cause health problems and withstand different harsh environments such as extreme pH, severe temperature, low water activity, and high salt concentration, *L. monocytogenes* is a considerable risk in every step of food processing, including refrigeration.

1.1.3 *Listeria* pathogenesis

Listeria pathogenesis follows the infectious pattern of attaching to and invading epithelial cells and subsequently spreading to other tissues. *Listeria* infection via contaminated food involves the traversing of bacterium through the gastric stomach to eventual sites of infection along the small intestine.

Attachment of the bacterium to epithelial cells is a critical step in infection. Various virulence factors may aid in attachment, including LAP, FbpA, Ami, and P60 (*Burkholder et al., 2010*). Located on the bacteria cell wall, LAP interacts with the epithelial receptor human heat shock protein 60 (Hsp60) and facilitates adherence and subsequent translocation (*Burkholder et al., 2010*). Acting as an adhesion protein, FbpA binds to host immobilized fibronectin, enhancing

adherence to host cells (*Osanai et al., 2013; Camejo et al., 2011*). Dramsi et al., (2004) also showed that as a chaperone protein, FbpA can facilitate secretion of specific bacterial virulence proteins of *L. monocytogenes* such as the invasion protein InlB and the lysis protein listeriolysin (LLO), which are important virulence factors in the following invasion and infection steps (*Dramsi et al., 2004*). Comprised of a N-terminal catalytic domain and a C-terminal cell wall-anchoring domain including multiple repeated dipeptide glycine/tryptophan (GW) modules, Ami protein also contributes to *L. monocytogenes* adherence to eukaryotic cells (*Milohanic et al., 2001*). As the amount of GW repeats increases, the anchoring effect of Ami increases (*Braun et al., 1997*). Located mostly in the supernatant, P60 may have the effect of disaggregating long bacterial cell chains, so that these disaggregated single cells can further invade nonprofessional phagocytic cells (*Kuhn et al., 1988*).

Once attached, *Listeria* employs mechanisms allowing invasion of the host cell. In addition to actively being engulfed by phagocytic cells, *L. monocytogenes* can also interact and infect non-phagocytic cells through the recognition and binding between bacteria and host cell virulence protein receptors (*Radoshevich et al., 2017*). The most well-characterized virulence proteins involved in *Listeria* invasion of non-phagocytic cells are internalin A (InlA) and B (InlB) of the internalin protein family. InlA interacts with host protein receptor E-cadherin, and induces receptor clustering, phosphorylation, and ubiquitination (*Radoshevich et al., 2017*). These conformation changes ultimately lead to invasion of *L. monocytogenes* of the host cell, and invasion promotes bacteria translocation through the intestinal tract to deeper tissues (*Lecuit et al., 2001*). Binding of InlB and host hepatocyte growth factor receptor (Met) activates proliferative signaling cascades, and mediates cytoskeletal rearrangements of actin in host cells to facilitate the uptake of bacteria by host cells (*Bierne et al., 2007*). At the same time, the interaction between soluble InlB and

complement component 1 Q subcomponent-binding protein (C1QBP), as well as glycosaminoglycans (GAGs), increases receptor clustering and aids in endocytosis (*Radoshevich et al., 2017*). Additionally, Vip binding with host receptor GP96 as well as LpeA is also required in *L. monocytogenes* invasion to epithelial cells (*Camejo et al., 2011*).

Once internalized into host cells, *Listeria* is usually present in vacuoles. *L. monocytogenes* escape from the vacuoles with the help of Listeriolysin O (LLO) and two phospholipases (*Radoshevich et al., 2017*). LLO, a secreted protein from the cholesterol-dependent cytolysin toxin family, contributes to *L. monocytogenes* escape from both primary vacuoles containing newly internalized bacteria and secondary vacuoles, which form during cell-to-cell spread (*Liang et al., 2014*). While phospholipase PlcA aids in primary vacuole lysis with LLO, PlcB cooperates with LLO on the lysis of secondary vacuoles (*Liang et al., 2014*). After escaping to the cytosol of the host cell, *L. monocytogenes* may replicate using resources from host cells. Subsequently, the bacteria induce accumulation of host F-actin in the cytoplasm for cytosol movement and cell-to-cell spread (*Liang et al., 2014*). Actin assembly-inducing protein Arp 2/3, after being activated by the bacteria surface protein Act A, stimulates the polymerization of actin for bacteria cell-to-cell motility (*Cossart et al., 2008*).

1.1.4 *L. monocytogenes* in food processing

L. monocytogenes is ubiquitous in nature. As a saprophyte, *L. monocytogenes* can be found in soil, animal feces, and skin from otherwise healthy animals (*Skovgaard et al., 1989)*. Coupled with its ability to survive under extreme conditions, *L. monocytogenes* can contaminate across different steps in food processing and is found in diverse food products, including meat, dairy, and seafood (*Thevenot et al., 2006*). Additionally, cross contamination is facilitated by poor sanitation of products, equipment, food handlers, and consumers. More specifically, contact between food and

various environmental surfaces, including refrigerator walls and shelves, food contact surfaces, non-food contact surfaces, and workers' hands, can increase *L. monocytogenes* contamination of food products (*Lianou et al., 2007*). Previous studies showed that *Listeria monocytogenes* was especially prevalent on non-food contact surfaces including drains (*Hoelzer et al., 2011*). *L. monocytogenes* contamination on non-food contact surfaces can occur through aerosolization, cleaning systems, or workers (*Gibson et al., 1999*).

Contamination of surfaces is controlled primarily through cleaning such as mechanical disruption by brushing, scrubbing, and high-pressure spraying in effort to remove organic matter, followed by disinfection to reduce viable bacteria (*Gibson et al., 1999*). However, there are still challenges associated with the control of *L. monocytogenes* in food processing environments. Wirtanen et al. (1992) showed that high pressure spraying was effective in cleaning *L. monocytogenes* contaminated surfaces (*Wirtanen et al., 1992*). The aerosol generated from high pressure spraying, however, can facilitate transfer of bacteria from non-food contact surfaces to food and vice versa (*Gibson et al., 1999*). Thus, mechanical measures may cause cross contamination of contact surfaces throughout the environment leading to contamination of food products.

In addition, planktonic *L. monocytogenes* cells attach to surfaces, on which they can generate extracellular polymeric substances (EPS) and form biofilms. Biofilms are believed to enable bacteria to respond to the environment stimuli as a community, instead of individually. Mainly comprised by polymerized substances such as polysaccharides, phospholipids, and protein, the existence of EPS in biofilms contributes nutrients and increases water activity and can also prevent access of disinfectants to individual bacterial cells (*Chmielewski et al., 2003*). Biofilms can be formed by *Listeria* on various surfaces in food processing environments, including food contact

surfaces such as stainless steel and plastic, and environmental surfaces such as conveyor belts, floor drains, or hand trucks (*Chmielewski et al., 2003*). Based on the complex environments in food industry, heterogeneous biofilms consisting of multiple bacteria species are possible and biofilms containing *L. monocytogenes*, *Pseudomonas fragi,* and *Staphylococcus xylosus* have been characterized (*Norwood et al., 2000*). As the biofilm persists, extracellular polymeric substances (EPS) generation increases and forms a more complex protective layer. These characteristics enable *L. monocytogenes* to survive antimicrobial treatment and persist in the environment.

Furthermore, it is generally accepted that, as is seen with antibiotics, the use of disinfectants can lead to selection of disinfectant resistant organisms. Lemaitre et al. (1998) and Aasc et al. (2000) each characterized *L. monocytogenes* that were resistant to chemicals commonly used in food processing, including benzalkonium chloride (BC) and quaternary ammonium compounds (QAC; *Lemaitre et al., 1998; Aase et al., 2000*). Lemaitre et al. showed that 14 of 208 strains of *Listeria* isolated from various environments were resistant to hexamidine diisethionate, ethidium bromide, and benzalkonium chloride (*Lemaitre et al., 1998*). Multi-resistance in these strains was conferred by a transferrable genetic element originally identified in *Staphylococcus*.

1.2 Phage Therapy

1.2.1 Phage biology

Bacteriophages were first discovered in the waters of the Ganges and Jumna rivers in India by Ernest Hankin, a British microbiologist in 1896. Shortly thereafter, Frederick Twort proposed that the organisms could be viruses. Felix d'Herelle observed and isolated phages when culturing fecal samples of *Shigella* infected patients. In these cultures, d'Herelle noticed clear plaques or holes in the bacterial lawns that were the result of phage-mediated lysis of the bacterial cells

(*Sulakyelidze et al., 2001*). d'Herelle termed the viruses "bacteriophages" (bacteria eaters) and, as such, d'Herelle is largely credited with pioneering bacteriophage therapy.

1.2.2 Taxonomy

Bacteriophages are viruses that infect bacteria. Most phages studied for therapeutic uses belong to Caudovirales order, under which there are 14 families including Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Cortioviridae, Lipothrixviridae, Plasmaviridae, Rudiviridae, Fuselloviridae, Inoviridae, Microviridae, Leviviridae, and Cytoviridae (*Salmond et al., 2015*).

Viruses are often classified based on the structure of their genomes, including double-stranded (ds) DNA, ds RNA, single-stranded (ss) DNA, and ss RNA. Phages have different morphology types including tailed, polyhedral, filamentous, and pleomorphic, with lipid or lipoprotein coverings on some of the phages (*Salmond et al., 2015*). The overwhelming majority (96%) of phages have ds DNA as nucleic material and have tailed structure (*Ackermann, 2011*).

1.2.3 Phage life cycles

Phages are often further classified based on their life cycles/replication methods into lytic and lysogenic phages. The infection process of both lytic and lysogenic phages starts with the interaction between phages and specific receptors on the host cell surfaces, followed by injection of phages genome into the host bacteria (*Salmond et al., 2015*). While the bacterial cells provide a variety of interaction receptors on their surfaces, phages with different entry models can adopt different organelles to interact with these receptors (*Lindberg, 1973*). The binding of phages with their specific receptors on host bacteria enables the absorption of phages into bacteria cell. This process allows phage infection to be highly specific for certain bacteria. For Gram-positive bacteria, most phage receptors are polymers located on the cell wall, such as teichoic acids in

Bacillus and *Staphylococcus*, teichuronic acids in *Bacillus*, and C-carbohydrates in *Streptococcus* (*Lindberg, 1973*). Teichoic acids as well as its substituents N-acetylglucosamine and rhamnose were shown to act as phage receptors for two *Listeria* phages A118 and A500 by Wendlinger et al. (*Wendlinger et al., 1996*). The importance of these receptors was corroborated by Huyen et al. who identified phage resistant *L. monocytogenes* 1/2a serotype isolates with no teichoic acid-associated N-acetylglucosamine in the bacterial cell wall components (*Tran et al., 1999*).

The life cycles of lytic and lysogenic phages diverge after insertion of the phage nucleic acid into the host bacteria cell. Lytic phages are able to use the host machinery to replicate their genomes, generate protein capsids, and assemble viral progeny (*Hobbs et al., 2016*). Lytic phages then initiate a lysis event, where higher osmotic pressure inside of host cell induces the release of intracellular substances including progeny virions. While most large bacteriophages have double-stranded DNA of >20 kb, some phages have smaller size genomes and simpler structures. Large genome-sized phages generally encode more than two lysis factors for host cell wall lysis, while smaller-sized genomes generally encode only one lysis protein. In *L. monocytogenes*, host proteins' endolysin Ply118 and Ply500 facilitate lysis of the host following phage replication (*Loessner et al., 2002*). Once released, the newly formed phages can bind to and invade adjacent bacteria, repeating the lytic cycle.

Unlike lytic phages, lysogenic phage may cause lysis, but may also integrate into the host genome forming a prophage, which does not directly cause lysis or death of host bacteria (*Salmond et al., 2015*). Incorporation of the phage genome into the host genome allows for phage replication as phage DNA is propagated as the bacterial cell replicates. As a prophage, phage DNA may also be transferred horizontally to like and non-like bacteria. Prophages, however, may be excised or otherwise become lytic in response to environment signals, including stress, UV light, or chemical

insult. In each case, the excision of the prophage is not always exact and bacterial DNA may be exchanged between like and non-like bacteria through transduction. As such, lysogenic phages can influence the physiology and evolution of bacteria. In some situations, the appending of prophage genes to host bacteria genetic material could aggrandize the toxin production and virulence of bacteria, such as the acquisition of CTXΦ phage gene by *Vibrio Cholarae*, which enables the bacterium to produce cholera toxin (*Waldor et al., 1996*).

1.2.4 History of phage therapy

Phage therapy is the application of bacteriophages, whole or in part, to treat bacterial infections. d'Herelle (1919) was also among the first to use phages as an antibacterial, applying them to individuals with dysentery (*Sulakyelidze et al., 2001*). Two years later, Richard Bruynoghe published a paper referencing the use of phages to treat skin infections caused by *Staphylococcus* (*Bruynoghe et al., 1921*). Soon thereafter, pharmaceutical companies, such as the precursor of French company L'oreal in Europe and the Eli Lilly Company (Indianapolis, Ind.) in US, took interest in phage therapy, developing several products to treat various bacterial infections (*Sulakyelidze et al., 2001*). With the emergence and widespread clinical introduction of antibiotics in 1940s, western scientists largely lost interest in phage therapy, whereas the (former) Soviet Union and Eastern European countries remained active in phage research and the development of phage therapeutics (*Sulakyelidze et al., 2001*).

Coinciding with the rise of antibiotic resistance and more systemic research in phage efficacy, phage therapy has gained renewed interest. Currently there are numerous groups aiming to optimize phage-based therapies for a multitude of applications ranging from human medicine to disinfection of surfaces or medical equipment. Much of this research has taken place in agricultural or food contexts. Phages have shown efficacy in preventing foodborne pathogen colonization in

market weight pigs (*Wall et al., 2010*), poultry (*Bardina et al., 2012*), and cattle (*Rozema et al., 2009*), among other livestock species. Likewise, several groups have demonstrated that phage treatment could eliminate or limit bacterial contamination of food products, including meat (*Bigwood et al., 2008*), eggs (*Hong et al., 2016*), dairy products (*Garcia et al., 2010*), among others.

In recent years, different groups have examined the efficacy of phage treatment in reducing *Listeria* specifically. Soni et.al found that *Listeria* phage, Listex P100, was effective in reducing *L. monocytogenes* contamination on the surface of fresh catfish (*Soni et al., 2010*). In those experiments, catfish samples were inoculated with approximately a 4.3 log CFU/g mixture of two *L. monocytogenes* strains and then treated with P100. P100 mediated reductions of *L. monocytogenes* were observed at both 4 and 10ºC, over a ten-day period. Leverentz et.al (2003) applied a mixture of bacteriophages to *L. monocytogenes* contaminated melons and apples (*Leverentz et al., 2003*) and observed significant reductions in *L. monocytogenes* on phage treated melon samples, ranging 2.0 to 4.0 log CFU/mL, but not on apple samples (0.4 log CFU/mL reduction). They concluded that the poor performance of phage on apple samples might be caused by the phage reduction and inactivity from low-pH apple slices. Additionally, Susanne et al., (2008) treated ready-to-eat foods with *Listeria* phage and found that phage treatment was effective in controlling *L. monocytogenes* contamination both in liquids (such as chocolate milk; 4-5 log CFU/mL reductions compared to untreated samples) and on solid foods (e.g., turkey meat and hot dogs; 1.5-4 log CFU/g reductions compared to untreated samples). Their results also showed that compared to foods of animal origin, phage concentrations and infectivity during storage were reduced more in foods of plant origin (more than 1.0 log PFU/g), and concluded that phytochemical-based interference with phage efficacy may be possible (*Guenther et al., 2009*).

Phage "cocktails" are mixtures of different types of phages, which contribute to wider host range (*Chan et al., 2013*). In 2006, the US Food and Drug Administration approved a six-*Listeria* phage product (ListShield, Intralytix, Baltimore, MD) for use as a food additive in controlling *Listeria* contamination in ready to eat meat and poultry products. The six-phage combination was previously shown to lyse 170 *L. monocytogenes* strains (*Peek et al., 2006*), and several research groups have since conducted studies of ListShield efficacy under different conditions. Meenu et al. observed significant reductions of *L. monocytogenes* on pre-contaminated lettuce (91% reduction), cheese (82% reduction), smoked salmon (90% reduction), and frozen entrees (99% reduction) after treating food samples with ListShield by spraying (*Perera et al., 2015*). When combined with the UV-C treatment, ListShield reduced *L. monocytogenes* concentrations an additional 1.2 log units on chicken breast samples compared to samples treated with UV-C alone (*Yang et al., 2017*).

In addition to directly contaminating food products, bacteria may indirectly cross contaminate foods via contaminated processing surfaces or other materials. To date, however, there is still limited research aimed at determining whether phage-based treatments can effectively decontaminate *L. monocytogenes* on food processing surfaces. Arachchi et al. (2013) compared the efficacy of three *Listeria* phages (LiMN4L, LiMN4p, and LiMN17), both individually and in combination, in reducing adhered LM cells on stainless steel coupons (*Arachchi et al., 2013*). Application of the poly-phage treatment reduced *L. monocytogenes* concentrations on stainless steel coupons by 3.8-4.5 CFU/cm^2 compared to non-treated coupons. Similar reductions were seen with application of individual phages.

Soni et al. (2010) further evaluated the ability of bacteriophage P100 in controlling biofilms formed by *L. monocytogenes* on stainless steel surfaces (*Soni et al., 2010*). A significant reduction

of 3.5-5.4 log CFU/cm^2 *L. monocytogenes* was observed in the phage treated surfaces compared with untreated surfaces. Sadekuzzaman et al. (2016) found similar results using ListShield to treat *L. monocytogenes* biofilms on stainless steel coupon and rubber surfaces, observing 1.9 – 2.4 log CFU/cm^2 and 1.0 log CFU/cm^2 reductions, respectively, after application of the phage treatment (*Sadekuzzaman et al., 2017*).

1.2.5 Phage as co-therapy

In recent years, several groups have explored incorporating bacteriophages as a co-therapy or as part of a multi-hurdle approach to limiting bacterial contamination or infection**.** Djurkovic et al. (2005) tested the synergistic effect between Cpl-1, a bacteriophage lytic enzyme, and several antibiotics against *Streptococcus pneumoniae* cell walls (*Djurkovic et al., 2005*). Cpl-1 had an additive effect with gentamicin in reducing contamination of one *Streptococcus pneumoniae* strain. Additionally, Cp1-1 combined with penicillin was effective in limiting growth of penicillin resistant strains. Their study also suggested that Cpl-1 may work synergistically with only some antibiotics, as not all the antibiotics tested increased Cp1-1 mediated reductions in *Streptococcus pneumoniae*.

Sagar et al., (2017) showed that application of *Pseudomonas Aeruginosa* specific phage vB_PaeM_P6 in combination with sub-MIC ciprofloxacin significantly reduced antibiotic resistant *Pseudomonas Aeruginosa* biofilm formation (*Sagar et al., 2017*). Similar synergistic effects of phage and ciprofloxacin against *Pseudomonas Aeruginosa* were reported by others. Oechslin et al. (2017) found that phage therapy alone was comparable with ciprofloxacin monotherapy, reducing *Pseudomonas Aeruginosa* by 2.5 log CFU/g in 6 hours. Phage in combination with ciprofloxacin, however, reduced bacterial concentrations by > 6 log CFU/g under the same conditions (*Oechslin et al., 2017*).

In addition to combining with antibiotics, phages have shown synergism with other chemicals in controlling bacterial contamination. Roy et al. (1993) observed a significant reduction of *L. monocytogenes* on pre-contaminated stainless steel and polypropylene surfaces when the coupons were co-treated with phage and quaternary ammonium compound (QUATAL), an active ingredient widely used in disinfectant products (*Roy et al., 1993*). Importantly, the same group found that the quaternary ammonium compounds did not impair phage stability or infectivity for up to four hours.

The combination of phages or phage products with other microbe-produced antibacterial proteins has received some attention as well. Garcia et al. (2010) assessed whether phage endolysin LysH5 and nisin, a bacteriocin, could act synergistically in inhibiting *Staphylococcus*, and together the two compounds reduced the minimum inhibitory concentrations of both the endolysin and nisin (*Garcia et al., 2010*). The combination treatment effectively cleared *Staphylococcus* in spiked, pasteurized milk, and was more effective than each treatment alone. Becker et al. (2008) showed similar results combining the staphylococcal bacteriophage K endolysin (LysK) with *Staphylococcus simulans* bacteriocin Lysostaphin to treat methicillin-resistant *Staphylococcus aureus* (*Becker et al., 2008*).

Being obtained from plant material, essential oils are often antibacterial and are believed to affect the stability of bacterial cell membrane and mitochondria (*Burt 2004*). As complex natural mixtures, essential oils can contain up to 60 components (*Bakkali et al., 2007*), of which the phenolic components are considered to chiefly contribute to the antibacterial properties of essential oils (*Burt 2004*). Based on their properties, essential oils have been considered as a natural potential antibacterial additive. Friedman et al. (2002), demonstrated that cinnamon cassia, in particular, inhibited growth of *L. monocytogenes* (*Friedman et al., 2002*). Limited studies, however,

have focused on the synergetic effect between bacteriophages and essential oils. In our studies, we tested the additive effects when phages were combined with essential oil.

1.2.6 Goal of the research

We hypothesized that bacteriophages may be effective in reducing *L. monocytogenes* on food processing surfaces under more realistic conditions as previously tested by others. Thus, the goal of this study was to measure the efficacy of *Listeria* phage in reducing *L. monocytogenes* on non-food contact surfaces under scenarios that might be seen in food processing environments. Sterilized stainless steel coupons were used to mimic non-food contact surfaces. These coupons were inoculated with *L. monocytogenes* and *Listeria* phage and other factors that could interfere with the effectiveness of the phage treatment, including competing bacteria, organic matter (soil), fat, and blood, among others. Our results indicate that, in general, phage treatment significantly reduces *L. monocytogenes* concentrations. Phage treatment was not effective when used to prevent *L. monocytogenes* contamination (i.e., phage applied to coupons several hours prior to *L. monocytogenes* inoculation). Additionally, the presence of blood on the coupons interfered with phage efficacy. Taken together, our data indicate that *Listeria* phages may be effective in reducing *L. monocytogenes* contamination of non-food contact surfaces in food processing environments. Under some conditions, however, phages may need some form of protection to ensure their efficacy (e.g., to prevent desiccation and inactivation).

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CHAPTER 2. EFFICACY OF *LISTERIA* PHAGE ALONE OR AS A CO-TREATMENT IN REDUCING *LISTERIA MONOCYTOGENES* CONTAMINATION OF NON-FOOD CONTACT SURFACES

2.1 Abstract

Introduction: Listeria monocytogenes is a foodborne pathogen able to flourish in different food processing environments. Bacteriophages are viruses that target bacteria and may be effective in controlling *Listeria monocytogenes* contamination. In this study, we evaluated the efficacy of bacteriophages in controlling *Listeria monocytogenes* growth on non-food contact surfaces under different conditions.

Methods: Stainless steel coupons or sterilized bags were inoculated with *Listeria monocytogenes* (LM94 or LM-GFP) and a cocktail of six *Listeria* bacteriophages under conditions that could limit or enhance phage treatment efficacy: pre-treatment of coupons with phage, protection of phages in oil, presence of various types of organic matter, and presence of competing bacteria. In each case, coupons or bags were incubated for 18 h at 21°C and *Listeria monocytogenes* concentrations were compared between phage-treated and untreated samples. Phage efficacy on *L. monocytogenes* growth was measured by ANOVA through comparing viable bacteria cell counts between phage treated and untreated group with two sample t-tests and WMW tests based on data distributions. Differences were considered statistically significant at $P < 0.05$.

Results: Phage treatment significantly reduced *Listeria monocytogenes* on stainless steel coupons co-inoculated with bacteria and bacteriophages ($P < 0.05$). Phage treatment remained effective in the presence of soil, fat, and competing (non-*Listeria*) bacteria ($P < 0.05$). Phage treatment was not effective in reducing bacterial concentrations in the presence of blood, as well as when used

as a preventative, i.e., phages applied to coupons 4 h prior to *Listeria* challenge. Protecting phages in oil prior to application did not significantly improve efficacy of the treatment.

Significance: Listeria phages may be effective in reducing *Listeria monocytogenes* contamination of non-food contact surfaces in food processing environments. Under some conditions, however, phages may need some form of protection to ensure their efficacy (e.g., to prevent desiccation and inactivation).

2.2 Introduction

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive, rod-shaped foodborne pathogen responsible for listeriosis, a serious infection mostly occurring in young, elderly, pregnant, and immunocompromised individuals. Infections with *L. monocytogenes* affect close to 1500 individuals in the US each year and result in 200 deaths (*Scallan et al., 2011*).

L. monocytogenes survives in what are otherwise adverse environments for bacteria, including extreme pH (4.0-9.6), low water activity, high salt concentrations, and wide-ranging temperatures (1-45°C). The probability of contracting listeriosis from a food source is low when *L. monocytogenes* loads in food are below 100 CFU/g and increases with increasing infectious dose (*Thevenot et al., 2004*). Due to the severity of listeriosis, US has zero tolerance for *L. monocytogenes* in prepared food, which includes both ready-to-eat and fully cooked foods that call for reheating prior to consumption, while the *L. monocytogenes* tolerance in Europe and Canada is 100 CFU/25g in ready-to-eat foods. Recalls due to potential *L. monocytogenes* contamination average an estimated $0.16 to $0.3 million in costs (*Ivanek et al., 2004*). Additional costs associated with listeriosis, including productivity losses and hospitalizations, approach $2.6 billion in US annually (*Chen et al., 2017*).

The ability of *L. monocytogenes* to survive under extreme environmental conditions enables it to contaminate food products across different steps in food processing. During processing, proper sanitation of food processing facilities and equipment is integral in preventing cross-contamination of food products. Cleaning and disinfection of non-food contact surfaces is also critical as pathogens can transfer from non-food contact surfaces to food via workers or mechanical disruption such as high-pressure spraying, a widely used cleaning method in food industries (*Gibson et al., 1999*). Kusumaningrum et al. (2002) observed that the transfer rate of foodborne pathogens from wet sponges to the stainless-steel surfaces and subsequently to the cucumber and chicken slices could be varied from 20% to 100% (*Kusumaningrum et al., 2002*). Disinfection of non-food contact surfaces is complicated by the ability of *L. monocytogenes* to attach to polypropylene, glass, stainless steel surfaces (*Silva et al., 2008; Blackman et al., 1996*) and form sanitizer-resistant biofilms. Wet conditions and lower temperatures often found in food processing environments can further contribute to bacterial growth (*Redfern et al., 2016*).

L. monocytogenes is endemic in many food processing facilities, highlighting the need for new and effective means to eliminate *L. monocytogenes* contamination. Bacteriophages are viruses that infect and replicate in bacteria cells and, in doing so, cause the bacterial cell to lyse. Attempts to employ bacteriophages as antimicrobials date to the early 1900's (*Fruciano & Bourne, 2007*). With the widespread clinical introduction of antibiotics in 1940s, most phage research was relegated to basic phage biology and the use of phages as reagents in genetics and molecular biology (*Sulakvelidze et al., 2001*). In recent years, phage-based antibacterials have gained renewed interest, not only to treat antibiotic resistant infections, but as a general means to control the transmission of bacteria in different systems (*Clark & March, 2006*; *Endersen et al., 2014*). In 2006, the US Food and Drug Administration approved the use of a poly-phage solution as a food

additive for the control of *L. monocytogenes* in different foods. The phage product is a mixture of six *Listeria* phages previously shown to have lytic activity in 170 *L. monocytogenes* strains (*Peek et al., 2006*).

Although bacteriophages have been studied as a means of limiting pathogenic bacteria in food products, there is currently limited research aimed at assessing whether phage can decontaminate food processing surfaces. Arachchi et al. (2013) compared the efficacy of three *Listeria* phages (LiMN4L, LiMN4p, and LiMN17), both individually and in combination, in reducing adhered *L. monocytogenes* cells on stainless steel coupons (*Arachchi et al., 2013*). Application of the poly-phage treatment reduced *L. monocytogenes* concentrations on stainless steel coupons by 3.8-4.5 log CFU/cm^2 compared to non-treated coupons. Similar reductions were seen with the application of individual phages. Additionally, Soni et al. (2010) found that bacteriophage P100 effectively reduced *L. monocytogenes* biofilms on stainless steel surface by 3.5-5.4 log CFU/cm^2 compared to untreated samples (*Soni et al., 2010*). In our study, we aimed to further examine the potential of employing phage to reduce *L. monocytogenes* contamination on steel surfaces under scenarios that might be seen in a food processing facility, including the presence of competing bacteria, organic matter (soil), fat, blood, among other factors.

2.3 Materials and Methods

2.3.1 Overall study design

Stainless steel coupons (SS coupons; Research Machining Services, Purdue University, IN, USA) were used as a facsimile of non-food contact surfaces in measuring efficacy of *Listeria* phage in reducing *L. monocytogenes* contamination under eight different conditions. In each case, the challenge *L. monocytogenes* was grown to log phase (OD_{600} = 0.35-0.42; SmartSpec Plus Spectrophotometer; BIO-RAD USA, Hercules, CA) in LB-MOPS (Fisher BioReagents, Fair Lawn,

NJ) liquid medium. For experiments using LM-GFP, all media contained chloramphenicol at 20 mg/L. The log phase LM94 or LM-GFP culture was then applied to stainless steel coupons as described below.

2.3.2 Bacterial strains and *Listeria* phage used in this study

LM94 and LM-GFP were both kindly provided by Dr. Haley Oliver (Department of Food Science, Purdue University, West Lafayette, IN USA). The initial bacterial inoculum concentration used in all experiments was 10^8 CFU/mL. The phage treatment was a mixture of six *Listeria* phages of equal concentration (ListShield; Intralytix, Inc.; Baltimore, MD) suspended in an isotonic buffer.

2.3.3 Measuring efficacy of phage in reducing *L. monocytogenes* on steel surfaces

Stainless steel coupons (n = 12 per treatment, replicated three times) were co-inoculated with LM94 (5 µL at 10^8 CFU/mL) and *Listeria* phage (5 µL at 10^9 PFU/mL). Inoculated coupons were incubated for 18 h at room temperature (21℃). Following incubation, individual coupons were suspended in 1 mL phosphate buffered saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO^4, pH 7.4) and vortexed for 1 min to remove bacteria. The resulting rinsate was serially diluted in PBS and individual dilutions (100 uL) were transferred to LB-MOPS agar plates for enumeration. Post-treatment LM94 concentrations were then compared across treatment groups.

2.3.4 Measuring efficacy of phage in reducing *L. monocytogenes* on pre-contaminated surfaces

Stainless steel coupons (n = 12 per treatment, replicated three times) were inoculated with LM94 (5 µL at 10^8 CFU/mL) and the applied bacterial suspension was allowed to dry at room temperature for 2 h. Individual coupons were then inoculated with *Listeria* phage (5 µL at 10^9

PFU/mL) and the coupons were incubated for 18 h at room temperature. Post-treatment concentrations of LM94 on each coupon were enumerated as previously described.

2.3.5 Measuring the efficacy of phage in reducing *L. monocytogenes* in complex microbial environments

Waste water (raw influent) was collected from a local wastewater treatment plant (West Lafayette, IN). Stainless steel coupons (n = 9 per treatment, replicated four times) were co-inoculated with LM-GFP (5 µL at 10^8 CFU/mL), waste water (5 µL), and *Listeria* phage (5 µL at 10^9 PFU/mL). Inoculated coupons were then incubated for 18 h at room temperature. Post-treatment concentrations of LM-GFP on each coupon were enumerated as previously described.

2.3.6 Measuring the efficacy of phage in preventing *L. monocytogenes* contamination

Stainless steel coupons (n =1 per treatment, replicated three times) were inoculated with *Listeria* phage (1, 5, or 10 µL at 10^9 PFU/mL) and allowed to dry at room temperature for 4 h. The applied phage solution was allowed to dry (4 h at room temperature) and the coupons were inoculated with LM94 (5 µL at 10^8 CFU/mL) and incubated for 18 h at room temperature. Post-treatment concentrations of LM94 on each coupon were enumerated as previously described.

2.3.7 Measuring the efficacy of phage in combination with essential oil in reducing *L. monocytogenes*

Stainless steel coupons (n =3 per treatment, replicated three times) were co-inoculated with *Listeria* phage (5 µL at 10^9 PFU/mL) and cinnamon cassia oil (5 µL at 5%; 100% pure cinnamon cassia oil; NOW Foods, Bloomingdale, IL) and allowed to dry for 4 h at room temperature. Individual coupons were then inoculated with LM94 (5 µL at 10^8 CFU/mL) and the coupons were incubated for 18 h at room temperature. Coupons with only *Listeria* phage or only cinnamon cassia oil were included as controls (all the coupons were allowed to dry at room temperature for 4 h

prior to application of LM94). Post-treatment concentrations of LM94 on each coupon were enumerated as previously described.

2.3.8 Measuring the efficacy of phage in reducing *L. monocytogenes* against organic matter backgrounds

First, *L. monocytogenes* was co-inoculated on stainless steel coupons with blood. Fresh cattle blood sample was kindly provided by Purdue University meat laboratory. Blood (5 µL, original sample) was added on stainless steel coupons (n = 3 per treatment, replicated three times) and dried for 30 minutes. LM-GFP (5 µL at 10^8 CFU/mL) and *Listeria* phage (5 µL at 10^9 PFU/mL) were inoculated on the coupons. Inoculated coupons were then incubated for 18 h at room temperature. Post-treatment concentrations of LM-GFP on each coupon were enumerated as previously described. Experiments were repeated first replacing blood with animal fat (n = 12 per treatment, replicated three times).

In separate experiments, sterilized plastic sample bags (n = 12 per treatment, replicated three times) were filled with organic soil (10 g; Nature's Care Organic & Natural Potting Mix; The Scotts Miracle-Gro Company, Marysville, OH), and sterilized water (5 mL). The contents of the bag were mixed by squeezing the bag repeatedly for 1 min. The soil/water mixture was then co-inoculated with LM-GFP (1 mL at 10^8 CFU/mL) and *Listeria* phage (1 mL at 10^9 PFU/mL). The contents of the bag were mixed as previously described. Inoculated bags were incubated for 18 h at room temperature. PBS (50 mL) was added into each bag and the contents in the bags were mixed as previously described. Post-treatment concentrations of LM-GFP in each bag were enumerated and compared across treatment groups.

2.3.9 Statistical analysis

Data were analyzed using SAS statistical software (version 9.4; SAS, Cary, NC, USA). Bacterial concentrations were transformed to log units prior to analysis. Phage efficacy on *L. monocytogenes* growth was measured by ANOVA through comparing viable bacteria cell counts between phage treated and untreated group with two sample t-tests and WMW tests based on data distributions. For the control (samples of only *L. monocytogenes*) and experiment (samples of *L. monocytogenes* at each condition together with *Listeria* phage) groups in which data did not follow the normal distribution, Wilcoxon rank-sum tests were conducted to compare differences in bacterial concentrations between control and experiment groups. Differences were considered statistically significant at $p < 0.05$.

2.4 Results

2.4.1 Efficacy of phage in reducing *L. monocytogenes* on steel surfaces

Preliminary experiments were designed to measure the efficacy of *Listeria* phage in reducing *L. monocytogenes* concentrations on stainless steel surfaces under non-complex conditions. Stainless steel coupons were co-inoculated with *Listeria* phage and LM94 and *L. monocytogenes* concentrations were measured after overnight incubation at room temperature. Final *L. monocytogenes* concentrations were significantly lower ($P < 0.0001$) on coupons co-inoculated with *L. monocytogenes* and *Listeria* phage (1.4 log CFU/mL) compared to final *L. monocytogenes* concentrations on coupons inoculated with only *L. monocytogenes* (untreated control; 5.3 log CFU/mL; Figure 2-1).

To test whether *Listeria* phage could reduce *L. monocytogenes* concentrations on previously contaminated steel surfaces, stainless steel coupons were inoculated with *L. monocytogenes* and allowed to dry for 2 h at room temperature before being treated with *Listeria* phage. After an 18 h

incubation at room temperature, final *L. monocytogenes* concentrations on coupons treated with *Listeria* phage (3.4 log CFU/mL) were significantly lower ($P < 0.0001$) than *L. monocytogenes* concentrations on coupons inoculated with only *L. monocytogenes* (untreated control; 5.0 log CFU/mL; Figure 2-2).

2.4.2 Efficacy of phage in reducing *L. monocytogenes* on steel surfaces in presence of potential inhibitors

We conducted a series of experiments measuring the efficacy of phage in reducing *L. monocytogenes* contamination in the presence of various potential inhibitors that might be found in food processing environments. In the first of a series of experiments, stainless steel coupons were inoculated with wastewater prior to inoculation with *L. monocytogenes* or *L. monocytogenes* and *Listeria* phage. Final *L. monocytogenes* concentrations on *Listeria* phage/wastewater treated coupons (below detection limit) were lower than *L. monocytogenes* concentrations on coupons inoculated with wastewater, but not treated with phage (untreated control; 5.1 log CFU/mL; Figure 2-3).

Similarly, phage efficacy in reducing *L. monocytogenes* in the presence of soil was tested by inoculating soil with *L. monocytogenes* or *L. monocytogenes* and *Listeria* phage in sterilized plastic sample bags. Bacterial concentrations were measured in each sample after overnight incubation. *L. monocytogenes* concentrations were significantly lower ($P < 0.0001$) in phage treated soil (5.9 log CFU/mL) compared to *L. monocytogenes* concentrations in untreated soil (7.1 log CFU/mL; Figure 2-4).

These experiments were repeated replacing soil with animal fat; stainless steel surfaces were co-inoculated with LM-GFP, *Listeria* phage, and fat. Bacterial concentrations were measured on each coupon following an overnight incubation. *L. monocytogenes* concentrations were

significantly lower ($P < 0.0001$) on phage treated coupons (1.9 log CFU/mL) compared to *L. monocytogenes* concentrations on untreated coupons (3.8 log CFU/mL; Figure 2-5).

Likewise, stainless steel surfaces were co-inoculated with LM-GFP, *Listeria* phage, and blood. Bacterial concentrations were measured on each coupon following an overnight incubation. There were no differences ($P = 0.0981$) in *L. monocytogenes* concentrations between phage treated coupons (3.6 log CFU/mL) and untreated coupons (4.0 log CFU/mL; Figure 2-6).

2.4.3 Efficacy of phage in preventing *L. monocytogenes* contamination

We were interested in determining the efficacy of *Listeria* phage in preventing *L. monocytogenes* contamination or colonization on steel surfaces. Coupons were inoculated with *Listeria* phage and incubated at room temperature for 4 h to allow the coupons to dry. Coupons were then inoculated with LM94 and bacterial concentrations were measured on each coupon following an overnight incubation. No significant differences in final *L. monocytogenes* concentrations were detected between phage untreated coupons (5.3 log CFU/mL) and coupons pre-inoculated with phage at any amount tested (1×10^6 PFU phage application: 5.5 log CFU/mL; 5×10^6 PFU phage application: 5.2 log CFU/mL; 1×10^7 PFU phage application: 4.8 log CFU/mL; Figure 2-7).

2.4.4 Efficacy of phage in combination with essential oil in reducing *L. monocytogenes*

In effort to provide some means of protection to phage and allow the treatment to work as a preventative, we combined *Listeria* phage with an essential oil. Stainless steel surfaces were co-inoculated with *Listeria* phage and essential oil and incubated at room temperature to allow the coupons to dry. At 4 h, each coupon was inoculated with LM94 and bacterial concentrations were measured on each coupon following an overnight incubation. The final *L. monocytogenes* concentrations on coupons inoculated with *Listeria* phage and essential oil (undetected; $P = 0.0031$)

or essential oil only (1.1 log CFU/mL; P = 0.0061) were significantly lower than *L. monocytogenes* concentrations on untreated coupons (4.7 log CFU/mL). Again, there were no significant differences in *L. monocytogenes* concentrations between untreated coupons (4.7 log CFU/mL) and coupons pre-inoculated with phage and allowed to dry prior to inoculation with LM94 (5.6 log CFU/mL; Fig. 8).

2.5 Discussion

In the present study, we assessed the capacity of a *Listeria* phage treatment to reduce *L. monocytogenes* contamination of non-food contact surfaces. We used stainless steel coupons as a facsimile of stainless steel surfaces often found in food processing facilities. Our initial results showed that *L. monocytogenes* concentrations on stainless steel surfaces were significantly lower when the surfaces were treated with *Listeria* phage. To create more production-like scenarios, we repeated those experiments adding different factors that: 1) might be found in processing; and 2) could limit the efficacy of the phage treatment. These factors included adding unrelated or background bacteria, organic material, fat, and blood to the coupon prior to phage treatment. Using this model, we found that, in most cases, phage treatment of steel surfaces remained effective at reducing *L. monocytogenes* contamination when the phage treatment was applied to surfaces contaminated with *L. monocytogenes* prior to phage application. Pre-treating steel surfaces with phage, however, did not effectively prevent *L. monocytogenes* contamination and there was no additive effect with the addition of essential oil as the oil itself was antibacterial.

Several groups have shown that phage-based interventions may be effective in controlling *L. monocytogenes* on non-food contact surfaces. Soni et al., (2010) reported that *Listeria* phage P100 significantly reduced *L. monocytogenes* on steel surfaces by 3.5 to 5.4 log CFU/cm^2 compared with untreated surfaces (*Soni et al., 2010*). In contrast to our study, Soni et al. applied phage to *L.*

monocytogenes biofilms. Biofilms are intense bacteria films surrounded by extracellular polymerized substances produced by *L. monocytogenes* and other bacteria. In our study, because of the timing of both bacteria and phage application to the steel coupons, it is not likely that any biofilm could form and our experiments are more likely measuring phage impact on planktonic cell populations. In such a scenario, phage treatment significantly reduced *L. monocytogenes* concentrations.

Additionally, phage treatment reduced *L. monocytogenes* concentrations on steel surfaces when applied two hours after the coupons were inoculated with *L. monocytogenes*. However, timing of phage application appeared to affect efficacy of the treatment in limiting *L. monocytogenes* contamination as efficacy was highest when phage was applied closer to or at the time of bacterial contamination/inoculation. Delayed application of *Listeria* phage could allow further replication of *L. monocytogenes* on coupon surfaces. With increased bacterial concentrations, the multiplicity of infection (MOI; ratio of phages particles to bacterial cells) would be lower than in co-inoculation experiments, which could influence the final bacterial concentrations. Nevertheless, coupled with the results of Soni et al., our results indicate that phage treatment could reduce *L. monocytogenes* at initial, early, and late (biofilm) stages of infection.

Food processing is normally a microbiologically complex environment with various microbial communities in different niches (e.g., drains, sinks, etc.). We sought to determine whether phage treatment remained effective on surfaces contaminated with various other--perhaps competing--bacteria, similar to what might be encountered in a food processing facility. Here, phage treatment significantly reduced *L. monocytogenes* contamination on steel surfaces also contaminated with human waste water. Historically, bacteriophages were originally isolated from sewage water, such as the isolation of Nocardiophages from wastewater sample (*Khairnar et al.,*

2014). Our results indicate that in this complex microbial environment, phages are highly host specific and are able to remain effective against their host in the presence of unrelated bacteria because of this high specificity.

Similar results (efficacy of phage treatment in microbiologically complex environments) were seen in our laboratory examining the impact of phage treatment on surrounding bacteria in the pig gut. Hong et al. (2016) applied a 10-*Salmonella* phage cocktail by oral gavage to pigs and found no global changes in the pig gut microbiome compared to untreated pigs. (*Hong et al., 2016a*). Hong concluded that the high specificity of the phages relegated them to only affecting their target bacterial population (i.e., *Salmonella*), which was not present in the pre-screened pigs.

Interestingly, in some cases, phage treatment appears to be more effective in reducing targeted bacteria in microbiologically complex environments versus environments with little to no unrelated bacteria. Previous research by Hong et al. (2016) showed that *Salmonella* phages were more effective in controlling *Salmonella* Enteritidis in ground meat versus liquid eggs (*Hong et al., 2016b*). Ground meat contains various microbial communities, while liquid eggs are functionally sterile. In the same study, phage resistance developed at much higher frequencies in phage treated eggs versus phage treated ground meat. Similar to antibiotic resistance, phage resistance often develops at the expense of other bacterial functions, either by mutating multi-purpose phage receptor proteins or requiring production of new proteins (*Hyman et al., 2010*). As such, Hong et al. concluded that the more complex environment of the ground meat could provide a more competitive environment, effectively limiting the growth of phage resistant bacteria. Thus, phage treatment in ground pork remained effective. A similar situation could be found when applying phage treatment against the background of wastewater where the competition for

resources tempered growth of any phage resistant *L. monocytogenes*. We, however, did not measure resistance development. As such, this conclusion remains speculation until further study.

We hypothesized that pre-application of *Listeria* phage could prevent the establishment of *L. monocytogenes* on steel surfaces. Similar preventative capacity of phages has been seen in live animals where feeding phages to pigs prior to exposure of *Salmonella* resulted in significant reductions in *Salmonella* colonization in the pig intestine (*Wall et al., 2010; Saez et al., 2011*). Similar research focused on the ability of phage to prevent contamination is still limited. Our results, however, indicated that pre-treatment of steel surfaces with phage did not effectively reduce *L. monocytogenes* contamination; thus, the preventative capacity of phage treatment is not monolithic and the type of bacteria and phage being tested coupled with the environment may influence results.

Other groups have shown that phage activity is easily influenced by environmental conditions, such as pH, water activity, or temperature (*Jończyk E. et al., 2011*). Yang et al. in 2017 demonstrated that *Listeria* phage activity under UV-C light was enhanced and thus led to greater reductions in *L. monocytogenes* contamination (*Yang et al., 2017*). In our case, it may be that the activity of phages was reduced due to desiccation as the phages were applied in a liquid solution and the coupons were allowed to dry for approximately 4 h. Walter (2003) tested the efficacy and durability of mixed *Bacillus anthracis* bacteriophages against *Bacillus anthracis* bacteria under different conditions and found that desiccation of the phage significantly reduced phage infectivity (*Walter, 2003*).

We designed a series of experiments to determine if protecting the phage in some manner could overcome the potentially inhibitory effect of desiccation. To do so, we pre-treated steel surfaces with a suspension of phage and essential oil (cinnamon cassia). Essential oils themselves

are often antibacterial and are believed to affect the stability of bacterial cell membranes. Friedman et al. (2002), demonstrated that cinnamon cassia, in particular, inhibited growth of *L. monocytogenes* (*Friedman et al., 2002*). In our studies, there were additive effects when phages were combined with essential oil. However, in the model we used, the essential oil was highly effective itself in reducing *L. monocytogenes* concentrations, and any additive effect could have been masked by the efficacy of the essential oil. It may be that more optimal combinations prove more effective, which warrants further study.

Besides unrelated bacteria, surfaces in food processing facilities may also be contaminated with various other types of organic matter, which could impair phage efficacy both physically or metabolically. In our study, *Listeria* phage effectively reduced *L. monocytogenes* contamination on steel surfaces in the presence of either fat or soil. In the case of fat, it appeared that the fat itself may have been somewhat antibacterial as final concentrations on non-treated steel surfaces containing fat (controls) were lower than what was seen in the controls of our other experiments. We could not, however, statistically compare results across experiments. Fatty acids may have antimicrobial activity based on their amphipathic property (*Rangel, 2017*) where fatty acids can bind to bacteria with their hydrophobic (nonpolar) sides, dissolving the bacteria cell walls. Thus, in our experiment, fat did not interfere with phage efficacy, but could have an additive effect.

The opposite was seen in experiments where steel surfaces also contaminated with soil. In these cases, *L. monocytogenes* concentrations on untreated coupons (controls) were generally higher than what was seen in controls of our other experiments. Again, we were unable to compare results across experiments. Botzlera et al. (1974) reported soil can act as a reservoir of *L. monocytogenes* (*Botzlera et al., 1974*), serving as medium for bacterial growth as soil, in general, contains organic matter, water, and minerals. The efficacy of phage treatment, namely lytic

capacity, can depend on the multiplicity of infection (MOI) or the ratio of phage particles to targeted bacterial cells in a solution (*Payne et al., 2003*). It is possible that the soil enhanced growth of *L. monocytogenes* resulting in a lower MOI and reduced bacterial lysis.

Finally, phage treatment did not reduce *L. monocytogenes* concentrations when coupons were also contaminated with blood. Blood contains numerous factors designed to identify and clear foreign bodies like both phage and bacteria. It is possible that both *L. monocytogenes* and phage could be targeted and lysed by immune cell products (e.g., antibodies, complement factors, etc.), reducing the concentration of *L. monocytogenes* in both phage treated and non-treated coupons.

We conclude that phage-based treatments have potential as a means of controlling *L. monocytogenes* on non-food contact surfaces. Under some conditions, however, phages may need some forms of protection to ensure their efficacy (e.g., to prevent desiccation and inactivation). Admittedly, there are limitations exist in our research. Our study design may have ignored some generally existing environmental factors in food processing such as humidity. With the request of frequent water cleaning, food processing environment are often quite humid. As high humidity is considered as a supportive factor for *L. monocytogenes* growth on stainless steel surfaces, *Listeria* phage efficacy may vary if humidity factor was considered. We also did not do multi-factorial testing (e.g., presence of both competing bacteria and fat, etc.).

Future research could focus on mimicking more scenarios in food processing, such as the efficacy of *Listeria* phage against multi-biofilms formed by *L. monocytogenes* and other common food pathogens on non-food contact surfaces. While our attempts to use cinnamon cassia oil as a co-therapy were not successful, it may be that other compounds could be used together with phage to enhance the antibacterial effect of each treatment.

2.6 Acknowledgements

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2.7 References

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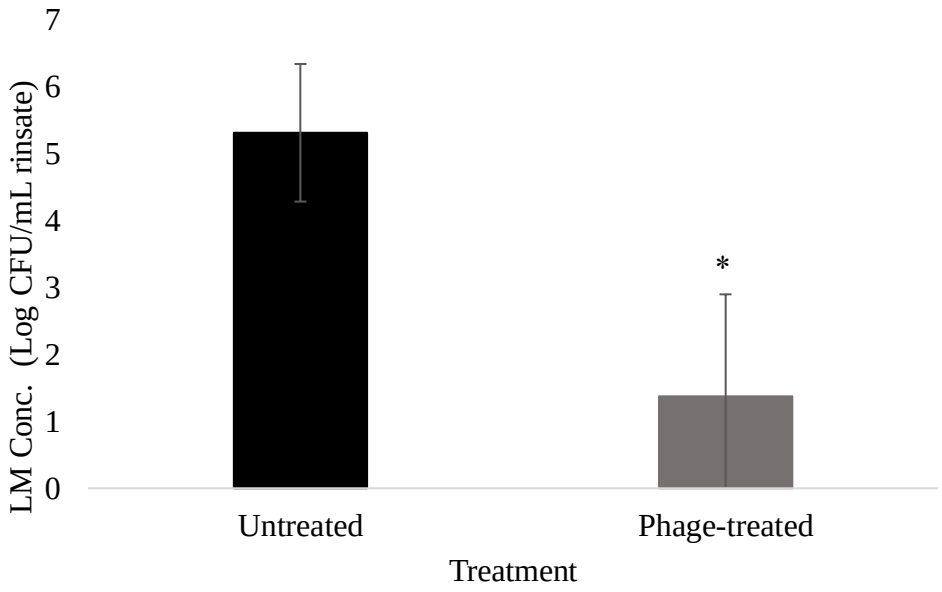

Figure 2-1 *L. monocytogenes* concentrations on stainless steel coupons co-inoculated with *L. monocytogenes* and *Listeria* phage**.**

Concentrations measured on inoculated coupons (n = 36) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

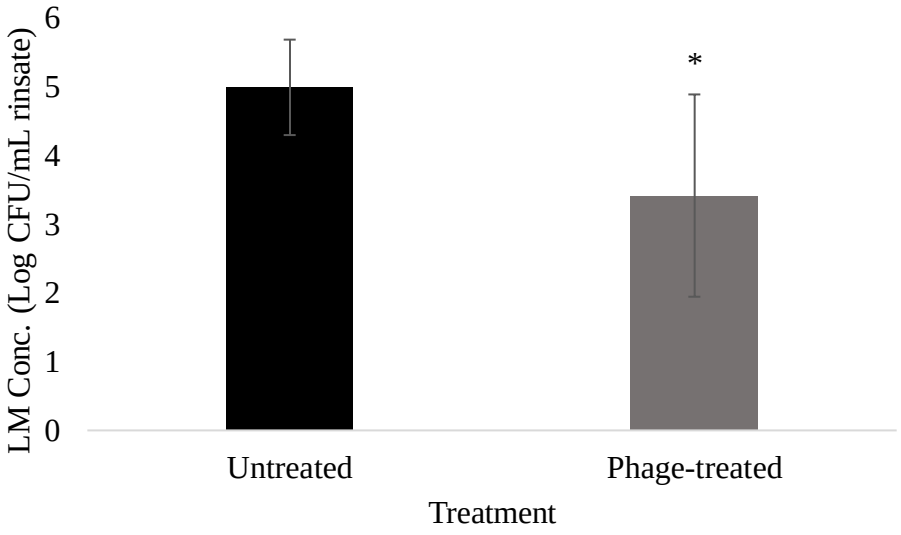

Figure 2-2 *L. monocytogenes* concentrations on stainless steel coupons inoculated with *Listeria* phage applied two hours after *L. monocytogenes* inoculation.

L. monocytogenes concentrations measured on inoculated coupons (n = 36) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

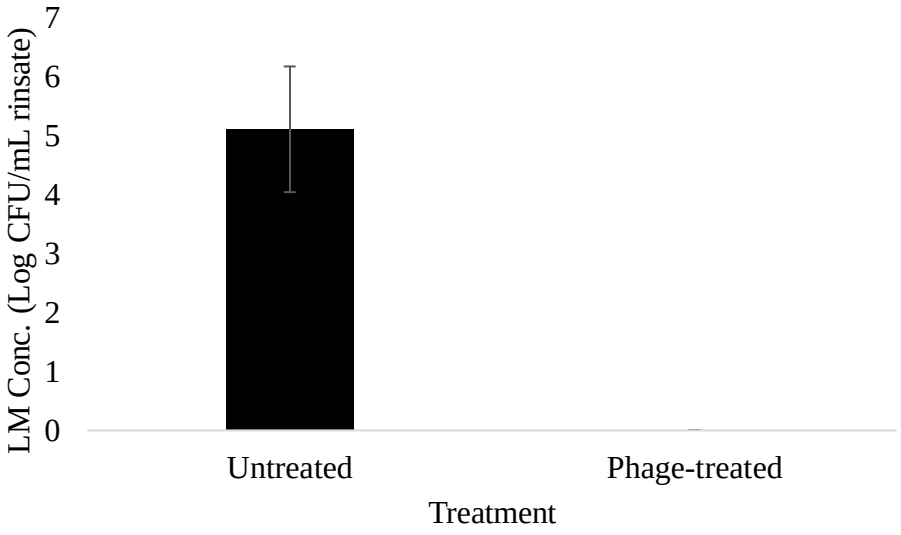

Figure 2-3 *L. monocytogenes* concentrations on stainless steel coupons inoculated with *Listeria* phage, *L. monocytogenes*, and waste water.

L. monocytogenes concentrations measured on inoculated coupons (n = 36) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

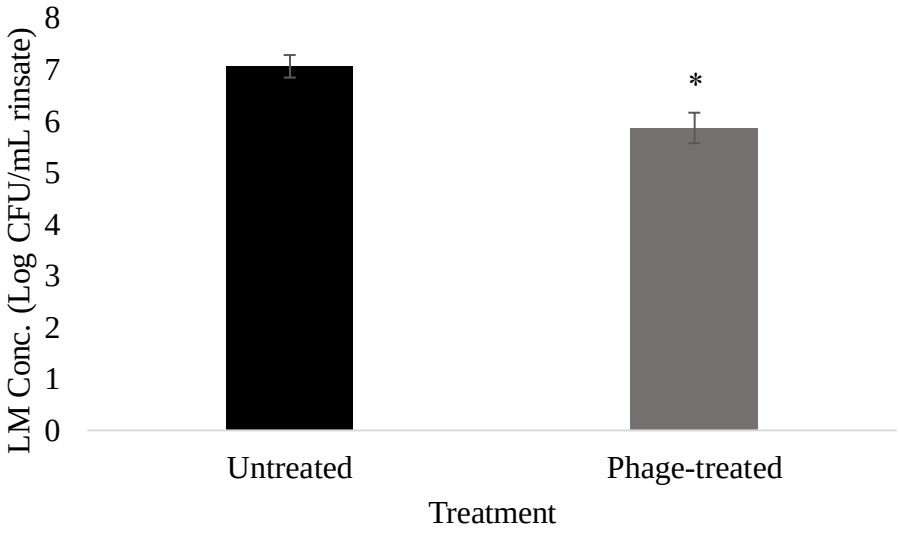

Figure 2-4 *L. monocytogenes* concentrations in soil inoculated with *Listeria* phage and *L. monocytogenes*.

L. monocytogenes concentrations measured in inoculated sample bags (n = 36) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

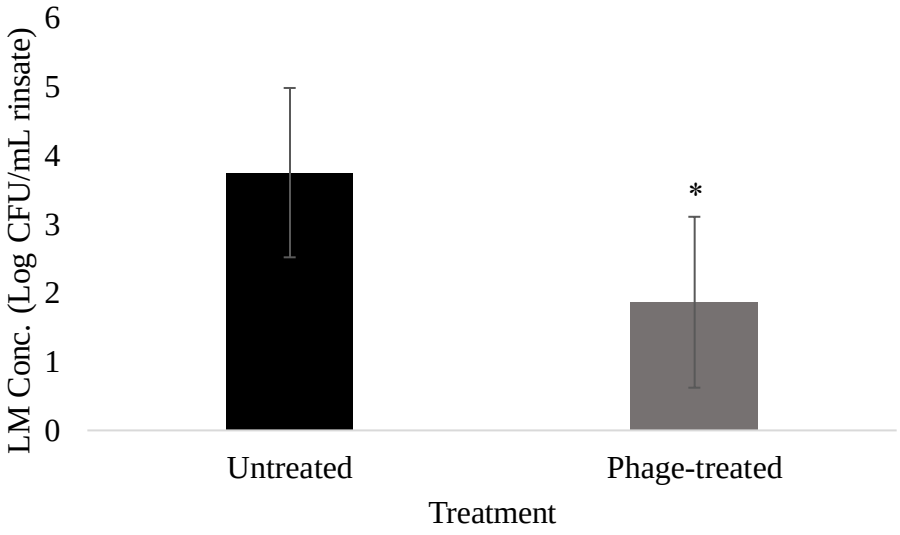

Figure 2-5 *L. monocytogenes* concentrations on stainless steel coupons inoculated with *Listeria* phage, *L. monocytogenes*, and animal fat.

L. monocytogenes concentrations measured on inoculated coupons (n = 36) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

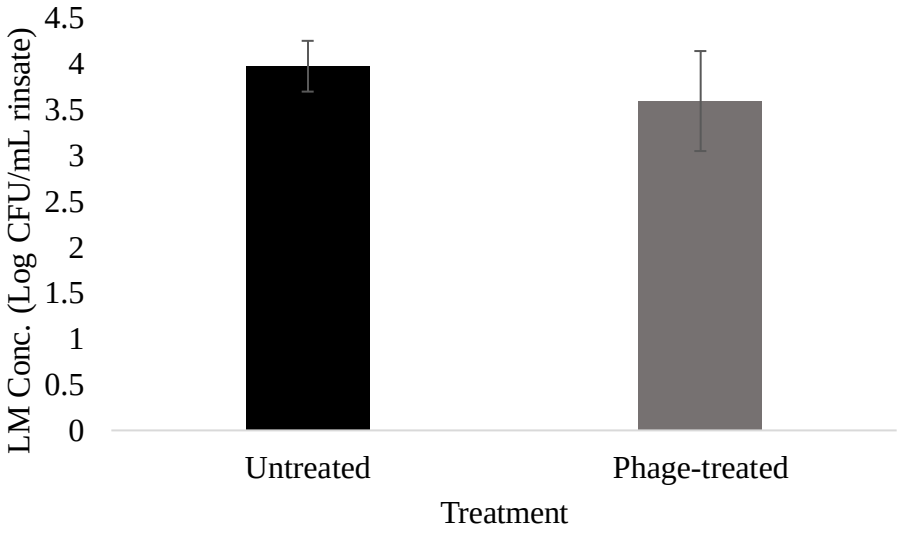

Figure 2-6 *L. monocytogenes* concentrations on stainless steel coupons inoculated with *Listeria* phage, *L. monocytogenes*, and blood.

L. monocytogenes concentrations measured on inoculated coupons (n = 9) after 18 hours incubation at 22ºC; * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

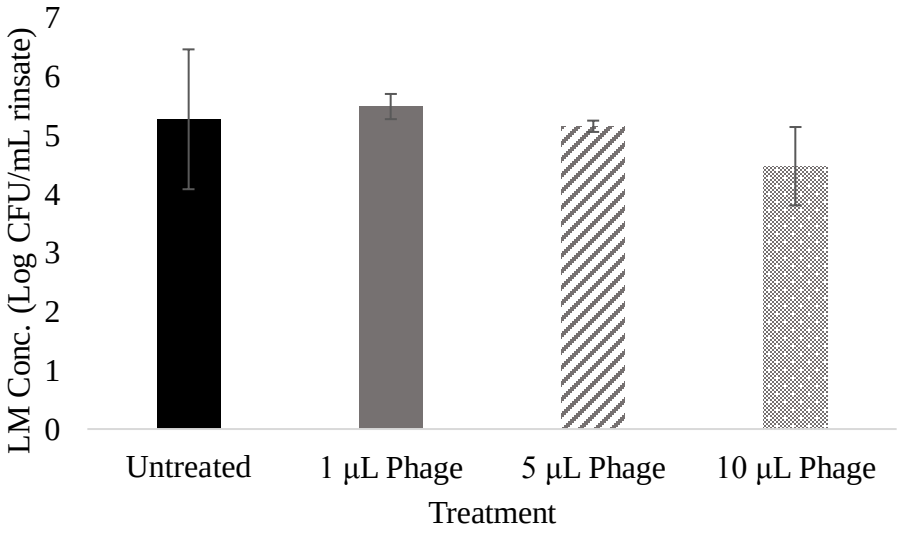

Figure 2-7 *L. monocytogenes* concentrations on stainless steel coupons pretreated with increasing amounts of phage.

L. monocytogenes concentrations measured after inoculation with *L. monocytogenes* (untreated), *L. monocytogenes* with 1 µL phage (10^9 CFU/mL), *L. monocytogenes* with 5 µL phage, or *L. monocytogenes* with 10 µL phage. LM = *L. monocytogenes*.

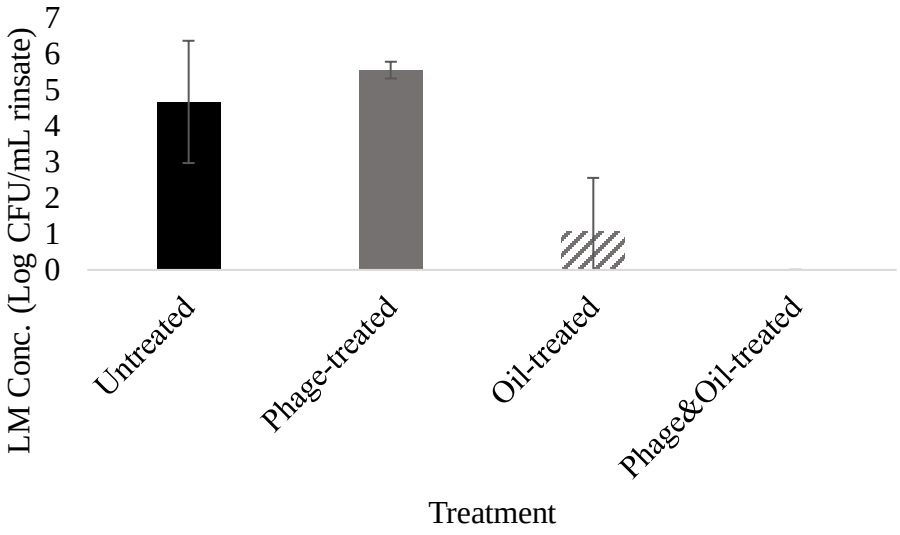

Figure 2-8 *L. monocytogenes* concentrations on stainless steel coupons inoculated with *Listeria* phage, *L. monocytogenes*, and/or volumes of essential oil.

Coupons were inoculated with phage (with or without oil) and allowed to dry for 4 hours at room temperature. Coupons were then inoculated with *L. monocytogenes* and *L. monocytogenes* concentrations were measured after incubation overnight at 22ºC. * = statistically significantly differences at $P < 0.05$. LM = *L. monocytogenes*.

CHAPTER 3. CONCLUSION

3.1 Conclusions of Research

The results presented in this thesis are part of a larger study we conducted to determine whether bacteriophages could be utilized to reduce *Listeria* spp. contamination in retail food processing establishments (e.g., delis, restaurants, etc.). In our previous studies, we measured the lytic spectrum of the six *Listeria* phages described in these studies against a library of 475 *Listeria* spp. isolates. Those results were previously reported in the thesis of Ajita Sundarram, Department of Food Science, Purdue University, as *Lytic capacity and spectrum of* Listeria *phage against* Listeria spp. *With varied genotypic and phenotypic characteristics* in August, 2017. Briefly, we found that 47.8% *Listeria* spp. isolates were moderately or highly susceptible to lysis by phage cocktail. Additionally, *Listeria* spp. isolates which were previously found to have a persistent phenotype were less susceptible ($P > 0.05$) to phage lysis compared to the non-persistent phenotype isolates. Additionally, lysis was temperature dependent with lytic capacity of the phages decreasing as temperature of incubation increased ($P < 0.05$).

Based on its capacity to lyse a wide range of *Listeria* spp. isolates, we hypothesized that the phage cocktail could be an effective tool in reducing *L. monocytogenes* in food processing. This led us to the subsequent experiments described here measuring the efficacy of *Listeria* phage in reducing *L. monocytogenes* on surfaces typically found in food processing environments.

Our results showed that phage treatment significantly reduced *Listeria monocytogenes* on stainless steel coupons co-inoculated with bacteria and bacteriophages ($P < 0.05$). Phage treatment remained effective in the presence of soil, fat, and competing (non-*Listeria*) bacteria ($P < 0.05$). Phage treatment was not effective in reducing bacterial concentrations in the presence of blood, as well as when used as a preventative, i.e., phages applied to coupons 4 h prior to *Listeria* challenge.

Protecting phages in oil prior to application did not significantly improve efficacy of the treatment. We concluded that phage-based treatments have potential as a means of controlling *L. monocytogenes* on non-food contact surfaces. Under some conditions, however, phages may need some forms of protection to ensure their efficacy (e.g., to prevent desiccation and inactivation).

There were, however, some limitations in our research. Our study design may have ignored some generally existing environmental factors in food processing such as humidity. In some food processing environments, humidity may be high, especially with repeated cleaning procedures, and humidity may impact growth of both *L. monocytogenes (Redfern et al., 2016)* and the subsequent lysis by phage. We also did not do multi-factorial testing (e.g., presence of both competing bacteria and fat, etc.).

Future research could focus on mimicking more scenarios in food processing, such as the efficacy of *Listeria* phage against multi-biofilms formed by *L. monocytogenes* and other common food pathogens on non-food contact surfaces. While our attempts to use cinnamon cassia oil as a co-therapy were not successful, it may be that other compounds could be used together with phage to enhance the antibacterial effect of each treatment.

3.2 Reference

Redfern, J. and J. Verran. 2016. Effect of Humidity and Temperature on the Survival of Listeria Monocytogenes on Surfaces. *Letters in Applied Microbiology*. DOI:10.1111/lam.12714