Application of Bacteriophage in Food Manufacturing Facilities for the Control of *Listeria monocytogenes* and *Listeria* spp.

Robert G. Reinhard

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Joseph D. Eifert[a], Committee Chair

Renee R. Boyer[a]

Susan E. Duncan[a]

R. Hartford Bailey[b]

[a] Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061, USA

[b] Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762-6100, USA

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Application of Bacteriophage in Food Manufacturing Facilities for the Control of *Listeria monocytogenes* and *Listeria* spp.

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ABSTRACT

The purpose of this research was to determine if bacteriophage (phage) could be used to treat and reduce the incidence of *Listeria* in food manufacturing facilities, and thereby reduce the risk of food products being cross-contaminated with *Listeria monocytogenes*. *Listeria* incidence in food manufacturing ready-to-eat environments was surveyed at 31 ready-to-eat (RTE) food plants. A total 4,829 samples were collected from all locations. Nine (29%) facilities had zero samples positive for *Listeria* spp., whereas 22 (71%) had one or more samples positive. The total incidence of *Listeria* spp. in all RTE food plants was 4.5%.

The effectiveness of phage against *Listeria* was determined when applied to stainless steel, polyurethane thermoplastic, and epoxy. Each material was inoculated with a cocktail containing *L. monocytogenes* and *L. innocua* (4 to 5-$\log_{10}$ CFU/cm^2) and treated with two different concentrations of phage ($2x10^7$ and $1x10^8$ PFU/cm^2). Treated samples were held at 4 or 20°C for 1 and 3h. After treatment with phage, *Listeria* reductions ranged from 1.27–3.33 $\log_{10}$ CFU/cm^2 on stainless steel, 1.17–2.76 $\log_{10}$ CFU/cm^2 on polyurethane thermoplastic, and 1.19–1.76 $\log_{10}$ CFU/cm^2 on epoxy. *Listeria* reduction occurred on all materials tested, under all conditions. Higher phage concentration, longer time, and higher environmental temperatures led to significantly ($P \leq 0.05$) greater reduction of *Listeria* on stainless-steel and polyurethane thermoplastic.

The effectiveness of a phage against *Listeria* spp. was evaluated in two food manufacturing facilities, operating at either 4°C or 20°C. First, a moderate application of a $2x10^7$ PFU/mL phage was applied once per day over three days and samples were collected and analysed for *Listeria* at 0, 24, 48 and 72 h. This phage treatment led to a decrease in the incidence of *Listeria* by 67%. A second application method was studied with phage being applied in the food manufacturing environment in an intensified manner (3 times in 18 hours) at a higher concentration of phage ($1x10^8$ pfu/mL). This intensified application led to a 32% overall reduction in the incidence of *Listeria* in the production environment. Applications of *Listeria* specific phage can be an additional intervention strategy for controlling pathogenic *Listeria* organisms in food production facilities.

Application of Bacteriophage in Food Manufacturing Facilities for the Control of *Listeria monocytogenes* and *Listeria* spp.

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GENERAL AUDIENCE ABSTRACT

Listeriosis is a serious illness caused by the bacterium *Listeria monocytogenes*. Annually in the United States it is estimated that 95.7% of all listeriosis illnesses are caused by the consumption of contaminated food, and it is generally recognized that *L. monocytogenes* is caused by cross contamination of ready-to-eat foods from an environmental source. The purpose of this research was to determine if food manufacturing facilities could use bacteriophage (phage) to treat and reduce the incidence of *Listeria* in food manufacturing plant, and thereby reduce the risk of food products being cross-contaminated with *L. monocytogenes*.

The incident rate of *Listeria* was surveyed by collecting 4,829 samples from 31 ready-to-eat (RTE) food plants across the United States. Nine (29%) facilities had zero samples positive for *Listeria* spp., whereas 22 (71%) plants had one or more samples positive. The total incidence of *Listeria* spp. in all RTE food plant samples was 4.5%.

Second, research was completed to determine if phage at different levels reduced *Listeria* on three common food manufacturing plant materials (stainless steel, polyurethane belting and epoxy flooring). After *Listeria* was attached to each material *(*4 to 5-$\log_{10}$ CFU/cm^2), they were treated with two different levels of phage ($2x10^7$ and $1x10^8$ PFU/cm^2) at two temperatures (4 or 20°C) for 1 and 3 hours. After treatment with

phage, *Listeria* reduction of 93.2 to 99.9% occurred on all materials tested, under all conditions.

Finally, two different methods of applying phage (moderate and intensified) in a food manufacturing plant was studied. The moderate application was a single treatment with lower concentration of phage ($2x10^7$ PFU/mL) once per day for three days, while the intensified treatment was the application of high phage concentrations ($1x10^8$ pfu/mL) three different times, all in a single day. Both application methods reduced the incidence of *Listeria* in the food manufacturing plant. The total reduction across all trials was 67% using the moderate application method and 32% using the intensified application of phage.

DEDICATION

I dedicate my dissertation work to all the teachers, coaches, mentors and professors who have ever challenged my peers and I to continuously and relentlessly pursue knowledge and learning. I have been fortunate, and I am grateful for the circumstances that allowed me to pursue a Doctor of Philosophy in Food Science several decades after first leaving college. While I will never reach a place where I have learned enough and where all the questions have answers, I will continue indefinitely in the pursuit of knowledge, understanding and wisdom.

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I. Introduction

Listeriosis is a serious illness caused by the bacteria *Listeria monocytogenes* and is of particular concern to susceptible populations - pregnant women, neonates, elderly adults, and individuals with a weakened immune system. Annually in the United States it is estimated that 95.7% of all human listeriosis illnesses are caused by the consumption of contaminated food, for a total of 1591 foodborne illnesses with a hospitalization rate at 94% and a death rate of 15.9% (Scallan et al., 2011). Since listeriosis is usually caused by the consumption of food contaminated with *L. monocytogenes*, extensive efforts are made to control the organism in food manufacturing.

Food manufacturing companies spend millions of dollars annually to control the potential contamination of pathogenic and non-pathogenic bacteria on food and surfaces within a manufacturing facility that contact food products during production. Generally, manufacturing firms use Good Manufacturing Practices (GMP) and Sanitation Standard Operating Procedures (SSOP), with verification of performance through microbiological testing within the manufacturing facility to assure the control of microbiological hazards. Companies often have specific programs to verify the control of *L. monocytogenes* in these areas of the manufacturing plant. These programs include robust environmental sampling for the presence of *Listeria* spp. (a potential indicator of *L. monocytogenes*) in the ready-to-eat (RTE) manufacturing areas of a plant, focusing on non-contact surfaces and sometimes including the verification sampling of food contact surfaces.

Persistence of *L. monocytogenes* in the RTE processing environment is the main cause of concern with regard to post-process contamination of RTE food products. Food safety industry leaders, Tompkin (2002) and Butts (2003), have outlined effective strategies for controlling *L. monocytogenes* and stress the importance of finding and eradicating potential indicators such as *Listeria* spp. in the RTE processing environment. By using the tactics outlined in their approaches, it is possible to identify and remove growth niches from food contact and non-contact surfaces.

There are also regulatory requirements for monitoring *L. monocytogenes* controls in a food plant. Since 2004 manufacturing facilities regulated by USDA FSIS have been required by regulation (9 CFR 430) to have programs in place that control the potential contamination of *L. monocytogenes* on RTE food. Additionally, FDA regulated facilities have seen recent changes to regulations. In 2012, U.S. Congress passed legislation and the president signed into law the Food Safety Modernization Act. This law has been followed by the publication of numerous new regulatory requirements for those facilities operating under FDA jurisdiction. Included in this sweeping regulatory change are requirements to monitor, through product and environmental testing for microbiological hazards, the production of safe foods.

The purpose of an environmental microbiological sampling program is to identify and reduce potential hazards before product becomes contaminated. Further, assurance that *L. monocytogenes* is not present in product can be achieved by a reduction or the elimination of *Listeria* spp. from the post-lethality RTE processing environment. This is accomplished with aggressive control and verification of control

through implementation of an environmental *Listeria* spp. sampling program. A microbiological sampling program has several parts:

1.1. To consistently monitor for the presence of *Listeria* spp. in the post-lethality RTE processing environment using reliable, accurate sampling methods.

1.2. To assess the effectiveness of environmental control procedures in preventing the formation of *L. monocytogenes* niches or other sites by examining monitoring data and taking preventive measures on a regular basis.

1.3. To assess, in a timely manner, whether the environment in which RTE foods are produced is under control (Sanitation Standard Operational Procedures, SSOP, Verification).

1.4. To promote rapid and effective mitigation responses when *Listeria* spp. is found in the production non-product contact environment and on product contact surfaces.

1.5. To verify that any identified *Listeria* spp. in the environment has been corrected and to provide data to facilitate short- and long-term assessment of *Listeria* control.

1.6. To provide benchmarking data for the development of “Best Practices”.

Listeria presence in the RTE processing environment is a cause of concern with regard to post-process contamination of RTE food products. Therefore, rapid and effective response to eliminate the persistence of *Listeria* in the RTE processing environment is a main focus. Once *Listeria* is identified food manufacturers initiate several different activities which can be disruptive to the production of product,

damaging to equipment, and costly. Facilities generally investigate the root cause of a positive *Listeria* spp. finding with additional sampling to find the source and focus on the plant's sanitation practices. Specifically, facilities often initiate a more thorough cleaning of the area and equipment and use chemical sanitizers on food contact and non-contact surfaces (i.e. peracetic acid, quaternary ammonium compounds, hypochlorite). However, not all food manufacturing equipment and the environment of the manufacturing area are easily cleaned. Some products and production areas need to be completely dry during manufacturing and application of water and sanitizers can delay food production and result in reduced inventory.

The use of novel technologies may be an alternative or an enhancement to traditional sanitation for the control of *Listeria* in food manufacturing. Specifically, bacteriophage, viruses that can infect and reduce the presence of a target bacterium, have potential for use throughout the food production supply chain. New commercially available bacteriophage against *Listeria* are available and reasonably priced. Studies have shown the effectiveness of bacteriophage against *L. monocytogenes* when applied directly to products (Chibeu et al., 2013, Gutierrez et al., 2017). Additionally, studies have shown the use of bacteriophage is more effective against *L. monocytogenes* than chemical disinfectants (Chaitiemwong et al., 2014) under certain conditions. However, the general use of bacteriophage for the control of *Listeria* spp. in a food manufacturing facility needs further study and development. Additionally, the concentration of bacteriophage applied for the control of *Listeria* spp. needs to be further researched.

Goals and Objectives

The overall present study consisted of three parts:

1. Evaluate the incidence of *Listeria* spp. in food manufacturing facilities that produce a variety of RTE foods. Facilities were all located in the contiguous United States and represent a diverse geographical distribution. This first part of the study had the following objectives:
    - Identify food manufacturing locations representing a diverse product mix and geographical location for environmental testing of the RTE processing environment.
    - Evaluate the presence of *Listeria* in a diverse population of food manufacturing facilities.
    - Determine the incidence of *Listeria* spp. in different food manufacturing plants during operations and after sanitation (pre-operational samples).

2. Determine the effectiveness of commercially available bacteriophage on the reduction of *Listeria* spp. on two -food contact and one non-food contact surfaces in food manufacturing facilities. This second component of the study had the following objectives:
    - Determine the susceptibility of *Listeria* spp. to bacteriophage on different food contact and non-contact surfaces when applied in a mist.
    - Determine the lethality of phage against *Listeria* spp. when different concentrations, diluted in water, are applied in a mist on different food contact and non-contact surfaces.
    - Determine the effect of two different environmental temperatures (4^{o}C and 20^{o}C) on phage efficacy against *Listeria* spp. when different phage concentrations are applied as a mist to food contact and non-contact surfaces.

3. Determine the effectiveness of bacteriophage on reducing the presence of *Listeria* spp. when applied as an additional intervention for the control of *Listeria* in the food manufacturing facility. This third phase of the study had the following objectives:
    - Determine the effectiveness of bacteriophage in reducing the incidence of *Listeria* spp. in RTE manufacturing facilities operating at two different operational temperatures (4°C and 20°C) when applied onto surfaces as a mist.
    - Determine effectiveness of bacteriophage in reducing the incidence of *Listeria* spp. using two different applications strategies.
        - Intensive – three applications within a 18 h period using a 5% phage solution
        - Moderate – single application in a 18 h period using a 1% phage solution

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II. Literature Review

1. Introduction

Consumers are increasingly turning to grocery store delicatessens and the prepared food departments for ready-to-eat (RTE) foods that are convenient, of high quality, and contain minimal preservatives. As a result of the increased demand food processors are using more natural ingredients and processing solutions that deliver unique flavors, as well as, provide a high level of safety (Roman et al., 2017). In addition to safety and organoleptic qualities, processors are also focused on reducing waste by utilizing reusable or recyclable packaging and employing preservation methods that lengthen product shelf life without compromising product integrity.

1.1. Ready-to-eat food production and microbial control

Many factors affect the prevalence of microorganisms in RTE foods ranging from cooking procedures, sanitization methods, packaging, and Good Manufacturing Practices (GMP). Pathogens occur naturally in the environment and can easily contaminate and establish harborage locations in a food production facility.

To maintain control of the microbial load in a food plant several basic principles are followed by personnel. Good Manufacturing Practices (GMP) and Good Hygiene Practices (GHP) are fundamental to food safety and quality and have been incorporated into food plant procedures for decades. This includes keeping materials from the raw manufacturing side of a facility separate from the RTE production area, wearing plant specific garments and footwear, placing restrictions on movement of people and equipment throughout the facility, having separate air handling systems, and providing

positive air pressure in zones where high hygiene is imperative. Separation of materials and personnel located on the raw production side from the RTE side is extremely important as these two areas have different requirements for levels of hygiene, and it is critical that control factors be implemented to prevent cross contamination.

1.2. Ready-to-eat pathogen control and sanitation practices

Effective sanitation procedures are central to maintaining control of the microbial community within a food processing facility. Cleaning procedures in food manufacturing facilities can vary greatly depending on the type of food produced. For example, the type of cleaning needed in a facility producing RTE meats will differ from cleaning requirements of facility producing bakery products. Facilities producing meat and meat products are prone to microbial contamination because the product is near neutral pH and has a high protein content. Cleaning and sanitation practices in meat plants follow a cleaning schedule detailed in the facilities sanitation program. The program will stipulate cleaning procedures, frequency, and chemical management necessary to maintain desired detergent strength and sanitizer efficacy. The main cleaning and sanitation method used in RTE meat processing facilities is wet cleaning followed by flood sanitizing. In summary, this process involves removal of food debris from equipment surfaces and floors, equipment disassembly, followed by application of a cleaning compound to remove soil, reassembly of equipment, then rinsing with an EPA approved sanitizer (Frank, 2000). Other cleaning and sanitizing programs that are present in meat production facilities include clean out-of-place (COP) systems that involve manual cleaning of equipment such as fittings, gaskets, valves, and product handling utensils.

In contrast, clean-in-place (CIP) involves removal of soil from the inside of pipes and tanks with high velocity agitation using spray or spray balls followed by sanitizer application.

While these facets are critical to producing a wholesome food product, other considerations are also important for maintaining a high level of hygiene. Production equipment should be designed such that it is easily cleanable, made of materials that are compatible with sanitizing and cleaning chemicals, accessible for maintenance and cleaning, and has no niches for microbial harborage (Kramer, 2010).

Floors in production areas of meat and poultry plants can be dry, damp or constantly wet due to manufacturing conditions. Given that moisture is needed to support transfer and growth of microorganisms, floors can become a perfect medium for pathogen growth and cross contamination (Tompkin, 2002). Floors composition can also impact the presence of pathogens, such as *Listeria monocytogenes*, as cracks or chips in brick, epoxy or tile can provide niche areas where nutrients and moisture can become trapped and allow establishment of microbial biofilm (Djordjevic et al.,2002). In an effort to control the establishment and persistence of microbial biofilms food processors have typically used sanitizing chemicals, such as peracids and quaternary ammonium compounds (QAC), applied to floors after all equipment has been cleaned and sanitized. Also, as a prophylactic measure powdered sanitizers, such as QAC and hydrogen peroxide, are applied at regular intervals throughout the production day to manage any incidental contamination (Ceylan, 2011). While this approach has proven effective, other non-chemical treatment options, such as bacteriophage application, are

gaining interest due to concerns of sanitizer tolerance and consumer desire for naturally produced food products (Ortega Morente et al., 2013; Roman et al., 2017).

2. Listeria monocytogenes

2.1. Pathogenicity and significance in processed foods

Listeria monocytogenes was first described in the literature in 1926, when Murray et al. (1926) isolated the organism from the blood of infected rabbits. The researchers reported a typical monocytosis in the infected animals, and isolated small Gram-positive bacillus, which they named *Bacterium monocytogenes*. The following year, Pirie (1927) reported similar findings out of South Africa, in which he isolated a bacterium from African jumping mouse and named the organism *Listerella hepatolytica*. After learning that these organisms were the same, the name was changed to *Listerella monocytogenes*, and was subsequently changed to *Listeria monocytogenes* in 1940 (Ryser et al., 1991). *L. monocytogenes* are short, Gram-positive, non-sporeforming, facultatively anaerobic rods, with a growth range of 3-45°C (Farber and Peterkin, 2000).

L. monocytogenes is pathogenic, causing listeriosis in animals and humans. Symptoms of listeriosis in humans can include fever and diarrhea in healthy individuals, or abortions and stillbirths in pregnant women, and sepsis, pneumonia and meningitis in newborns, the elderly, and immunocompromised individuals (Painter and Slutsker, 2007). While 13 serotypes have been identified, the majority of human listeriosis cases are caused by serotypes 1/2a, 1/2b and 4b (Cossart, 2001). Although the incidence of listeriosis is low, the case-fatality rate can be as high as 20-30% (Todd and Notermans, 2011).

L. monocytogenes was first identified as a foodborne pathogen in 1981 (Todd and Notermans, 2011). Since that time, many outbreaks linked to the consumption of refrigerated, RTE foods including hot dogs, deli meats, smoked seafood, cheese, pate, coleslaw, raw and insufficiently pasteurized milk have been reported (Tompkin, 2002). Since *Listeria* are psychrotrophic, and able to multiply at refrigeration temperatures, growth and persistence in the refrigerated RTE food production environment, and subsequent contamination of food is the leading cause of listeriosis outbreaks (Tompkin, 2002). The U.S. Centers for Disease Control and Prevention (CDC), for the period from 1998-2008, reported 24 confirmed foodborne listeriosis outbreaks resulting in 359 illnesses and 38 deaths (Cartwright et al., 2013).

2.2. Incidence and historical prevalence

Several large foodborne outbreaks in the 1980's lead the U.S. Food and Drug Administration (FDA) and the U.S Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) to establish a "zero-tolerance" policy for *L. monocytogenes* in RTE foods (Shank et al., 1996). The policy deemed a RTE food to be adulterated if *L. monocytogenes* was detected in a 25-g sample (≥0.04 CFU/g) (Smoot et al., 1997). At that time, FSIS established microbiological testing programs for RTE meat and poultry products produced at federally inspected facilities. Prevalence data reported out by FSIS for the 10-year period from 1990 to 1999 show a cumulative positive rate of 0.52% to 5.16% in meat and poultry samples collected from federally inspected establishments during this time period, with the greatest prevalence from sliced lunch meats (Levine et al., 2001). In 2000, FDA and FSIS collaborated on a quantitative risk assessment of the

relative risk to public health from foodborne *L. monocytogenes* among various categories of RTE foods. A total of 23 food categories were included in the risk assessment, including seafood, produce, dairy (cheese, pasteurized and unpasteurized milk), meat and deli-type salads. Deli meats were found to pose the greatest risk of listeriosis among all RTE foods (US FDA and USDA-FSIS, 2003). Subsequently, additional risk assessments were conducted to compare the relative risk of deli meat sliced at federally inspected establishments compared to deli meat sliced at retail and found higher prevalence for *L. monocytogenes* in retail sliced deli meat than for manufacturer sliced and packaged lunch meat (Endikrat et al., 2010, Gombas et al., 2003, USDA-FSIS, 2010). FSIS monitoring data shows that the prevalence of *L. monocytogenes* in RTE meat and poultry products collected from federally inspected establishments continues to decline, including the most recently published data from 2017, which reports a positive rate of 0.2% (USDA-FSIS, 2018).

2.3. Monitoring programs, regulatory requirements and persistence in the food processing environment

In 2003, the USDA-FSIS promulgated regulations for the control of *L. monocytogenes* in post-lethality exposed RTE products, otherwise known as the *Listeria* Rule (USDA-FSIS, 2003). These regulations were designed using a science-based approach, based on the joint risk assessment published earlier the same year. This approach identified three alternatives, based on risk, for classification of an establishment's RTE meat and poultry products. Alternative 3 products pose the greatest risk to public health, and control of *L. monocytogenes* is based solely on GMP's and sanitation SOP's (SSOP's). Alternative 2 products either contain an

antimicrobial agent that inhibits growth of *L. monocytogenes* to less than 2-logs throughout the refrigerated shelf life, or a post lethality treatment in the final package that inactivates *L. monocytogenes* levels by at least 1-log. The highest level of risk reduction and the best control practice according to FSIS for *L. monocytogenes* is Alternative 1. Alternative 1 product are processed with a post lethality treatment that inactivates *L. monocytogenes* and contain an antimicrobial to prevent its growth. Additionally, with any alternative choice, each establishment must implement an environmental monitoring program (EMP) to detect and identify transient and persistent *Listeria* spp. in the RTE production environment.

The RTE meat and poultry industry had implemented EMP's in the mid to late 1980's, as a result of the identification of *L. monocytogenes* as a foodborne pathogen and the numerous foodborne outbreaks linked to consumption of contaminated food products. Tompkin et al. (1992) reported on one company's efforts to minimize product contamination in RTE meats, and their strategies to control the production environments in 12 processed meat plants and comply with regulatory policy. Data from over a 3-year period was shared and showed improved control over time, but that floors were difficult to control, and if positive, increased the risk of contaminated packaging lines. Based on industry experience with control of *L. monocytogenes* in the RTE environment to prevent post-process contamination of RTE foods, industry leaders published guidance on preventing post-process contamination of RTE foods (Tompkin et al., 1999). This guidance provided information on common harborage sites for *L. monocytogenes*, sanitation principles and frequency of cleaning, design elements of an EMP to verify control, and response to a positive finding.

Certain strains of *L. monocytogenes* have been reported to persist in a food production environment for months, or even years. A summary of this data shows serotypes 1/2a, 1/2b, 3b and 4b persisting in food processing environments from 4 months to 12 years (Tompkin, 2002). Serotype 4b, an outbreak strain from a cooked deli poultry product, matched a previously isolated strain from the same plant, 12 years later (Wenger et al., 1990). Data from a 2015 listeriosis outbreak in ice cream showed that the PFGE patterns from isolates from 6 people with listeriosis from 2010 to 2014 were indistinguishable from isolates from ice cream cups made in the food processing facility linked to the outbreak (CDC, 2015).

2.4. Control strategies

One of the control strategies used by industry to prevent post-process contamination of *L. monocytogenes* in RTE foods is by eliminating *Listeria* from the RTE food production environment. Persistent strains of *L. monocytogenes* have been identified as the most common cause of post-process contamination in RTE foods, and the cause of listeriosis outbreaks (Malley et al., 2015). Robust environmental sampling programs that include procedures for investigating, identifying, and eliminating the root cause of contamination, also known as “Seek & Destroy,” were first implemented in the RTE meat and poultry industry. When implemented properly, these programs result in facilities that are able to successfully maintain control of the *L. monocytogenes* hazard in their RTE production environments (Butts, 2003, Tompkin, 2002). These programs are designed to identify and manage growth niches by improvements in equipment and

facility design, employee GMP's and traffic patterns, maintenance and tool control, operational conditions, and effective sanitation.

As previously described, the collaborative risk assessment for *L. monocytogenes* identified deli meats as the highest risk for listeriosis among the 23 RTE food categories evaluated (US FDA and USDA-FSIS, 2003). This model was updated to include data on pre-packaged vs. retail sliced deli meats, and data for the use of growth inhibitors in these products. The updated model estimated that retail sliced deli meat without growth inhibitors would account for 69.8% of total deaths (Endrikat et al., 2010). There are numerous studies that demonstrate the effect of various growth inhibitors in RTE meat and poultry products. These studies demonstrate that the use of lactate-diacetate blends, benzoate, propionate, sorbate and cultured sugar-vinegar blends, with or without synthetic or naturally sourced sodium nitrite (cure) can be effective growth inhibitors in processed meat products. Each of the ingredients can limit *L. monocytogenes* growth to <2-logs and meet FSIS Alternative 2 status in these products (Glass et al., 2007, Golden et al., 2014, Porto et al, 2002, Samelis, 2002). Additionally, inhibition is enhanced and can limit *L. monocytogenes* growth to less than 1-log with higher concentrations added to the product (Glass et al., 2007, Golden et al., 2014).

Another strategy used by the industry for controlling *L. monocytogenes* in RTE foods is the use of high-pressure processing (HPP). Researchers have demonstrated that the use of HPP at 600 Mpa for 180 sec can inactivate between 2 and 4-log of *L. monocytogenes* in RTE meat products (Hayman et al., 2004, Youart et al., 2010). These treatments would meet the requirements for FSIS Alternative 2 status as an in-package post lethality treatment (PLT).

3. Bacteriophage

Bacteriophage (phage) are viruses that specifically infect bacteria and are harmless to humans, animals, and plants (Greer, 2005). They are considered the most abundant organism in the biosphere, and their role in the environment is generating much interest in the agricultural, food and healthcare industries (Sulakvelidze et al., 2001, Manrique et al., 2017, Fernandez et al., 2018). When used as antimicrobial agents in clinical medicine, phages offer several advantages over conventional antibiotic treatment, including efficacy against drug resistant organisms, specificity for target organisms, and patient safety (Sulakvelidze et al., 2001). In the food industry, phages used as antimicrobials offer several similar advantages including high target specificity, low toxicity, and are relatively inexpensive (Sillankorva et al., 2012). The two main areas for investigation of phage application in the food industry are as pre and post-harvest interventions. In primary production phages have been studied for control of *Salmonella* and *Campylobacte*r in poultry, control of *Salmonella* in swine, and *E.coli* O157:H7 in cattle. Research in post-harvest application has focused on similar applications including *Listeria* control on fruits and vegetables plus RTE meats (Endersen et al., 2014).

3.1. Mechanisms

Bacteriophages are obligate parasites and do not have their own metabolism and must rely on their host to multiply. Studies have revealed that not all phages replicate similarly and that there are important differences in the replication cycles. These phages are categorized as lytic and lysogenic. Both phages multiply by taking over the

host cells protein machinery. Lysogenic phage multiplication leads to integration of phage genetic information into the bacterial chromosome without host cell death. Lytic phage multiplication ends in cell lysis and death for the host organism, and for this reason, lytic phages are of interest for the control and elimination of bacterial pathogens in the food industry (Sillankorva et al., 2012).

Lytic phage reproduction cycle consists of five stages. The first is recognition of the host and adsorption, followed by penetration or injection of phage nucleic acid then a latent period whereby phage proteins necessary for genome replication are expressed. During the latent phase multiple copies of phage DNA are synthesized and lysozyme that is found in the phage tail is used to disrupt the bacterial cell wall. The next phase in the reproduction cycle is maturation. Phage maturation is when new phage components are assembled into virions. The final stage is lysis, the release of phage from the cell as specific enzymes hydrolyze the host bacterial cell wall from inside, thus releasing infectious phage particles that can find a new host and can initiate the cycle again (Sulakvelidze et al., 2001).

3.2. Development of commercial phage preparations

The first FDA clearance for a phage preparation for direct food contact was obtained in 2006 for a *L. monocytogenes* specific cocktail called ListShield™. Since that initial introduction of phage used directly in the food supply, regulatory approvals issued for phage products used for food safety related applications has gradually increased (Moye et al., 2018). Phage preparations have been developed to control several pathogens with assorted applications. These include phage specific for *Xanthomonas*

campestris to control bacterial spot on tomatoes and food safety applications for the control of *Shigella* spp., *Salmonella* spp., and *Listeria* spp. in food (Moye et al., 2018). While the ability of phages to infect and lyse host cells is proven, there are challenges to implementing the technology in food production. Namely, phages must first come in contact with susceptible cells to initiate the lytic cycle. As phages are non-motile the effectiveness of treatments is dependent on concentrations used. Also, due to the nature of the lytic cycle, phage replication should result in an exponential increase in particles once applied to food. However, studies have suggested that phage particles do not increase substantially after application indicating repeated lytic cycles and consequently exponential increases in phage populations may not occur (Oliveira, et al., 2014; Soni, et al., 2012). This limiting factor may explain the fact that once phage is applied to food, the level of target organisms decreases initially, but very little or no further reduction is observed (Kang et al., 2013).

3.3. Stability of phage

Various external factors, such as temperature, acidity, and salinity determine the incidence and viability of phages and can inactivate a phage particle by damaging its physical attributes (head, tail, envelope) or by altering its nucleic acid structure (Jończyk et al., 2011). Temperature is a critical factor for phage survivability, playing a central role in the phage's reproductive cycle. At sub-optimal temperatures, low temperatures, phage genetic material is less able to penetrate bacterial host cells, therefore, lower level of phage will be produced in the multiplication cycle. At higher temperatures the latent stage time increases, and lower level of phage are produced (Tey et al. 2009).

Another important factor influencing stability of phage is the pH of the environment (Jończyk et al., 2011). Researchers have shown that pH can influence attachment, infectivity, intracellular replication and multiplication of phages and that lytic activity of phages decreases at values less than 5 and over 10 while the optimum pH is around a neutral pH of 6–8 (Silva et al., 2013). Osmotic shock has also been shown to inactivate bacteriophages. A rapid change in osmotic pressure may cause phage nucleic acid to extrude from the tail or their heads to break (Jończyk et al., 2011).

3.4. Bacteriophage in food manufacturing

Intervention strategies using bacteriophage have been developed to control foodborne pathogens in pre- and post-harvest foods including fruit, vegetables, and fresh and RTE meats. Control using phage is gradually being accepted as a natural technology (Moye et al., 2018; Endersen et al., 2014). While lytic phages for biocontrol provide several advantages, the technology does have its limitations and drawbacks. Chemical sanitizers can inactivate phages and reduce their effectiveness necessitating the application of phage and sanitizer separated by time. If there are several pathogen types present in a food, then due to their specificity, phage will only reduce prevalence of the targeted pathogen (Moye et al., 2018).

3.5. Bacteriophage – *Listeria* specific

Thus far, over 500 *Listeria* phages have been isolated and characterized. Lysogeny is common in *Listeria* and most *Listeria* phages identified belong to the family *Siphoviridae,* which are considered lambda-type with characteristic long, flexible tail and

isometric head. All members of this group are temperate and encode a lysogeny control region (Klumpp and Loessner, 2013). While most *Listeria* phages are temperate and have a limited host range, several strictly lytic *Listeria* phages in the *Myoviridae* family have been identified, namely A511 and P100 (Carlton et al., 2005). *Listeria* phages A511 and P100 are similar but not genetically identical, have a broad host range and can infect *L. monocytogenes* of the serovar groups 1/2 and 4 (Carlton et al., 2005; Klumpp and Loessner, 2013). While they are similar at the primary sequence level, they differ in composition of some structural proteins. Their broad host range and lytic capability have made them suitable for use as antimicrobials in food safety systems to control *L. monocytogenes*. Bacteriophage preparation PhageGuard LISTEX™ uses a single phage, P100, and is approved for use as a processing aid for use in raw and RTE foods to eliminate *L. monocytogenes* in several countries including the USA, Canada, Australia and the Netherlands (Anonymous, 2019). Other phage products approved by FDA and USDA for use on food and surfaces in production facilities consists of phage cocktails that contain mixtures of up to 14 distinct phages (Leverentz et al., 2003).

Lytic particles called monocins, have been described by Zink and Loessner (1994) as substances produced by *Listeria* cultures that inhibit other *Listeria* spp. monocin structure is similar to that of phage tails and contain tail associated lytic proteins used for cell wall penetration that are toxic to specific *Listeria* strains (Klumpp and Loessner, 2013). Presently there is renewed interest in the antimicrobial properties of monocins and phage lysins for biocontrol of pathogens (Brussow, 2017).

3.6. Mutation and resistance to bacteriophage

To combat predation of bacterial cells by phage, different resistance mechanisms have evolved so that bacteria are able to protect themselves against a phage attack. These include, preventing phage adsorption, preventing DNA entry, modifying phage nucleic acids, and abortive infection systems (Labrie et al., 2010). The initial step of infection involves adsorption of phages onto host surface receptor sites. To prevent phage adsorption, bacteria have evolved adsorption blocking mechanisms. For example, *Escherichia coli* cells use lipoproteins to block phage receptors. Outer membrane protein TraT, disguises or changes the conformation of protein A which is a receptor for many *E. coli* phages (Labrie et al., 2010). Phage adsorption can also be prevented by extracellular polymers produced by bacteria providing a physical barrier between phages and their receptors. However, phages have developed enzyme systems to overcome barriers by producing lyases and hydrolases to breakdown bacterial polysaccharides (Labrie et al., 2010).

Bacteria contain restriction enzymes that recognize and cut foreign DNA. Phages shield their DNA from the endonucleases by modifying their own DNA by altering or avoiding specific sequences typically recognized by the enzymes. In some instances, inherent enzymes that protect bacterial DNA from its own endonucleases are utilized by phage DNA to modify the structure so that phage DNA will no longer be recognized as foreign and phage infection cycle can run to completion (Hagens and Loessner, 2010). Some bacteria have abortive infection mechanisms (AIM) which lead to death of the infected cell without release of progeny phage. Typically, AIM target a critical step in the phage multiplication cycle such as replication, transcription or translation. For example,

in *E. coli* an inactive form of the sensor protein, RexA, is activated by the phage protein-DNA complex that forms as a replication intermediate after phage infection. Two activated RexA proteins will stimulate the membrane-bound protein RexB which operates as an ion channel and allows transport of monovalent cations through the inner membrane damaging membrane potential and destroying the cell (Labrie et al., 2010).

3.7. PhageGuard Listex™ specific review - development, use and research

The first approval for a commercially available phage preparation to be applied directly to food was issued in 2006 by the FDA. The product, ListShield™ (Intralytix, Inc., USA), consisted of a cocktail of phages with broad spectrum efficacy. Since that initial product offering several other phage preparations have been commercialized, such as EcoShield (Intralytix, Inc., USA), Agriphage (Omnilytics, Inc., USA), and PhageGuard Listex P100 (Micreos Food Safety, The Netherlands). PhageGuard Listex™ is USDA/FDA GRAS approved and is accepted as a processing aid in Australia, New Zealand, Israel, Switzerland, The Netherlands (EU), and Canada. It is certified organic by the Organic Materials Review Institute (OMRI, USA) and by and SKAL Biocontrole in The Netherlands (SKAL, EU).

PhageGuard Listex™ is a concentrated solution of phage P100 characterized by its efficacy against a broad host range of *Listeria* spp. It can be used as a processing aid to treat food products that are prone to contamination with *L. monocytogenes*. Effectiveness of Listex™ has been evaluated using several food product types indicating efficacy varies and is dependent on multiple factors including application

method, food surface and phage concentration. Soni and Nannapaneni (2010) observed a 1.8 to 3.5 log reduction of *Listeria* on raw salmon fillets stored at 4 or 22°C after using 10^8 PFU/g of Listex™. Chibeu et al., (2013) compared efficacy of antimicrobials potassium lactate and sodium diacetate with Listex™ in RTE roast beef and turkey stored at 4 and 10°C. Their results showed that Listex causes an initial reduction of *L. monocytogenes* of between 1.7 and 2.1 log CFU/cm². Oliveira et al., (2014) contaminated melon and pear slices with *L. monocytogenes*, treated with Listex™ and stored at 10°C. After 2 days they observed a 1.5 log reduction on slices and reported reductions of between 2.5 and 4.0 log in melon and pear juice. However, under the same treatment conditions Listex had little effect on lower pH apple slices and apple juice. Their results indicate that Listex™ could be used to inhibit *Listeria* growth on fresh-cut and in fruit juices with high pH during storage at 10°C.

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Incidence of *Listeria* spp. in Ready-to-Eat Food Processing Plant Environments Regulated by the U.S. Food Safety Inspection Service and the U.S. Food and Drug Administration

Robert G. Reinhard [a], Robin M. Kalinowski [c], Peter W. Bodnaruk*[c], Joseph D. Eifert [a], Renee R. Boyer [a], Susan E. Duncan [a], and R. Hartford Bailey[b]

[a] Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061, USA

[b] Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762-6100, United States

[c] Tyson Foods, Inc., 400 S. Jefferson St., Chicago, IL 60607

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Abstract

A multiyear survey of 31 ready-to-eat (RTE) food processing plants in the United States was conducted to determine the incidence of *Listeria* spp. in various RTE production environments. Samples were collected from 22 RTE plants regulated by the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) and from 9 RTE food plants regulated by the U.S. Department of Health and Human Services' Food and Drug Administration (FDA). Only non-food contact surfaces in the RTE manufacturing areas with exposed RTE product were sampled. Each sample was individually analyzed for the presence of *Listeria* spp. by using a PCR-based rapid assay. In total, 4,829 samples were collected from various locations, including freezers, equipment framework, floors, walls, wall-floor junctures, drains, floor mats, doors, and cleaning tools. Nine (29%) of the facilities had zero samples positive for *Listeria* spp. in the production environment, whereas 22 (71%) had one or more samples positive for *Listeria* spp. The total incidence of *Listeria* spp. in all RTE food plants was 4.5%. The positive rate in plants regulated by the FSIS ranged from 0 to 9.7%, whereas the positive rate in plants regulated by the FDA ranged from 1.2 to 36%.

Listeriosis is a serious illness caused by the bacterium *Listeria monocytogenes*. This illness is of particular concern to susceptible populations such as pregnant women, neonates, the elderly, and individuals with a weakened immune system. Annually in the United States it is estimated that 95.7% of all listeriosis illnesses are caused by the consumption of contaminated food, for a total of 1,591 foodborne illnesses with a hospitalization rate of 94% and a death rate of 15.9% (11). Because listeriosis is usually caused by the consumption of food contaminated with *L. monocytogenes*, extensive effort is made to control the organism in food manufacturing. There are numerous governmental entities within the United States that have some responsibility in ensuring the safety of the food supply, and the majority of the regulatory oversight is split between two agencies: the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) and the U.S. Department of Health and Human Services' Food and Drug Administration (FDA). In total, the FDA has responsibility for approximately 80% of all food consumed at home in the United States, whereas the FSIS has regulatory oversight for the remaining 20%. This includes the responsibility for oversight and inspection of approximately 64,300 manufacturing establishments of which 58,000 are under FDA jurisdiction and 6,300 are under FSIS rules and regulations (7).

Since the late 20th century, both the FDA and the FSIS have had a greater awareness of product contamination by *L. monocytogenes* and the public health impact of food contamination on susceptible populations. Both regulatory agencies have established regulatory requirements related to the presence and control *L. monocytogenes* in food, and both have provided guidance to industry on the control of *L. monocytogenes* in a food manufacturing environment (14, 16, 17). Guidance from the

regulatory agencies to manufacturers that has proven successful includes defined sanitation practices for routine cleaning; use of self-draining food contact surfaces and food contact surfaces made from corrosion-resistant, nontoxic, nonabsorbent materials; and close monitoring of good manufacturing practices.

In addition, the FSIS has incentivized, through its inspection activities, the development of postprocessing technology and the use of growth inhibitors of *L. monocytogenes* in the products it regulates. This incentive has had a positive impact on the potential risk of listeriosis; and over the past 2 decades, numerous studies have been conducted by researchers in industry and academia to validate the efficacy of antimicrobial ingredients, such as sodium diacetate and sodium lactate, or to develop product treatments that are applied postpackaging. Product surface treatments, such as octanoic acid and lauric arginate, have also been validated and approved for use on ready-to-eat (RTE) products as well as postpackage treatments, such as high pressure processing. These treatments, too, have contributed to minimizing the risk potential for *L. monocytogenes* on RTE foods (2–5).

Although regulatory oversight has certainly helped reduce some of the risk of *L. monocytogenes*, industry and industry organizations have developed the leading best practice and intervention strategies for controlling *Listeria* in food manufacturing. Practices developed by industry include setting up barriers to prevent cross-contamination, validation of antimicrobial treatments used in product formulation and processing, and mitigation strategies through the use of different chemical interventions, cleaners, and sanitizers. It was industry that first recognized the value of environmental monitoring as part of a food manufacturing facility's *Listeria* control program. Tompkin

(12), Tompkin et al. (13), and Butts (1) each outlined that the key strategy for controlling *L. monocytogenes* in the RTE processing environment was testing to find it, and then eradicating this environmental pathogen. Historical data have demonstrated that the prevalence of *Listeria* is highest in the processing environment, and sampling nonfood contact surfaces (NFCS) for this organism is a more effective approach for assessing control than product testing (12). Microbiological monitoring of the food processing environment and equipment is critical to identify and eradicate harborage sites within food manufacturing facilities.

Potential sources of *Listeria* in the RTE production environment have been well documented and include equipment framework, motor housings, maintenance tools, forklifts, cleaning tools, freezers, drains, door seals, floor mats, and cracks in floors and walls that can retain moisture (12, 13, 17). Lappi et al. (9) conducted studies in smoked fish plants and identified floor mats as the most likely source of *L. monocytogenes* persistence. Even after intervention strategies were implemented, including soaking mats in quaternary ammonia sanitizer overnight, the same subtype continued to be isolated from these mats (9). Other common positive sites included barrels and totes, plastic curtains, forklifts and pallet jacks, freezer doors and seals, drains, floors, drip pans, steps and stairs, walls, curbing, and tools (3, 5, 12, 13, 17). The industry has made significant progress in controlling the prevalence of *L. monocytogenes* in manufacturing environments and on food products in recent years. However, vigilance regarding control strategies is critical due to the ubiquitous nature of this organism. An essential part of any organization's *Listeria* control strategy is to determine the level of risk by continually sampling the environment for *Listeria* and implementing corrective

actions to remove the risk when found. The objective of this study was to determine the overall prevalence of *L. monocytogenes* in multiple RTE food production facilities and to identify any trends with respect to harborage potential.

Materials and Methods

Food Processing Plants

Thirty-one food processing establishments were surveyed, including 22 FSIS- and 9 FDA-regulated facilities. Establishments included in the study produced numerous RTE foods ranging from sliced lunchmeat and hot dogs to corn chips and various other refrigerated and frozen RTE foods. Facilities were all located in the contiguous United States and represented a diverse geographical distribution. Access to federally inspected RTE food production plants for a survey such as this is limited due to the proprietary nature of the data. Thus, details of the specific product types manufactured at each of these facilities are not reported.

Sample Collection

Survey samples were collected from the 31 establishments by a team of trained microbiologists. Each facility was evaluated once during this survey, and the evaluation included both production and preoperational (pre-op) samples. The same team was responsible for collecting samples across all locations. Production samples were collected after the facility had been manufacturing products for a minimum of 3 h, and pre-op samples were collected after cleaning and sanitation before resuming production. In most cases, production samples were collected, and the same sites were

sampled the following day after cleaning and sanitation and identified as pre-op samples. In five of the nine FDA-inspected plants, pre-op samples were not collected owing to the sanitation schedule and the practice of running numerous days between sanitation events. A minimum of 20 samples from each RTE area was collected at each sampling time, but the total number of samples collected was variable as some facilities have only one processing line, whereas other facilities have several. EZ Reach sponge (polyurethane) samplers (with 10 mL of HiCap neutralizing broth with attached glove pack, 24-oz [710-mL] sample bag; World Bioproducts, Mundelein, IL) were used for sample collection. Premoistened sponge samplers were aseptically removed from the sample bag, and an approximate area of 12 by 12 in. [30 by 30 cm] was swabbed by applying pressure and swabbing in a horizontal direction, followed by swabbing in a vertical direction. If the specific dimensions of the location being sampled was less than 12 by 12 in., all available area on the target site was sampled following the horizontal then vertical sampling technique. The sponge sample was then transferred back to the sample bag, and the attached handle was twisted off and discarded. All samples were stored and shipped refrigerated ($\leq$7°C) overnight to an ISO 17025–accredited testing laboratory. Testing was started within 36 h of sample collection.

Microbiological Analyses

At the laboratory, 60 mL of prewarmed *Listeria* special broth (Bio-Rad Laboratories, Inc., Hercules, CA) was added to each sample bag. The liquid was then expressed through the sponges at least 10 times by hand squeezing, after which all samples were incubated at 30°C for 25±1 h. DNA extraction and PCR plate preparation

were performed using the iQCheck Prep automated sample handler (Bio-Rad Laboratories, Inc.). Samples (100µL) were dispensed into a deep well plate that was transferred onto the sample handler. Bacterial genomic DNA was extracted from the enriched samples by using the iQ-Check *Listeria* spp. kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. In brief, 100µL of lysis reagent (reagent A plus reagent F) was added to each sample well and then the plate was sealed using sealing film. The deep well plate was incubated at 95-100°C for 15-20 min at 1300 rpm in a plate agitator-incubator. A five-µL aliquot of each extracted DNA sample was suspended in 45-µL of PCR solution containing fluorescent probes and amplification solution for the real-time PCR reaction. The PCR plate was removed from the IQ-Check Prep, sealed and transferred into the CFX thermal cycler. Prescription and control of the cycler was carried out by CFX manager™ IDE software (Bio-rad Laboratories, Inc. Hercules, CA). Results were interpreted by the IDE software (algorithm analysis of the quantification cycle (Cq) values of each sample). Values obtained that were Cq≥10 were considered to be positive for *Listeria* spp.

Results

In total, 4,829 samples were collected from 31 facilities and tested for the presence of *Listeria* spp., with a total positive rate for all 31 RTE locations of 4.5%. Nine (29%) of the facilities had 0 positive samples, whereas 22 (71%) of the plants tested had at least one positive *Listeria* spp. sample site. Samples collected during production of RTE food (operational samples) resulted in a 5.7% positive rate, and 3.1% of

samples collected after cleaning and sanitation of the facility (pre-op samples) were positive.

Twenty-two of the 31 facilities were regulated by the FSIS. From these plants, a total of 3,832 samples were collected and tested for the presence of *Listeria* spp., for a total positive rate of 2.2% (Table 1). Nine of these plants had zero positives within the samples collected, and 13 (59%) of the plants had at least one positive sample site. Samples collected during operations resulted in a positive rate of 1.9%, whereas pre-op samples were positive in 2.6% of the samples (Table 1).

The remaining nine plants sampled were FDA-regulated RTE plants. There was a total of 997 samples collected and tested for the presence of *Listeria* spp., for a total positive rate of 13% (Table 2). At least one positive sample was obtained from each plant evaluated (100%). Operational samples resulted in a positive rate of 16.6%, whereas pre-op samples were positive in 5.8% of the samples (Table 2). The distributions of positive *Listeria* sample locations for all plants are summarized in Table 3. Positive rates for a specific sample location varied from 1.1 to 21.6%.

Sample sites with the greatest percentage of *Listeria* spp. positives included floor mats (21.6%), barrels and totes (18.4%), plastic curtains (18.2%), pallet jacks (8.9%), and freezer doors and door seals (8.5%). No positive samples were detected from railings and hand rails, fans, electrical boxes and conduit, and maintenance tools and toolboxes.

Discussion

The persistence of *L. monocytogenes* in the RTE processing environment is a cause for concern with regard to postprocess contamination of RTE food products. Previous studies have summarized published data on the persistence of *L. monocytogenes* in various food processing environments, including dairy, seafood, and meat processing plants, with persistent strains detected after months and even up to 8 years (3, 8, 12). *Listeria* in the environment increases the likelihood that the pathogen will contaminate processing and packaging lines, which increases the risk of product contamination (1, 12). Therefore, microbiological monitoring of the food processing environment and equipment is critical to identify and eradicate harborage within these facilities.

Strategies for controlling and eradicating *L. monocytogenes* in the RTE processing environment have been outlined by Tompkin (12) and Butts (1). By using the approach detailed by Butts (1), termed seek and destroy, it is possible to identify and remove growth niches in the RTE production area, thereby preventing product contamination (1). Malley et al. (10) have detailed the science-based strategy for the seek-and-destroy method and the importance of a supportive regulatory climate that encourages aggressive sampling in the RTE processing environment. Historical data have demonstrated that environmental prevalence of *Listeria* is highest on NFCS followed by product contact surfaces, with the lowest incidence on the actual food product (12). Data published by the USDA-FSIS on the incidence of *Listeria* on NFCS for calendar years 2012 through 2016 indicate a prevalence ranging from 6.42 to 9.14% (15). In this study, the NFCS positive rate for the 22 meat and poultry plants evaluated

was 2.2%, indicating a lower incidence than current industry performance. We were unable to find any published data on the incidence of *Listeria* in RTE plants regulated by the FDA.

An objective of this study was to sample NFCS of 31 RTE food processing facilities and to understand problematic areas across all facilities so that engineering controls or other intervention strategies could be put into place to remove these niches from the processing environment. As the intent was to detect *Listeria* in these environments, the team collecting samples focused on areas that had the potential to serve as a harborage for psychrotrophic microorganisms. Potential sources of *Listeria* contamination in the RTE production environment have been well documented and include equipment framework, motor housings, maintenance tools, forklifts, cleaning tools, freezers, drains, door seals, floor mats, and cracks in floors and walls that can retain moisture (6, 9, 12, 13, 17). In this study, some items such as floor mats and barrels and totes were positive at rates of 21.6 and 18.4%, respectively (Table 3). Other positive sites included plastic curtains, forklifts and pallet jacks, freezer doors and seals, drains, floors, drip pans, steps and stairs, and walls and curbing. Common positive sites detected did provide data to each processing facility on specific areas that needed attention, as well as indicate overall trends across the enterprise where food safety resources could be best used. Finally, several sample sites tested were never positive and included railings, electrical boxes and conduit, fans, and maintenance tools and toolboxes. Tools have frequently been reported in the literature as potential sources of contamination (12, 17). Tools were sampled on 69 occasions in this study, and none were positive for the presence of *Listeria*.

The RTE meat and poultry industry in the United States has demonstrated continuous improvement with regard to the prevalence of *L. monocytogenes* isolated from products, decreasing from 4.6% in 1990 to 0.39% in 2016 (15). Although the FSIS has monitored the prevalence of *L. monocytogenes* in RTE meat and poultry products and their processing environments since 1990 and 2006, respectively, the FDA has not had similar testing programs in place for RTE foods that support growth. The food industry, the FSIS, and the FDA have preferred strategies for controlling postprocessing contaminants. The strategy most accepted in the RTE meat and poultry industry is finding and eradicating *Listeria* spp. in the production environment. This approach has proven more effective than end product testing that may detect contaminated product on occasion but that will not lead to elimination of the hazard at its source.

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Table 1. Incidence of *Listeria* spp. in 22 ready-to-eat (RTE) meat and poultry plants regulated by FSIS

	Pre-Operational Sampling			Operational Sampling			Grand totals All Samples		
Facility No.	No. Samples	No. positive	% positive	No. Samples	No. positive	% positive	Total Samples	No. positives	% positive
1	101	3	3.0	100	4	4.0	201	7	3.5
2	80	0	0.0	80	0	0.0	160	0	0.0
3	24	1	4.2	25	0	0.0	49	1	2.0
4	40	0	0.0	76	2	2.6	116	2	1.7
5	30	0	0.0	30	0	0.0	60	0	0.0
6	20	0	0.0	20	0	0.0	40	0	0.0
8	78	0	0.0	79	0	0.0	157	0	0.0
10	99	8	8.1	90	4	4.4	189	12	6.3
11	60	0	0.0	60	0	0.0	120	0	0.0
12	174	2	1.1	200	4	2.0	374	6	1.6
13	118	14	11.9	120	9	7.5	238	23	9.7
14	130	1	0.8	130	1	0.8	260	2	0.8
15	40	0	0.0	40	0	0.0	80	0	0.0
16	150	3	2.0	150	1	0.7	300	4	1.3
17	179	2	1.1	180	7	3.9	359	9	2.5
18	61	3	4.9	61	2	3.3	122	5	4.1
20	25	0	0.0	25	0	0.0	50	0	0.0
22	120	5	4.2	120	2	1.7	240	7	2.9
23	60	0	0.0	60	0	0.0	120	0	0.0
25	169	4	2.4	91	0	0.0	260	4	1.5
30	40	4	10.0	40	0	0.0	80	4	5.0
31	129	0	0.0	128	0	0.0	257	0	0.0
Totals	1927	50	2.6	1905	36	1.9	3832	86	2.2

Table 2. Incidence of *Listeria* spp. in nine ready-to-eat (RTE) food plants regulated by FDA

Facility No.	Pre-Operational Sampling			Operational Sampling			Grand totals All Samples		
	No. Samples	No. positive	% positive	No. Samples	No. positive	% positive	Total Samples	No. positives	% positive
7	0	0	-	140	43	30.7	140	43	30.7
9	0	0	-	26	3	11.5	26	3	11.5
19	40	6	15.0	40	9	22.5	80	15	18.8
21	0	0	-	34	12	35.3	34	12	35.3
24	0	0	-	91	12	13.2	91	12	13.2
26	127	1	0.8	127	2	1.6	254	3	1.2
27	121	5	4.1	121	4	3.3	242	9	3.7
28	40	7	17.5	40	8	20.0	80	15	18.8
29	0	0	-	50	18	36.0	50	18	36.0
Totals	328	19	5.8	669	111	16.6	997	130	13.0

Table 3: Distribution of positive *Listeria* samples all RTE plants

Sample site	No. samples	No. positive	% positive
Floor mats	37	8	21.6
Barrels and totes	38	7	18.4
Plastic curtains	11	2	18.2
Pallet jacks	45	4	8.9
Freezer doors/door seals	235	20	8.5
Steps/stairs	175	11	6.3
Drains	421	30	7.1
Floors	715	39	5.5
Sinks	19	1	5.3
Drip/catch pans	58	3	5.2
Motor housings	84	4	4.8
Cooling water pipes	151	7	4.6
Walls/curbing	734	32	4.4
Carts/wheels	167	7	4.2
Cleaning tools	119	5	4.2
Equipment legs/feet	334	10	3.0
Control panels	137	4	2.9
Door frames/seals	430	11	2.6
Equipment framework	468	8	1.7
Hoses	95	1	1.1
Ladders	87	1	1.1

IV. Running title: Fate of *Listeria* on various surfaces when treated with bacteriophage (Research Paper)

Fate of *Listeria* on various food contact and non-contact surfaces when treated with bacteriophage

Robert G. Reinhard [a*], Robin M. Kalinowski [c], Peter W. Bodnaruk [c], Joseph D. Eifert [a*], Renee R. Boyer [a], Susan E. Duncan [a], and R. Hartford Bailey [b]

[a] Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061, USA

[b] Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762-6100, USA

[c] Tyson Foods, Inc., 400 S. Jefferson St., Chicago, IL 60607 USA

Keywords: *Listeria*, bacteriophage, food contact surface

*Corresponding author: +1 (312) 614-8058; Email: Bob.Reinhard@tyson.com

Abstract

Studies were conducted to determine the effectiveness of a bacteriophage (phage) suspension against *Listeria* spp. when applied for a short period of time on three common types of materials used in food manufacturing facilities, the materials included two food contact materials (stainless steel and polyurethane thermoplastic belting) and one non-contact material (epoxy flooring). Coupons of each material were inoculated with a cocktail containing *L. monocytogenes* and *L. innocua* (4 to 5-$\log_{10}$ CFU/cm^2) and allowed to attach on the surface. Two phage concentrations and a control, 0, $2x10^7$ and $1x10^8$ PFU/cm^2 were evaluated. Treated samples were held at either 4 or 20°C for 1 and 3h to determine the effect of temperature and treatment time on the level of reduction on each surface. Reductions in *Listeria* populations ranged from 1.27 – 3.33 $\log_{10}$ CFU/cm^2 on stainless steel, from 1.17 – 2.76 $\log_{10}$ CFU/cm^2 on polyurethane thermoplastic belting, and from 1.19 – 1.76 $\log_{10}$ CFU/cm^2 on epoxy resin flooring. Higher phage concentration ($1x10^8$ PFU/cm^2), longer treatment time (three hours), and temperature of the environment (20°C) showed a significantly ($P \leq 0.05$) greater reduction of *Listeria* on the stainless-steel and polyurethane thermoplastic belting coupons. Overall, *Listeria* reduction by phage treatment occurred on all food facility materials tested, under all conditions.

Highlights

- Treatment of hard surfaces, commonly found in food plants, with phage P100 can provide a reduction in *Listeria* populations.
- For stainless steel, reductions in *Listeria* ranged from 1.27 – 3.33 log_{10} CFU/cm^2
- *Listeria* reductions on polyurethane thermoplastic belting ranged from 1.17 – 2.76 log_{10} CFU/cm^2
- On epoxy resin flooring reductions in *Listeria* ranged from 1.19 – 1.76 log_{10} CFU/cm^2
- A 3-hour treatment time with bacteria phage was minimally more effective than a 1-hour treatment time on three different food plant surface material coupons.
- A phage concentration of 10E8/mL (vs. 2x10E7/mL) led to ~0.7 log greater reduction of *Listeria* on stainless steel and polyurethane thermoplastic belting coupons
- Reduction of *Listeria* after bacteria phage treatment were greater ($P \leq 0.05$) with longer treatment time and higher phage concentrations on stainless steel and polyurethane thermoplastic belting coupons but not on epoxy flooring coupons.

Introduction

Bacteriophage (phage) are viruses that specifically target and infect a host microorganism but are harmless to humans and non-microbiological hosts. The potential application of phage, as a novel food safety technology has been proposed by many authors, and numerous phage studies have been conducted looking at either the direct application of phage into food, or the use of phage to overcome bacterial biofilms (Gilbreth, et al. 2005; Gutiérrez, et al. 2016; Labrie, et al. 2010; Sillankorva, et al. 2012; Simões, et al. 2010; Srey, et al. 2013). However, research focused on the practical application of phage in the food manufacturing environment, as an antimicrobial, to reduce the presence of *Listeria* species, is very limited.

Listeria monocytogenes is a psychrotrophic pathogen that causes illness primarily in immune-compromised individuals, the elderly and pregnant women (Gilbreth, et al. 2005). Food products contaminated with *L. monocytogenes* have included ready-to-eat (RTE) foods such as dairy products, deli meats and precut, packaged fresh produce. As the pathogen is ubiquitous and can survive and grow at refrigeration temperatures, food processors have devoted extensive resources to prevent and control the presence of the organism in the manufacturing environment. To maintain the hygienic status of the plant environment, food processors implement Good Manufacturing Practices (GMP) and Sanitation Standard Operating Procedures (SSOP) with verification of performance through microbiological testing. Many companies also include additional specific programs to verify the control of *L. monocytogenes* in RTE areas of the manufacturing plant. These programs include robust environmental sampling for the presence of *Listeria* spp. (a potential indicator of *L. monocytogenes*) in

the RTE manufacturing areas of a plant, focusing on non-contact surfaces and sometimes including the verification sampling of food contact surfaces (Tompkin, 2002).

Persistence of *L. monocytogenes* in the RTE processing environment is the main cause of concern with regard to post-process contamination of RTE food products. Once a persistent strain of *Listeria* is identified as a potential contaminant, a rapid and effective response to eliminate the organism from the RTE processing environment becomes a main focus of those responsible for production (Butts, 2003; U.S. Department of Agriculture, 2014). This response will include several different mitigation strategies which can be disruptive to product manufacture, may damage equipment (i.e. water into electrical systems), and can be costly. Facilities generally investigate the root cause of a positive *Listeria* spp. finding with additional sampling to identify the source and focus on the plant's sanitation practices. Specifically, facilities will initiate a more intensive cleaning of the affected area which can include processing equipment and the immediate environment. Use of chemical sanitizers on food contact and non-contact surfaces (i.e. peracetic acid, quaternary ammonium compounds, hypochlorite) follows physical cleaning to eliminate microbial contaminants. A survey conducted by Reinhard et al. (2018) reported *Listeria* prevalence in the environment of U.S. meat and poultry facilities at between 0.0 - 9.7% and 1.2 to 36.0% in non-meat facilities regulated by the U.S. Food and Drug Administration. These data indicate that elimination of *Listeria* from the RTE manufacturing environment continues to be a major food industry challenge with contributing factors such as accessibility of equipment and production areas for ease of cleaning. (Keto-Timonen, et al. 2007).

The use of novel technologies may be an alternative to or an enhancement of traditional sanitation methods used for the control of *Listeria* in a food manufacturing environment. Specifically, bacteriophage have a potential for use throughout the food production supply chain to be used to control bacteria of human health concern. Recently, several bacteria phage containing solutions have become commercially available for use in the food manufacturing industry, including P100 for use against *Listeria* which is offered by Micreos Food Safety B.V., (Wageningen, The Netherlands). Studies have shown the effectiveness of P100 against *L. monocytogenes* when applied directly to food products (Chibeu, et al. 2013; Gutierrez, et al. 2017). Additionally, studies have shown the use of bacteriophage P100 is more effective against *L. monocytogenes* than chemical disinfectants under certain conditions (Chaitiemwong, et al. 2014). However, the use of bacteriophage for the control of *Listeria* spp. in a food manufacturing environment needs further study and development. The objective of this research was to examine the efficacy of phage P100 against *L. monocytogenes* and *Listeria* spp. on three different hard surfaces commonly found in a food manufacturing facility.

2. Materials and Methods

2.1 Listeria Strains and Inoculum Preparation

One strain of *L. monocytogenes* (ATCC 13932) and one strain of *Listeria innocua* (ATCC 51742) were obtained from Microbiologics (St. Cloud, MN, USA). Each culture was streaked onto a brain heart infusion agar plate (BHIA; Hardy Diagnostics Springboro, OH, USA) and incubated at 35°C for 18-20 h. A single isolated colony of

each strain was resuscitated in 10 mL of BHI broth (BBL, Becton Dickinson, Sparks, MD, USA) and incubated at 35°C for 48 h. The two *Listeria* strains were pooled by adding equal volumes (9.5 mL) of each to a sterile test tube with one mL of fetal bovine serum (Life Technologies Corp, Carlsbad CA, USA) to achieve a 5% concentration (ASTM E1153-14, 2014). The inoculum suspension was serially diluted in Butterfield's phosphate diluent (BPD: Hardy Diagnostics Springboro, OH, USA) and plated on Ottavani and Agosti agar (ALOA; bioMerieux, Durham, NC, USA) to verify cell concentration. The cell density of the inoculum preparation was determined following incubation of ALOA plates at 35°C for 48 h.

2.2. Commercial Bacteriophage Preparation

Phage P100, a *Listeria* specific bacteriophage, was obtained from Micreos Food Safety B.V., (Wageningen, The Netherlands) with a titer of $2x10^{11}$ plaque-forming units per mL (PFU/mL). The phage concentrate was diluted in sterile Milli-Q™ (MilliporeSigma, Burlington MA, USA) water to achieve concentrations of $2x10^{7}$ and $1x10^{8}$ PFU/mL. Use of MilliQ™ or spring water is necessary as P100 is sensitive to low level chlorine present in potable drinking water.

2.3. Test Coupons

Three different materials used in food manufacturing facilities were used to construct either 8 x 8 cm (64 cm^2) or 10 x 10 cm (100 cm^2) coupons (test squares), depending on the material type. Materials included in this study were stainless steel (314 with MD80 finish) (Alro Steel Corporation, Jackson, MI, USA), polyurethane

thermoplastic belting (Mol Belting Systems, Grand Rapids, MI), and epoxy flooring commonly used in food plants (TUFCRETE part A - polyol and TUFCRETE part B-MDI, Tufco Int. Gentry AR, USA). Coupon surfaces were treated with 70% alcohol, rinsed with deionized water, and exposed to ultraviolet light for at least 1 hour in a biosafety hood to prior to inoculation. After sanitization, test coupons were placed in separate, sterile Petri dishes.

2.4. *Coupon Inoculation and Treatment.*

Duplicate test coupons of each material were inoculated by applying the inoculum suspension with a pipette and spreading across the entire surface of the coupon using a sterile plastic spreader. Each coupon was inoculated with 2.5 µl/cm^2 of the inoculum to target a level 10^6 CFU/cm^2. Inoculated coupons were incubated at 37°C and approx. 40% relative humidity until visibly dry for 20 to 40 min, depending on the material type, to allow for attachment of the bacterial cells to the surface of the coupons (ASTM E1153-14, 2014).

Test coupons were treated with three different levels of phage P100: control (Milli-Q™ water only), 2x10^7 PFU/cm^2 (1%) and 1x10^8 PFU/cm^2 (5%). Water or diluted phage preparations, 10 µl/cm^2, were applied to the inoculated coupons using a micropipettor and distributed across the surface using a sterile spreader. Treated coupons were held at 4 or 20°C for both one and three hours. At 30 min intervals during the treatment time, 0.25 mL Milli-Q™ water was mist sprayed over the coupons (single spray 1 mL liquid aspirator over 4 coupons) to keep the surface moist during storage and to maintain humidity in the incubator to simulate a food manufacturing environment.

2.5. Microbiological Analyses

Each test and control coupon were aseptically transferred to a sterile sample bag to which either PBS (controls) or PBS containing dead *Listeria* cells was (treated) added. The coupons were rinsed with 50 mL of buffer and the outside of the sterile bag was massaged by hand for 30 s to remove adhered cells. Serial dilutions were prepared, and aliquots plated onto AOLA selective agar plates to obtain *Listeria* counts on each test coupon. Plates were incubated at 35°C for 48 h and typical colonies counted and recorded.

2.6. Preparation of Dead Listeria Cells

A cell suspension of dead *Listeria* cells was prepared according to the procedure outlined below to neutralize any residual phage activity present in coupon rinsate (Chaitiemwong, et al., 2014). An overnight culture of *L. innocua* (ATCC 51742) was prepared by inoculating BHI broth (400 mL) with incubation at 35°C for 18-24 h. The overnight culture was separated into 50 mL conical tubes and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded, and each pellet re-suspended in 20 mL phosphate buffered saline (PBS). All suspensions were pooled, and PBS buffer added to bring the total volume to 500 mL. The cell concentration of this suspension was 10^8 CFU/mL. The cell suspension was autoclaved at 121°C for 20 min to inactivate the cells.

2.7. Statistical Analysis

The Generalized Linear Mixed Models procedure was used to analyze the main effects and interactions of material type, treatment concentration, and storage temperature on the differences in mean microbiological numbers (SAS Institute Cary, NC).

3. Results

Log reductions of *Listeria* from bacteriophage on three coupon surfaces are summarized in Tables 1-3. Overall reductions ranged from 1.27 – 3.33 $\log_{10}$ CFU/cm^2 on stainless steel, 1.17 – 2.76 $\log_{10}$ CFU/cm^2 on polyurethane thermoplastic belting, and 1.19 – 1.76 $\log_{10}$ CFU/cm^2 on epoxy flooring. *Listeria* reduction from bacteriophage treatment was observed on all three materials, under all conditions evaluated.

Two environmental temperatures, refrigerated (4°C) and ambient (20°C), were evaluated to determine if there was an effect from the environmental conditions on the effectiveness of bacteriophage. Temperature did not have an effect on the level of reduction in *Listeria* levels on epoxy flooring. However, greater reductions ($P \leq 0.05$) were observed on stainless steel and thermoplastic belting when held at ambient temperature compared to refrigeration (Table 4).

High ($1x10^8$ PFU/mL) and low ($2x10^7$ PFU/mL) phage concentrations were evaluated on all three coupon types. The high phage concentration resulted in significantly greater reductions ($P \leq 0.05$) in *Listeria* levels on the stainless steel and belting materials. The level of reduction on the epoxy flooring material at high and low phage concentrations were not significantly different (Fig. 1). Initial inoculum levels of

coupons were targeted at approximately $1x10^6$ log_{10} CFU/cm^2.Reductions from water spray and through the treatment process without bacteriophage (control) are shown in Table 1.

Two treatment times, one and three hours, were evaluated for all three materials. While the interactions between time and temperature, and time and phage concentration on epoxy were not statistically significant, dwell time was significant, with the longer dwell time of 3 hours resulting in higher log-level reductions (Fig. 2).

Interactions between time and temperature and time and phage concentration on stainless steel were significant, with greater reductions as treatment time, phage concentration, and temperature were increased. Stainless steel coupons treated with high phage concentration for three hours and stored at 20°C resulted in the greatest reductions compared to the other treatments. The interaction between phage concentration and temperature was not significant on stainless steel. Interactions between time and temperature, and phage concentration and temperature on the belting material were significant, with greater reductions as phage concentration and temperature increased. The interaction between time and phage concentration was not statistically significant on the belting material.

4. Discussion

The food industry has been addressing the presence of *L. monocytogenes* in food manufacturing facilities since the early-1980's. A number of different techniques have led to significant reductions in the presence of the pathogenic organism and have led to vastly improved food safety outcomes (Butts, 2003; U.S. Department of

Agriculture, 2014). Many improvements have focused on, been developed for, and implemented by the meat and poultry industry in the United States. These improvements were driven by regulatory oversite and the risk associated with human illness from these foods when they are contaminated with *L. monocytogenes*. Different processes to mitigate *L. monocytogenes* were used in red meat and poultry facilities included i) sanitary standard operational procedures, ii) the implementation of regulatory HACCP in the mid-1990's, iii) sanitary design principles for equipment and facilities circa 2004, iv) advancement of antimicrobials in the early part of this century, and v) sanitizer development/optimization from early 1990's to the present. Additionally, industry environmental sampling programs, product testing and advancement of laboratory techniques have enabled improved identification and targeting of the hazard in the animal based-product supply chain. Similar advancements have been used across all food and beverage manufacturing, however, the techniques used are not applicable to all product categories, and major infrastructure changes to improve sanitary design of equipment and facilities is a lengthy and expensive process.

The outcomes of this study show the potential effectiveness of applying bacteriophage on common surface materials used in a food manufacturing plant to control *L. monocytogenes.* Reductions in *Listeria* levels were observed on all three materials, at refrigerated and ambient temperature conditions, and at high and low phage concentrations for both 1 and 3-h treatment times. Effectiveness of bacteriophage at both refrigerated and ambient temperatures is important to the food industry, as many food production plants are refrigerated, as in the case of RTE meat and poultry. However, many others production facilities are not refrigerated. One

important factor to consider when making decisions on intervention strategies in the food production environment is contact/treatment time of an antimicrobial like bacteriophage on the food contact or non-contact surface. Additional considerations include whether bacteriophage application occurs during production or during sanitation and what is reasonable in a food production environment. Previous studies that evaluated bacteriophage on food manufacturing facility surfaces used contact times that are not practical in a food production setting (Soni and Nannapaneni. 2010). Use of bacteriophage during and immediately after sanitation will not result in reduced pathogen incidence as phage are inactivated in the presence of chemical sanitizers. Keeping RTE food environments dry has long been one of the tools used in the industry for *Listeria* control. However, in this study preliminary data indicated that phage efficacy is enhanced if surfaces were kept moist by delivering a fine mist of MilliQ™ water every 30 min. Bacteriophage efficacy on hard surfaces in the production environment can follow this same process and be enhanced in dry areas by maintaining low levels of moisture on treated surfaces.

5. Conclusion

The use of bacteriophage applied into the environment for the reduction and control of *Listeria* in a food manufacturing facility is a novel and relatively inexpensive intervention that could be undertaken by food manufacturers. However, additional research is required. Specifically, the techniques used for application of the bacteriophage needs to be optimized. The methods used in this study, in a laboratory setting, gave a good indication of what could be set-up relatively simply and

demonstrate the reduction of *Listeria* on food facility materials by treatment with bacteriophage. Additionally, the robustness of using bacteriophage in a food plant, considering all the other antimicrobials already in use, needs to be further understood, along with the effectiveness of the technology in the presence of a wider range microbiological conditions.

Finally, the presence and absence of *L. monocytogenes* or *Listeria* spp. within the ready-to-eat manufacturing environment has always been the focus of the food industry as it relates to the pathogen's control. However, from a risk and hazard reduction standpoint the occurrence of *Listeria* in the food production environment needs to be better understood. The use of bacteriophage in a food manufacturing facility to reduce the incidence of *Listeria* and to achieve better public health outcomes has great promise and potential to be used in addition to the other technological advancements developed in the past 20 years.

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Figure Legends

Figure 1. Phage concentration (log PFU/cm^2) and log CFU/cm^2 reduction *Listeria* on different materials. Means with different letters were significantly different ($P<0.05$).

Figure 2. Treatment time and log CFU/cm^2 reduction *Listeria* on different materials. Means with different letters were significantly different ($P<0.05$).

Figure 1.

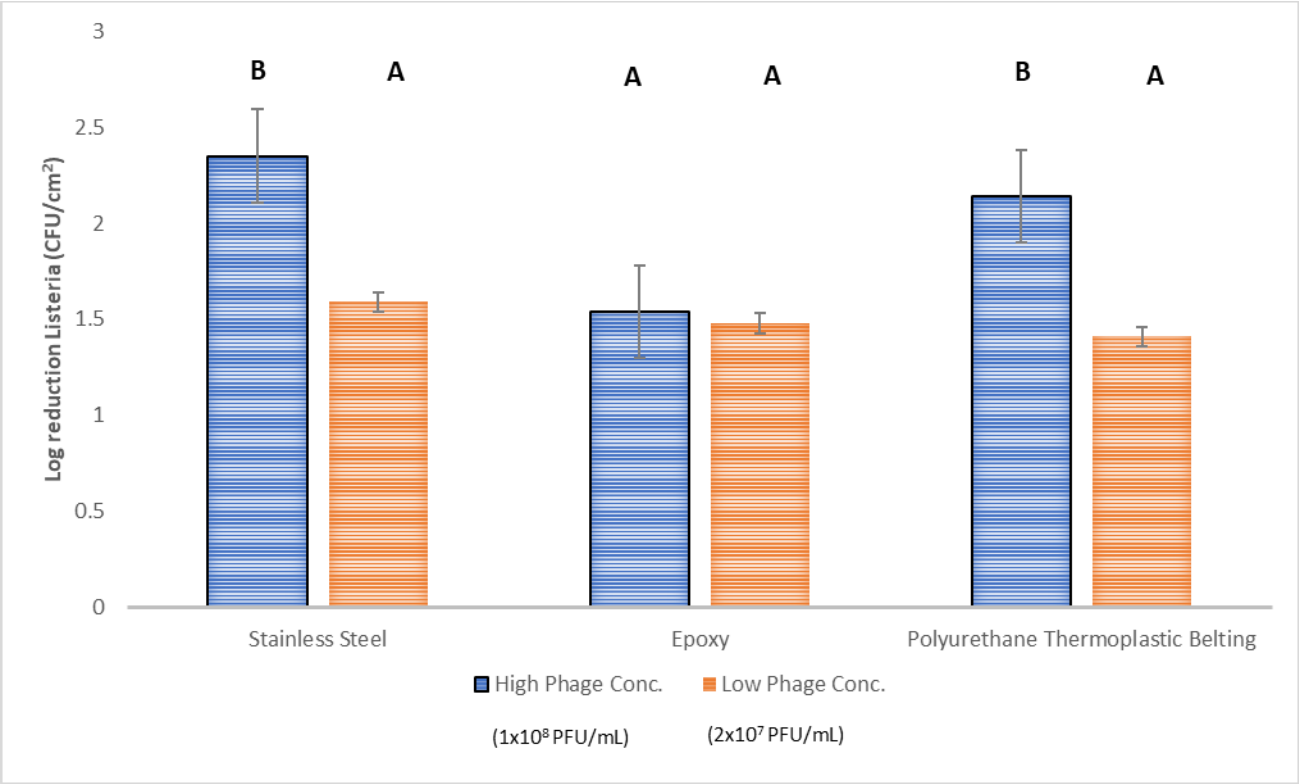
3
2.5
2
1.5
1
0.5
0
Log reduction Listeria (CFU/cm²)
B
A
A
A
B
A
Stainless Steel
Epoxy
Polyurethane Thermoplastic Belting
High Phage Conc. (1x10⁸ PFU/mL)
Low Phage Conc. (2x10⁷ PFU/mL)

Figure 2.

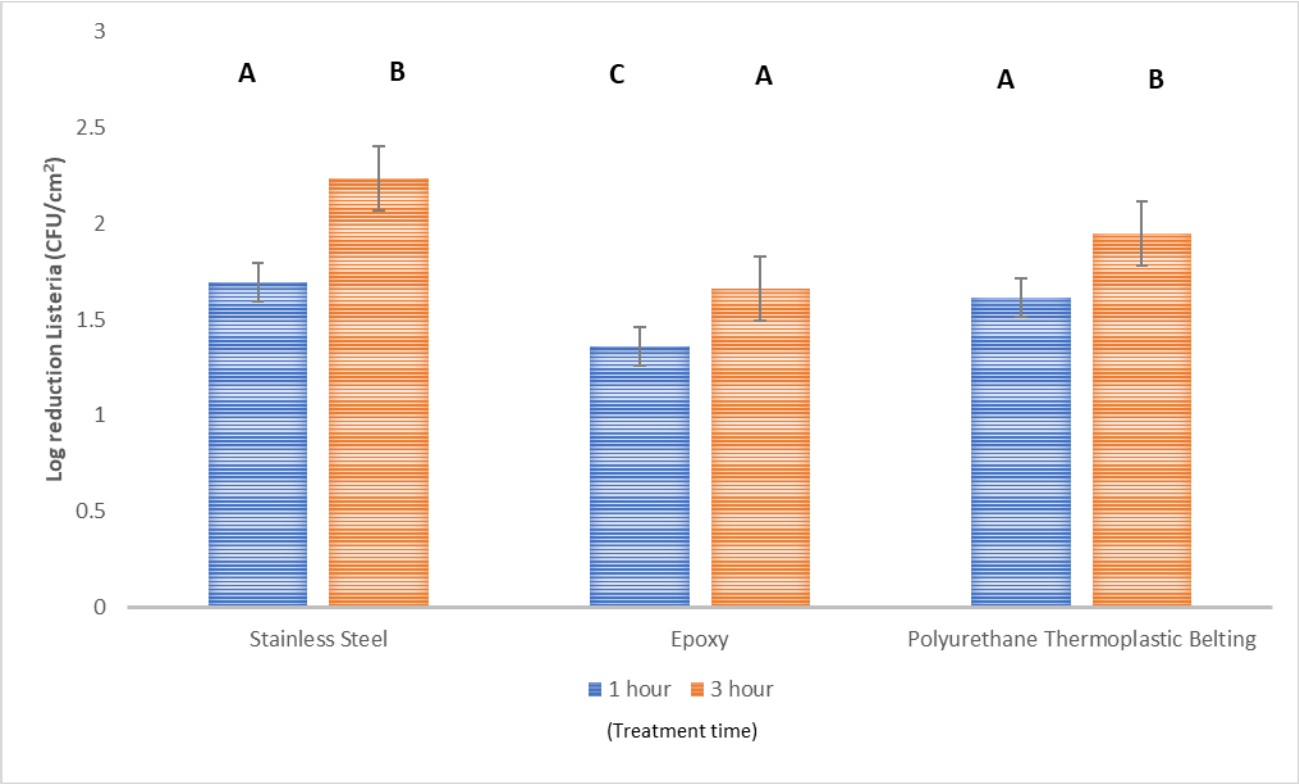
A
B
C
A
A
B
3
2.5
2
1.5
1
0.5
0
Log reduction Listeria (CFU/cm²)
Stainless Steel
Epoxy
Polyurethane Thermoplastic Belting
1 hour
3 hour
(Treatment time)

Table 1. *Listeria* levels on stainless steel coupons treated with bacteriophage

Phage Concentration (PFU/mL)		0	2×10^7	1×10^8
Storage Temperature (°C)	Dwell Time (h)	Log_{10} CFU/cm^2 recovered (control)	Log_{10} CFU/cm^2 reduction	Log_{10} CFU/cm^2 reduction
4	1	4.37[a]	1.36	1.68
4	3	4.17	1.27	2.15
20	1	4.98	1.53	2.21
20	3	5.25	2.19	3.33

[a]Mean of 6 replicates (duplicate samples and three independent trials)

Table 2. *Listeria* levels on epoxy coated flooring coupons treated with bacteriophage

Phage Concentration (PFU/mL)		0	2×10^7	1×10^8
Storage Temperature (°C)	Dwell Time (h)	Log_{10} CFU/cm^2	Log_{10} CFU/cm^2 reduction	Log_{10} CFU/cm^2 reduction
4	1	4.50 [a]	1.40	1.46
4	3	4.41	1.83	1.54
20	1	4.23	1.19	1.40
20	3	4.09	1.50	1.76

[a]Mean of 6 replicates (duplicate samples and three independent trials)

Table 3. *Listeria* levels on polyurethane thermoplastic belting coupons treated with bacteriophage

Phage Concentration (PFU/mL)		0	2×10^7	1×10^8
Storage Temperature (°C)	Dwell Time (h)	Log_{10} CFU/cm^2	Log_{10} CFU/cm^2 reduction	Log_{10} CFU/cm^2 reduction
4	1	4.60 [a]	1.28	1.81
4	3	4.56	1.44	1.81
20	1	4.74	1.17	2.19
20	3	4.76	1.75	2.76

[a]Mean of 6 replicates (duplicate samples and three independent trials)

Table 4. Interaction of coupon material versus storage temperature on reduction of *Listeria* (log CFU/cm^2)

Material	Storage Temperature (°C)	log CFU/cm^2 reduction
Polyurethane Thermoplastic Belting	4^o C	1.59^c
Polyurethane Thermoplastic Belting	20^o C	1.97^b
Epoxy Flooring	4^o C	1.56^c
Epoxy Flooring	20^o C	1.46^c
Stainless Steel	4^o C	1.62^c
Stainless Steel	20^o C	2.32^a

^{abc} Means within the same column with different superscripts were significantly different ($P \leq 0.05$).

V. Running title: Application of bacteriophage for control of *Listeria* spp. in food plants

Practical application of bacteriophage in food manufacturing facilities for the control of *Listeria* spp.

Robert G. Reinhard [a*], Robin M. Kalinowski [c], Peter W. Bodnaruk*[c], Joseph D. Eifert [a], Renee R. Boyer [a], Susan E. Duncan [a], and R. Hartford Bailey[b]

[a] Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061, USA

[b] Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762-6100, United States

[c] Tyson Foods, Inc., 400 S. Jefferson St., Chicago, IL 60607

Keywords: *Listeria*, Bacteriophage, Food manufacturing

*Corresponding author: +1 (312) 614-8058 Email: bob.reinhard@tyson.com

Abstract

Studies were conducted to determine the effectiveness of bacteriophage (phage) (Listex™) spray application against *Listeria* spp. in the ready-to-eat (RTE) environment at eight different food manufacturing facilities, four facilities with a refrigerated operational temperature (4°C) and four facilities with an ambient operational temperature (20°C). The first method evaluated was a moderate phage application applied in the environment as a single application over three days (once every 24 h), at a concentration of $2x10^7$ PFU/mL phage. The second method evaluated was an intensified application applied three times (once every 6 h) in a single day at a concentration of $1x10^8$ PFU/mL. To evaluate both applications, environmental non-food contact surface (NCFS) samples were collected and analyzed for the presence of *Listeria* spp. before and after treatment. Before the moderate phage treatment, the incidence of *Listeria* spp. in the production plants at 4 and 20°C was 67.5 and 52.5%, respectively. When the moderate treatment protocol was applied the incidence of positives decreased to 22.5% in the 4°C environment and 17.5% in the 20°C production area. This represented a decrease of *Listeria* spp. by 67% in both production RTE room environments ($P<0.05$). For the intensified phage treatment method, the initial positive rate in the 4°C environment ranged from 5 – 47.5%, with an overall 43% reduction in *Listeria* spp. In the 20°C facility, initial environmental *Listeria* spp. ranged from 15 – 50%, with an overall reduction of 32% after treatment with the *Listeria* specific phage.

1. Introduction

Listeria monocytogenes is a pathogen of significant public health concern and food manufacturing facilities are required to operate under conditions that prevent contamination of ready-to-eat (RTE) foods with this organism. The primary vector for contamination of food by *L. monocytogenes* is cross-contamination from the manufacturing environment (Butts, 2003; U.S. Department of Agriculture, 2014). To mitigate the risk of cross-contamination, food manufacturers have developed numerous intervention strategies for the control of *L. monocytogenes* in post-lethality exposed RTE products. These strategies include, the development of Good Manufacturing Practices (GMP), Sanitation Standard Operating Procedures (SSOP), facility design and equipment design criteria for effective sanitation and to prevent environmental harborage of *Listeria* spp. Additionally, food manufacturers have implemented Environmental Monitoring Programs (EMP's) for detection of *Listeria* spp. in the manufacturing environment, so they can implement mitigation strategies when the organism is found (Tompkin, 2002). However, a recent study by Reinhard, et al. (2018) reported *Listeria* spp. prevalence in different RTE food manufacturing plants ranged from 0 to 36.0%. This rate of *Listeria* spp. findings indicates the food industry needs to continue to investigate innovative risk mitigation methods/technologies for the reduction of *Listeria* spp. in the RTE manufacturing environment.

Bacteriophage (phage) are viruses that infect specific bacteria and are harmless to humans, animals, and plants (Greer, 2005). Phage are the most abundant organism on earth, and their role in the environment is generating interest in the food and healthcare industries (Sulakvelidze, et al., 2001; Manrique, et al., 2017; Fernández, et

al., 2018). Intervention strategies using phage have been developed to control foodborne pathogens in pre and post-harvest foods including fruit, vegetables, fresh and RTE meats. Control using phage is gradually being accepted as a natural technology (Moye, et al., 2018; Endersen, et al., 2014). Phage application in the food industry has been studied and shown effective by several authors, and published research studies have looked at direct application of phage into or onto food (Gutiérrez, et al., 2017; Sillankorva, et al., 2012; Chibeu et al., 2013). However, research looking at the use of phage to target specific environmental pathogens has not been studied. The purpose of this study was to identify and evaluate two different phage application methods that could be used by food manufacturers to reduce the presence of *Listeria* spp. in the RTE manufacturing environment.

2. Materials and Methods

2.1 Food Processing Plants

Eight food processing establishments were evaluated that operated at two different environment temperatures (4 and 20^{o}C). Four facilities were inspected by the United States Department of Agriculture, Food Safety Inspection Service (FSIS) while the other four facilities were regulated by the U.S. Food and Drug Administration (FDA). The FSIS regulated establishments produced RTE meat and poultry products, and the FDA regulated establishments produced RTE bakery items and assembled RTE sandwiches. All facilities were in the Midwest region of the United States and all facilities were operating with a *Listeria* environmental monitoring program in place to

meet the regulatory requirements of either the FSIS or FDA. All facilities selected for this study had recently identified the presence if *Listeria* spp. in the RTE production environment.

2.2 Commercial Bacteriophage Preparation and Treatment Method

PhageGuard Listex™, a *Listeria* specific bacteriophage, was procured from Micreos Food Safety B.V. (Wageningen, The Netherlands), with a titer of $2x10^{11}$ PFU/ml. Phage concentrate was diluted in spring water to achieve concentrations of approx. $2x10^{7}$ and $1x10^{8}$ pfu/mL. Since phage are sensitive to low level chlorine present in potable drinking water, unchlorinated spring water had to be used and verified for the study.

The prepared phage suspension was sprayed directly into the RTE food manufacturing environment using a commercially available hand sprayer (Braber Equipment Ltd. Abbotsford, BC Canada). Ten (10) liters of the phage suspension was sprayed into the RTE processing environment, covering 1,000 ft^2, until the area was saturated (visibly wet). For the moderate method of application, the RTE manufacturing environment was treated with a $2x10^{7}$ PFU/mL phage suspension once per day for three applications (immediately after sample collection, 24 and 48h). For the intensified method of application trials, the RTE environment was treated with a high concentration of $1x10^{8}$ PFU/mL phage suspension every six hours for three applications (immediately after sample collection, 6 and 12h).

2.3 Sample Collection

For the moderate phage application trial, two different production facilities were studied: one operating at refrigeration temperature (4°C) and one operating at ambient temperature (20°C). A total of 160 NFCS sponge samples were collected from each production facility. Forty samples were collected before the initial treatment, and the same sites were sampled prior to each subsequent treatment (@ 24 and 48 h) and 24 h after the last treatment (@ 72 h). For the intensified application method, a total of six different trials per production facility were studied. A total of 80 NFCS samples were collected from each production facility. Forty samples were collected before initial treatment and at the same sites 6 hours after final phage treatment was applied.

EZ Reach™ Sponge Samplers with a polyurethane Sponge, 24 oz sample bag, 10 ml HiCap Neutralizing Broth and attached glove pack (World Bioproducts, Mundelein, IL) were used for sample collection. Pre-moistened sponge samplers were aseptically removed from the sample bag, and an approximate area of 12x12 inches was swabbed by applying pressure and swabbing in a horizontal direction, followed by swabbing in a vertical direction. If the specific dimensions of the location being sampled was less than 12x12 inches, all available area on the target site was sampled following the horizontal and vertical sampling technique. The sponge sample was then transferred back to the sample bag and the attached handle twisted off and discarded. All samples were stored and shipped refrigerated (≤7°C) overnight to the testing laboratory. Testing was started within 36 hours of sample collection.

2.4 Microbiological Analyses

At the laboratory, 60 ml of pre-warmed *Listeria* Special Broth (Bio-rad Laboratories, Inc. Hercules, CA) was added to each sample bag. The liquid was then expressed through the sponges at least ten times by hand-squeezing, after which all samples were incubated at 30°C for 25±1 h. DNA extraction and PCR plate preparation were performed using the Bio-Rad iQ-check™ Prep automated sample handler. Samples were dispensed into a deep well plate, which was transferred onto the sample handler. Bacterial genomic DNA was extracted from the enriched samples using the iQ-check™ *Listeria* spp. kit according to manufacturer's instruction (Bio-rad Laboratories, Inc. Hercules, CA). A five-µL aliquot of each extracted DNA sample was suspended in 45-µL of PCR solution containing fluorescent probes and amplification solution for the real-time PCR reaction. The PCR plate was removed from the IQ-Check Prep, sealed and transferred into the CFX thermal cycler. Prescription and control of the cycler was carried out by CFX manager™ IDE software (Bio-rad Laboratories, Inc. Hercules, CA). Results were interpreted by the IDE software (algorithm analysis of the quantification cycle (Cq) values of each sample). Values obtained that were Cq≥10 were considered to be positive for *Listeria* spp.

2.5 Statistical Analysis

The Generalized Linear Mixed Models procedure was used to analyze the main effects and interactions of sampling location, treatment intensity and room temperature on the differences in mean percent positive results (SAS Institute Cary, NC).

3. Results

Moderate phage application method involved treating the RTE production area once per day for three consecutive days and analyzing for *Listeria* spp. after each treatment. Figure 1 shows the decline by day with modified phage treatment and that the decline occurred each day during the trials. Analysis shown in Table 1 indicate a 66% reduction in the number of positive results at 4°C and a 65% reduction at 20°C. Statistical analysis indicated treatment of the RTE production area with phage following the moderate application method had a significantly reduced incidence ($P \leq 0.05$) of positive findings from initial sample results at both operational conditions (4 and 20°C). There was no difference ($P > 0.05$) observed in the reduction of *Listeria* spp. between manufacturing locations (operational temperature of 4 and 20°C) when treating with phage following the moderate application method.

Intensive application of phage comprised of treating the RTE production area every six hours over the course of one production day. Results in figures 2 and 3 show there was a decrease in the incidence of positive *Listeria* spp. findings at all six manufacturing facilities after intense application. The reduction after treatment occurred at all locations when phage was applied by the intensified application method. Analysis of data for each of three manufacturing plants operating at 4°C (Table 2) showed mixed significance in the reduction of *Listeria* spp. In the first plant operating at 4°C there is a significant ($P \leq 0.05$) reduction in the incidence *Listeria* spp. but in the two subsequent plants no significant difference ($P \leq 0.05$) was observed despite a reduction. In food manufacturing facilities operating at 20°C there was a significant reduction in *Listeria*

spp. after the intensified treatment method in two of the three manufacturing plants (table 3).

The results of each treatment by sample location are shown in table 4. This data shows information on the effectiveness of each treatment in reducing *Listeria* spp. incidence by site type. Reduction of *Listeria* spp. for the intensified sampling showed site reduction rate for *Listeria* spp. from 46 to 100%, while for the intensified application method these reductions ranged from 23 to 100%. This information could be useful for a food manufacturer when deciding what type locations to potentially treat with phage using either application method.

4. Discussion

To our knowledge, the use of bacteriophage for reducing the incidence of *Listeria* spp. in RTE food plants has not been investigated. The data from this study demonstrates that the application of *Listeria* specific phage in RTE food production environments by either the moderate or intensified application method can be an additional intervention strategy for controlling this pathogen on NFCS. Since the two application methods resulted in similar *Listeria* spp. reductions in the manufacturing area, a facility could implement either as an additional hurdle in their sanitation programs for the control of *Listeria* spp. in the RTE food production environment. This could reduce the risk of product contamination with *L. monocytogenes* and reduce the risk of an outbreak or recall due to product contamination. The moderate method would be appropriate in on-going control circumstances since the application is once per day and less resource intensive than the intensified application. The intensified application could be used when immediate outcomes are required.

There are specific areas in some food plants where the use of this phage treatment could be beneficial and effective. As an example, spiral freezers can be problematic as a source for the harborage of *Listeria*. The use of phage treatment on the inside (floor and wall seams, cooling coils) and outside of freezers may be an effective control strategy for preventing the development of niches within these units. Another utility of treating the environment with phage could be in environments in which it is difficult to keep the floors dry, such as in a hot dog peeling operation and in some vegetable processing facilities. Repeated applications of phage during the production cycle, such as the intensified treatment evaluated in this study, could serve to prevent the ingress of *Listeria* in these areas, and to further areas downstream in the process. Additionally, the use of phage in food production facilities is a novel approach and could be viewed as a more natural, biological alternative to chemical based solutions.

While the results are favorable and show reductions in the incidence of *Listeria* after both phage treatment methods, there are limitations for use in some food production environments. First, the presence of chemical based sanitizers will inhibit the efficacy of the phage, as these viruses are as susceptible to these chemicals as bacteria, and the phage will be inactivated if they come in contact with these sanitizers (Woolston, et al., 2013). Therefore, the use of phage in a food manufacturing facility should occur after cleaning and sanitation. Second, phage are more effective when used in moist environments (Moye, et al., 2018). One element of controlling *Listeria*, used in RTE food production environments, is to keep the area, especially floors, as dry as possible. Therefore, phage treatment in dry areas would require the manufacturing plant to assure treatment areas remained moist when applying a phage treatment.

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Figure Legends

Figure 1. *Listeria* spp. incidence in two different plant food production plant RTE environments before and after moderate phage treatment, when treated with a single application of a $2x10^7$ PFU/mL phage concentration and tested at 24, 48 and 72 h (n=40)

Figure 2. *Listeria* spp. incidence in three different food production plants operating at refrigerated (4°C) temperature in the RTE area before and after intensified phage treatment, when treated with three applications of a $1x10^8$ PFU/mL phage concentration and tested 6 h post final phage application (n=40)

Figure 3. *Listeria* spp. incidence in three different food production plants operating at ambient (20°C) temperature in the RTE area before and after intensified phage treatment, when treated with three applications of a $1x10^8$ PFU/mL phage concentration and tested 6 h post final phage application (n=40)

Figure 1

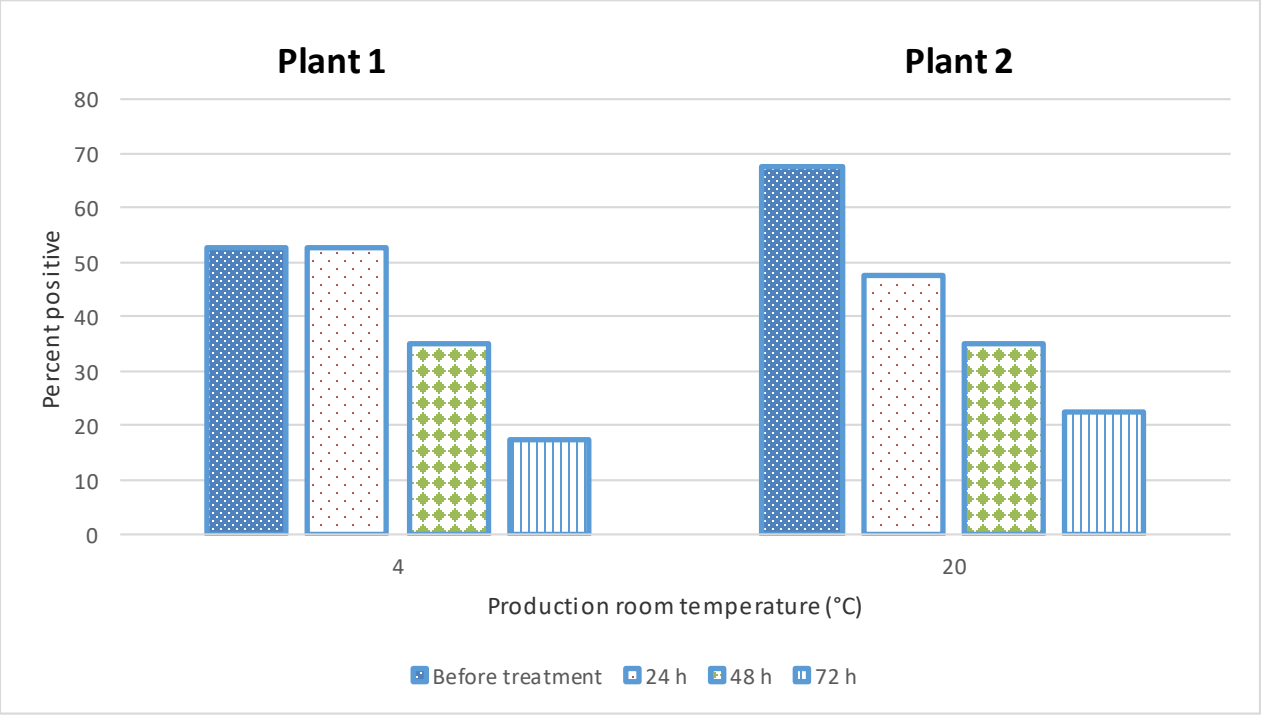
Plant 1
Plant 2
80
70
60
50
40
30
20
10
0
Percent positive
4
20
Production room temperature (°C)
Before treatment
24 h
48 h
72 h

Figure 2

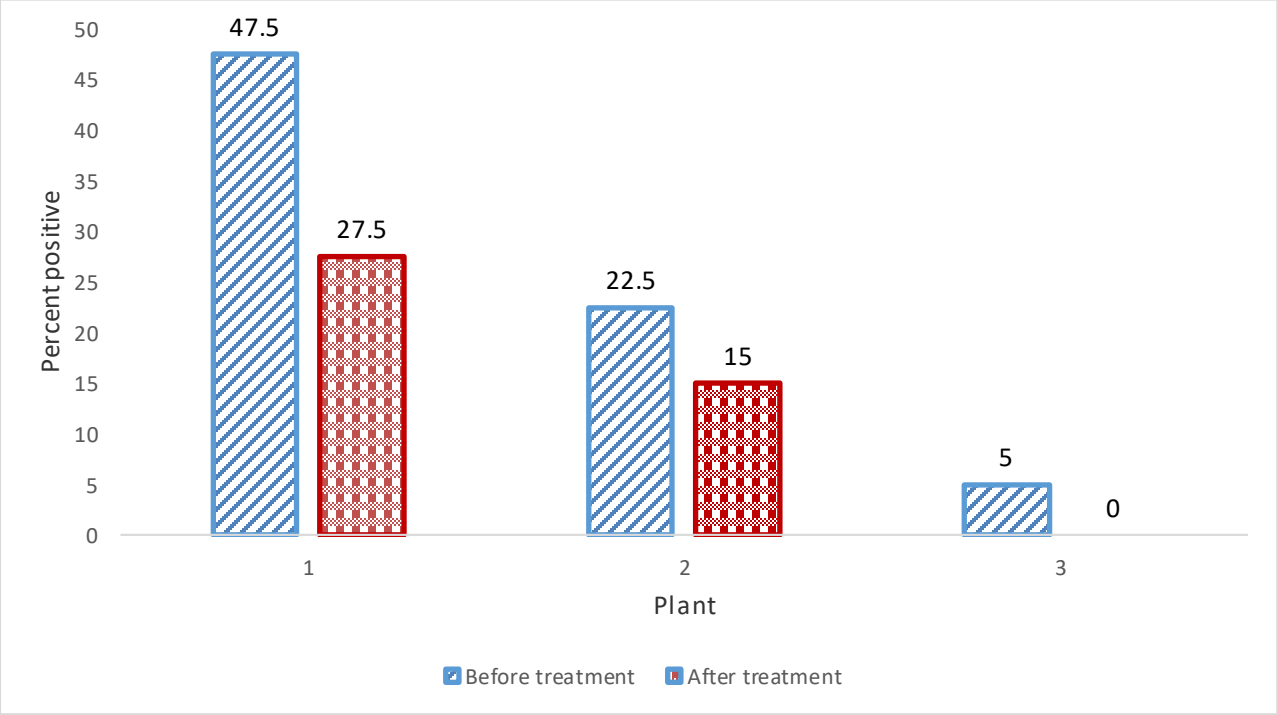
50
45
40
35
30
25
20
15
10
5
0
Percent positive
47.5
27.5
22.5
15
5
0
1
2
3
Plant
Before treatment
After treatment

Figure 3

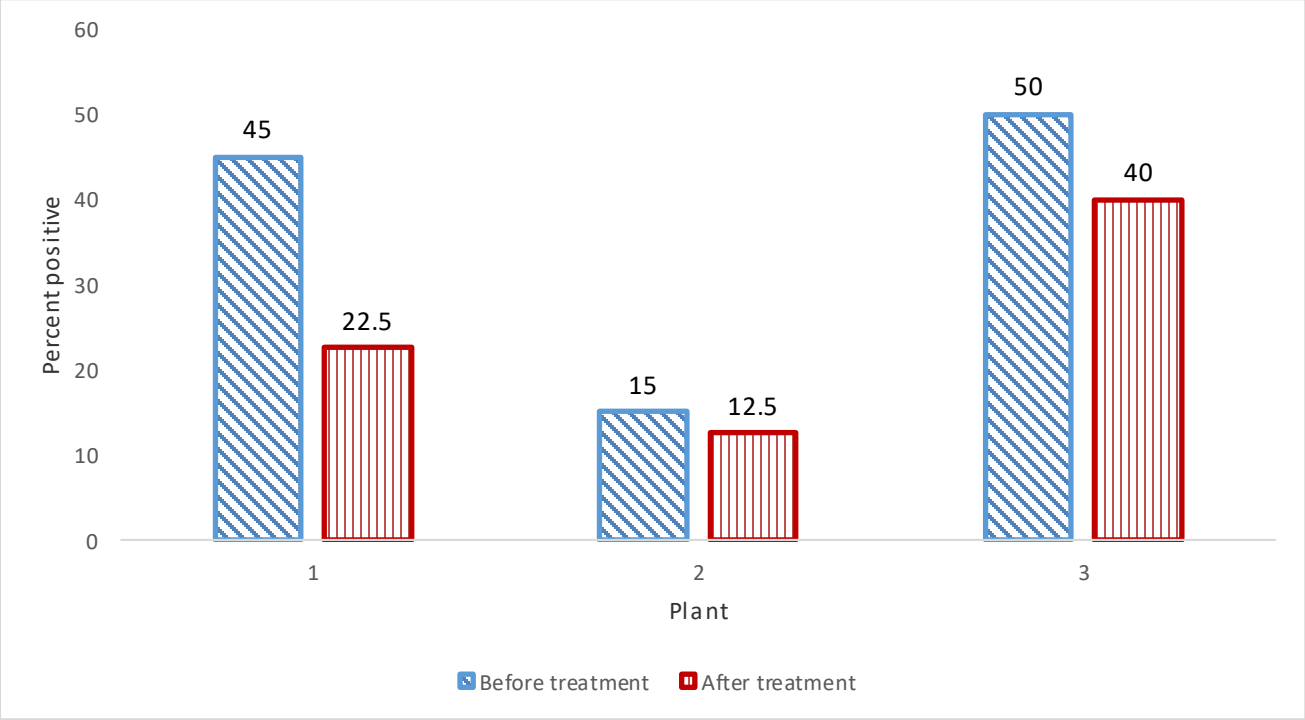
60
50
40
30
20
10
0
Percent positive
45
22.5
15
12.5
50
40
1
2
3
Plant
Before treatment
After treatment

Table 1. Incidence (%) of *Listeria* spp. following moderate phage application

	Room temperature	
	4°C	20°C
Before phage application	51.3[ac]	67.5[ac]
After three phage treatments	17.5[bc]	23.1[bc]

[a, b] – percentage within the same column with different superscripts were significantly different (P≤0.05)
[c] – percentage within the same row with same superscripts were not significantly different (P>0.05)

Table 2. Incidence (%) of *Listeria* spp. following intensive phage application in facilities operating at 4°C.

	Plant		
	1	2	3
Before phage application	47.5[a]	22.5[a]	5.0[a]
After three phage treatments	27.5[b]	15.0[a]	0.0[a]

[a, b] – percentage within the same column with different superscripts were significantly different (P≤0.05)

Table 3. Incidence (%) of *Listeria* spp. following intensive phage application in facilities operating at 20°C.

	Plant		
	1	2	3
Before phage application	45.0[a]	15.0[a]	50.0[a]
After three phage treatments	22.5[b]	12.5[a]	40.0[b]

[a, b] – percentage within the same column with different superscripts were significantly different (P≤0.05)

Table 4: Distribution of select positive *Listeria* sample sites and percent reduction after moderate and intensified bacteriophage application.

		Moderate[1] application (2 plants)				Intensive[2] application (6 plants)	
	Sample site	No. positive before treatment	No. positive after treatment (% reduction)			No. positive before treatment	No. positive after treatment (% reduction)
			24 h	48 h	72 h		
4°C	Walls/windows/curbing	5	4	3	3 (40)	12	8 (33)
	Drains	-	-	-	-	7	5 (28.5)
	Freezer doors/door seals	-	-	-	-	5	2 (60)
	Door frames/seals	-	-	-	-	3	1 (66)
	Equipment framework	4	3	2	2 (50)	1	0 (100)
	motors	-	-	-	-	1	0 (100)
	floors/curbing	15	10	8	5 (66)	-	-
	catch pan	1	1	0	0 (100)	-	-
	water pipe	1	0	0	0 (100)	-	-
	barrel	1	1	1	0 (100)	-	-
	TOTAL	27	19	14	10 (63)	30	17 (43)
20°C	Walls/windows/curbing	2	2	2	0 (100)	9	6 (33)
	Drains	3	3	2	0 (100)	2	1 (50)
	Door frames/seals	-	-	-	-	4	3 (25)
	Equipment framework	3	3	2	0 (100)	14	9 (32)
	wheels/casters	-	-	-	-	2	1 (50)
	floors/curbing	13	13	8	7 (46)	13	10 (23)
	TOTAL	21	21	14	7 (66)	44	31

[1] Moderate application = One application per day for three consecutive days

[2] Intensive application = Three applications every 6h over a 24h period

VI. Conclusions and Future Research

1. Conclusions

A prudent *Listeria* control program as defined by the U.S. Department of Agriculture, Food Safety Inspection Service (FSIS) (1) includes weekly environmental monitoring of each RTE food manufacturing line, post lethality processing, and weekly non-food-contact sampling (NFCS) in the area around each RTE manufacturing line. Generally, food manufactures collect 3-5 food contact *Listeria* spp. sponge samples on each production line and 5 to 10 environmental NFCS samples in the area around each RTE line. In total 8 to 15 *Listeria* spp. samples are collected weekly in a routine *Listeria* monitoring program at a cost of approximately $25 per sample. In a small manufacturing facility with 5 RTE manufacturing lines this equates to an annual spend of $52,000 to $97,500 for routine *Listeria* spp. monitoring.

In addition to the routine collection of RTE *Listeria* spp. environmental samples, a facility should collect additional samples each time a routine sample is positive for *Listeria* spp. to find and eliminate the route cause or source niche of *Listeria* to eliminate the origin conditions that allow the presence of the hazard. This investigative sampling can range from 20 to 100 additional *Listeria* spp. samples above and beyond the routine sampling. Plants with very good sanitation performance aim for 1% or less positive in the routine *Listeria* spp. sampling plan and at this baseline level, investigative sampling in a 5 RTE line manufacturing facility costs an additional $10,500 to $97,500 annually. In a plant with poor sanitation outcomes and higher base line levels of *Listeria* spp. in the RTE processing environment, say a 10% environmental rate, this investigative sampling to find and eliminate the cause of the *Listeria* spp. positive costs from

$104,000 to $975,000, using the same 5 RTE line plant example. In total a 5-line RTE manufacturing facility, the total annual costs they could expect for *Listeria* spp. in a prudent and well performing establishment ranges between $62,500 to $195,000 and in a facility that has poor sanitation performance in the control of *Listeria* spp. from $114,500 to $1,072,500.

Over the past three decades, the food manufacturing sector has made tremendous progress in dramatically reducing the likelihood of *Listeria monocytogenes* contaminating ready-to-eat foods. However, as shown in this research, elimination of *Listeria* spp. in food manufacturing facility RTE areas is a constant struggle and recalls of food products for *L. monocytogenes* contamination are costly to industry. Recall data (table 1) compiled from the FSIS website (2) since 2008 shows the potential impact to industry and the risk to consumers who could become infected with *L. monocytogenes* from the consumption of contaminated product.

Table 1: Pounds RTE Foods inspected by FSIS recalled due to *Listeria monooxygenase* contamination.

2008	349,000
2009	47,000
2010	384,000
2011	525,000
2012	439,000
2013	784,000
2014	270,000
2015	82,000
2016	47,000,000
2017	546,000
2018	4,100,000

These recalls were caused by high level of *Listeria* spp. in the RTE manufacturing area and subsequent *L. monocytogenes* contamination of finished RTE product, and the goal

of the routine *Listeria* spp. sampling program would be to find and prevent this contamination.

Recalls result in several financial burdens on the manufacture including the direct costs of the product recall. Direct costs include expenditures and administrative costs of product returned because of the recall. This is generally a portion of the value of the product recalled (i.e. recall 100,000 pounds, 30% returned to supplier, the value of 30,000 pounds product). If we estimate the direct product costs of all FSIS recalls, over 10 years (table 1), excluding 2016 because a single recall of not-ready-to-eat product accounted for that year's total pounds recalled, this represents 7.5 million pounds in 9 years. Applying a product estimated value per pound equal to $3 and assuming 100% of the product was returned, the approximate total cost of all product recalled would have been $22.3 million dollars.

However, manufacturers generally do not receive a claim on all product recalled because often product has been consumed and is not recovered. Returned product from a recall ranges from 10% to 30%, on average, and this median volume recovered is depended on several factors, including type of distribution (food service, retail, international) and timing between product production and the recall event (3). Low recovery dramatically reduces the direct product cost of a recall. Using the FSIS recall data, the recalls completed over the past 10 years were completed by 116 different manufacturing firms. At a 10% to 30% recovery of recalled product and using an estimated total product value at $3 per pound, each firm faced an estimated cost of $192,000 per event on average.

A second financial impact of *Listeria* spp. and food manufacturing firms' financial concerns related to *Listeria* control is the potential for business disruption. Food manufacturing is a high volume, low margin business. Therefore, disruption of production can put a manufacturing facility at risk for survival. Business disruption can happen in several ways. The first threat to business disruption is from food regulatory agencies. Both FSIS and Food Drug Administration (FDA) have extensive regulatory requirements for the control of *L. monocytogenes*, and either regulatory authority could act against a firm not meeting these required standards. These regulatory requirements include the potential for a manufacturing firm to be shut down when their plant's *Listeria* spp. monitoring program is finding higher levels of the organism in the RTE manufacturing environment. Robust regulatory requirements for *Listeria* control have been in place for meat and poultry facilities (FSIS regulated establishments) since 1999 and for FDA regulated locations since 2015. A second major threat to business disruption related to *L. monocytogenes* is market driven. The food manufacturing sector is very diverse in the United States and there are many customer and consumer choices. When a firm is not producing a specific product because of *Listeria* actions being taken at their plant by regulatory authorities, or as a result of a product contamination and recalls, customers and consumers simply change purchase behavior. They stop purchasing the manufacturing firm's products and this dramatically decreasing sales. Additionally, if the public perceives a general food safety risk in the category of product produced by the firm having *Listeria* related challenges, sales can decrease in an entire commodity sector, from all manufactures, making financial recovery difficult as alternative products are purchased by the public.

Finally, there is the economic impact of foodborne illness that has a large financial impact on manufacturers if they are not controlling *Listeria* in the RTE food manufacturing area of the plant. These costs fall in 3 areas (i) Direct Healthcare Costs (DHC), (ii) Direct Non-Healthcare Costs (DNHC), and (iii) Indirect Non-Healthcare Costs (INHC). Based on a recent study published on the cost of a *L. monocytogenes* recall with an outbreak study (4), it was estimated a single *L. monocytogenes* illness costs between $8,840 and $20,110 without including the cost of premature loss of human life. If there is loss of life, the financial costs, usually calculated as a disability-adjusted life year, DALY, the public health costs increase dramatically to between $2,032,018 and $3,654,232 per case.

In order to be successful at eliminating *Listeria* in RTE food manufacturing and to prevent the cross contamination of food by *L. monocytogenes*, the production location must implement aggressive monitoring programs and take corrective action when *Listeria* spp. is identified. This has been described in the literature as “seek and destroy”. Seek and destroy is an on-going process and part of a prudent companies’ food safety system and sanitation standard operating procedures. In addition, *Listeria* control programs have been expanded over the years to include, sanitary design of equipment and sanitary design of facilities. Current costs for building a state-of-the-art food manufacturing facility with excellent sanitary design is between $300 and $600 per square foot. With appropriate Listeria control programs, a food manufacturing facility can achieve extremely low levels of environmental *Listeria* spp. and even reach a point of none detected with an aggressive microbiological sampling program.

Our research reinforced the challenge and the issues industry faces to control *Listeria* in the RTE food manufacturing environment. We surveyed of 31 RTE food processing plants and collected a total of 4,829 samples, demonstrating a total incidence of *Listeria* spp. of 4.5%. The positive rate in plants regulated by the FSIS ranged from 0 to 9.7%, whereas the positive rate in plants regulated by the FDA ranged from 1.2 to 36%. The use of *Listeria* specific phage is a novel technology that could assist food manufacturers in the reduction of the environmental hazard. Bacteriophage have specific advantages, they are considered natural, specific to the targeted organism, and available commercially from at least 4 different manufacturers. Additionally, since phage are abundant and naturally occurring, food manufacturers could isolate and produce their own phage from their production environments. In our studies, the moderate and intensified phage application procedure cost $750 and $1200, respectively, for this intervention to be used in a 1000 ft^2 RTE production area.

In all the studies conducted for this manuscript, *Listeria* specific phage showed effectiveness in reducing the incidence of *Listeria* spp. Additionally, the application of phage, in a format that industry could adapt and utilize was shown to be effective. Specifically:

- Treatment of hard surface coupons made from materials commonly found in food plants, with phage, can provide a reduction in *Listeria* populations. For stainless steel, reductions in *Listeria* ranged from 1.27 – 3.33 log_{10} CFU/cm^2. *Listeria* reductions on polyurethane thermoplastic belting ranged from 1.17 – 2.76 log_{10} CFU/cm^2 and on epoxy resin flooring reductions in *Listeria* ranged from 1.19 – 1.76 log_{10} CFU/cm^2.

- Moderate phage application method in a food manufacturing facility, applying $2x10^7$ PFU/mL phage once per day for 3 consecutive days showed a decline in positive *Listeria* spp. findings results after every treatment (17.5 - 22.5% reduction). Additionally, an intensive application of higher levels of phage, $1x10^8$ PFU/mL, applied three times on one day, showed a similar reduction in the presence of the organism (32% reduction across all trials).

However, there are challenges in the use of *Listeria* phage in food manufacturing environments. Food manufacturing facilities use antimicrobial compounds and chemical sanitizers that inhibit the phage efficacy. Additionally, phage is not effective in dry environments. To overcome these challenges, the use of phage in a food production facility should occur after cleaning and sanitation and phage treatment should be kept moist throughout the treatment cycle.

2. Future Research

Research looking at new technologies and mitigation activities for *Listeria monocytogenes* control is of tremendous value. A few areas for future research related to the use of pathogen specific bacteriophage are:

1. Investigate the use of *Listeria* specific bacteriophage to prevent ingress and harborage development on product contact surfaces (i.e. slicer blades and sausage peelers).
2. Investigate the use of *Listeria* specific bacteriophage as a product surface treatment to understand applicability as an option to achieve USDA FSIS

Alternative 2 status in meat products that are not formulated with a validated inhibitor, or Alternative 1 status in meat products formulated with a validated antimicrobial system.

3. Evaluate the efficacy of *Listeria* specific bacteriophage in an RTE food plant over an extended period (several months) to understand if reductions in the incidence of *Listeria* are demonstrated with routine and long-term use.
4. Evaluate the use of *Listeria* specific bacteriophage for hand-dips and foot-baths commonly used in food manufacturing facilities. Determine if the intervention is effective in preventing the spread of transient *Listeria* with the manufacturing location.
5. Evaluate the use of *Salmonella* specific bacteriophage in raw meat and poultry products for the reduction of risk associated with human illness from *Salmonella*.

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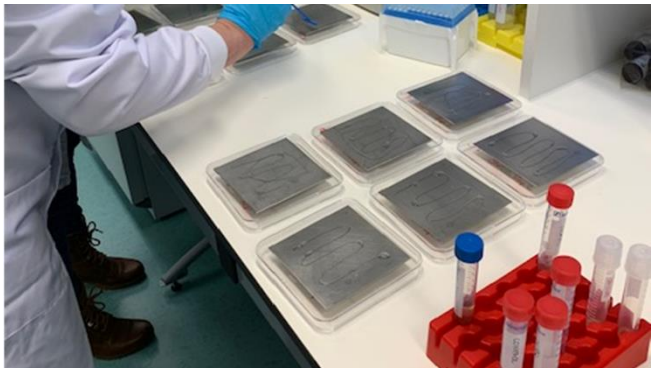
5. **VII. Addendum**



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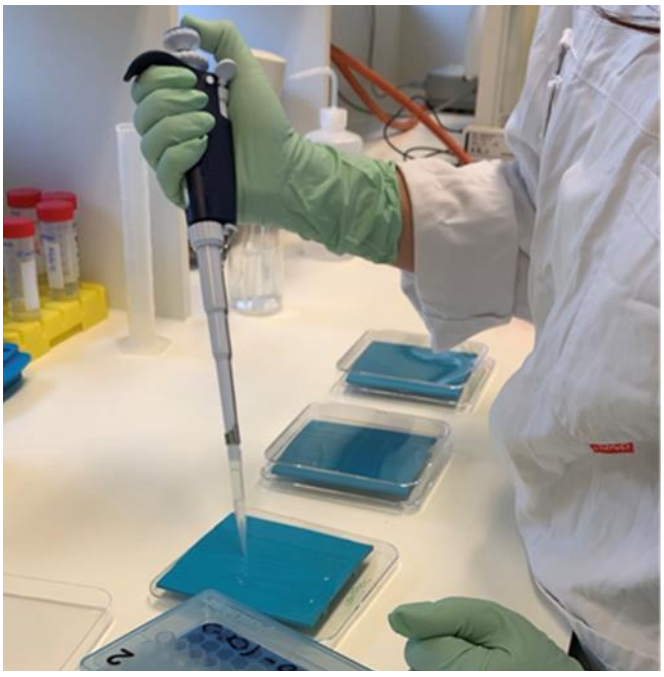

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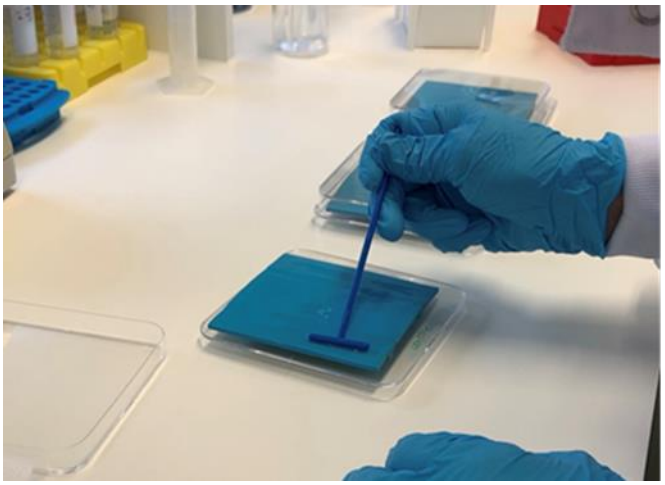
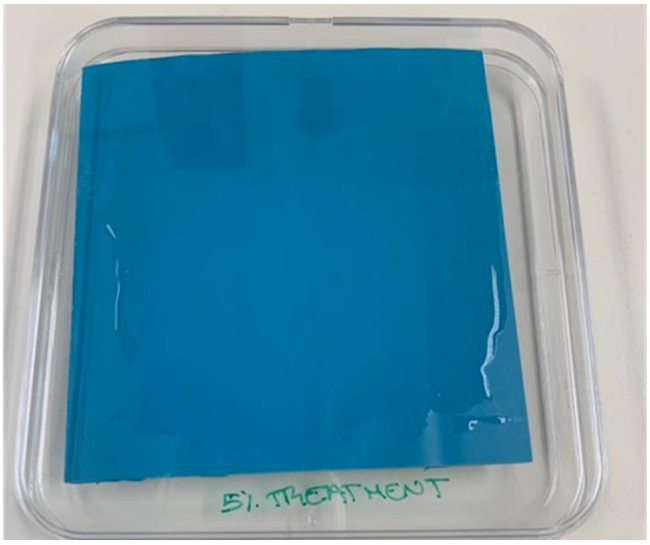


Spreading inoculate on polyurethane thermoplastic belting

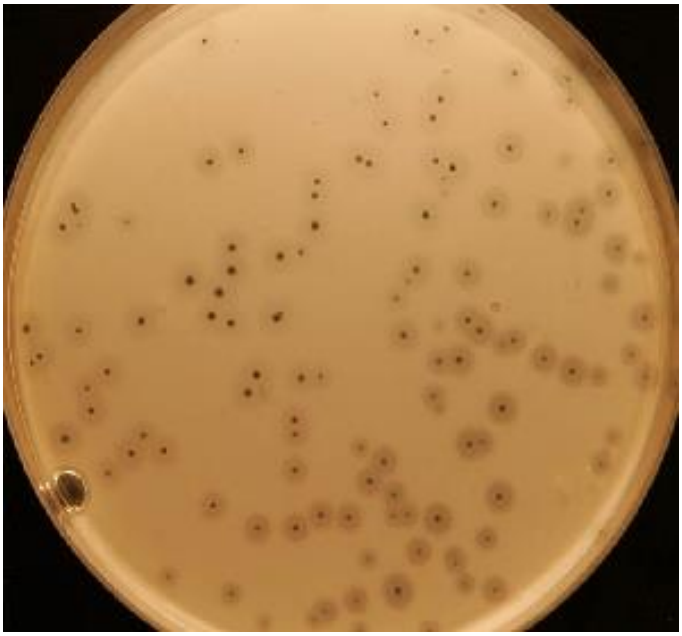


Polyurethane thermoplastic belting prior to attachment

Environmental survey at a sandwich plant

Environmental survey of RTE meat cooking area

Environmental Survey of RTE meat packaging



Bacteriophage titer plate (i.e. PFU)

Phage application at a RTE meat facility