

ORIGINAL ARTICLE

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

Robert G. Reinhard^{1,2}  | Robin M. Kalinowski² | Peter W. Bodnaruk² |
Joseph D. Eifert¹  | Renee R. Boyer¹ | Susan E. Duncan¹ | R. Hartford Bailey³

¹Department of Food Science and Technology, Virginia Tech, Blacksburg, Virginia

²Department of Food Safety and Quality Assurance, Tyson Foods, Inc., Chicago, Illinois

³Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi

Correspondence

Robert G. Reinhard and Joseph D. Eifert, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061.
Email: bob.reinhard@tyson.com (R. G. R.) and jeifert@vt.edu (J. D. E.)

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Abstract

Study objective was to determine efficacy of a bacteriophage suspension against *Listeria* spp. when applied to three common types of materials used in food manufacturing facilities. Materials included two food contact materials (stainless steel and polyurethane thermoplastic belting) and one noncontact material (epoxy flooring). Coupons of each material were inoculated with a cocktail containing *L. monocytogenes* and *L. innocua* (4 to 5-log₁₀ CFU/cm²). Two phage concentrations and a control, 0, 2 × 10⁷ and 1 × 10⁸ PFU/cm² were evaluated. Treated samples were held at 4 or 20°C for 1 and 3 hr to determine the effect of temperature and treatment time. Reductions in *Listeria* populations ranged from 1.27 to 3.33 log₁₀ CFU/cm² on stainless steel, from 1.17 to 2.76 log₁₀ CFU/cm² on polyurethane thermoplastic belting, and from 1.19 to 1.76 log₁₀ CFU/cm² on epoxy resin flooring. Higher phage concentration (1 × 10⁸ PFU/cm²), longer treatment time (3 hr), and processing area temperature of 20°C showed a greater ($p \leq .05$) reduction of *Listeria* on the stainless-steel and polyurethane thermoplastic belting coupons. Overall, *Listeria* reduction by phage treatment occurred on all three materials tested, under all conditions.

1 | 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, Rodríguez-Rubio, Martínez, Rodríguez, & García, 2016; Labrie, Samson, & Moineau, 2010; Sillankorva, Oliveira, & Azeredo, 2012; Simões, Simões, & Vieira, 2010; Srey, Jahid, & Ha, 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* sp. (a potential indicator of *L. monocytogenes*) in the RTE manufacturing areas of a plant, to include food contact and non-contact sites, with the focus on product contact surfaces (Tompkin, 2002). Each production facility is unique, and the sampling sites for

each facility should evolve based on the data and should include areas that have been found to be good indicators of control.

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. The cleaning and sanitation of food contact and noncontact surfaces consists of cleaning with an appropriate detergent combined with physical cleaning (hand scrubbing). Chemical sanitizers (i.e., peracetic acid, quaternary ammonium compounds, hypochlorite) are then applied to clean surfaces 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 to 9.7% and 1.2 to 36.0% in nonmeat 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, Tolvanen, Lunden, & Korkeala, 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 Micros 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, Hazeleger, & Beumer, 2014). P100 is desirable for biocontrol applications because it is a strictly lytic bacteriophage, that is, phage that always kill their host cell and does not transfer genetic material to the host, like transducing phage (Hagen & Loessner, 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* serotype 4b (ATCC 13932), and one strain of *L. innocua* (ATCC 51742) were obtained from Microbiologics (St. Cloud, MS). These strains were selected as they are commonly available as quality control strains, and *L. innocua* is commonly detected in RTE food production environments. Additionally, serotype 4b was included as it has been identified as the cause of the majority of human listeriosis outbreaks. Each culture was streaked onto a brain heart infusion agar plate (BHIA; Hardy Diagnostics Springboro, OH) and incubated at 35°C for 18–20 hr. A single isolated colony of each strain was resuscitated in 10 ml of BHI broth (BBL, Becton Dickinson, Sparks, MD) and incubated at 35°C for 48 hr. The two *Listeria* strains were pooled by adding equal volumes (9.5 ml) of each to a sterile test tube with 1 ml of fetal bovine serum (Life Technologies Corp, Carlsbad CA) 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) and plated on Ottavani and Agosti agar (ALOA; bio-Merieux, Durham, NC) to verify cell concentration. The cell density of the inoculum preparation was determined following incubation of ALOA plates at 35°C for 48 hr.

2.2 | Commercial bacteriophage preparation

Phage P100, a *Listeria* specific bacteriophage, was obtained from Micros Food Safety B.V., (Wageningen, The Netherlands) with a titer of 2×10^{11} plaque-forming units per milliliter (PFU/ml). The phage concentrate was diluted in sterile Milli-Q™ (MilliporeSigma, Burlington MA) water to achieve concentrations of 2×10^7 and 1×10^8 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 × 8 cm (64 cm²) or 10 × 10 cm (100 cm²) 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), 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). Coupon surfaces were treated with 70% alcohol, rinsed with deionized water, and exposed to ultraviolet light for at least 1 hr in a biosafety hood to prior to

inoculation. After sanitization, test coupons were placed in separate, sterile Petri dishes.

2.3.1 | 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 $2.5 \mu\text{l}/\text{cm}^2$ of the inoculum to target a level 10^6 CFU/ cm^2 . Inoculated coupons were incubated at 37°C and ~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), 2×10^7 PFU/ cm^2 (1%) and 1×10^8 PFU/ cm^2 (5%). Water or diluted phage preparations, $10 \mu\text{l}/\text{cm}^2$, 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 3 hr. 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 four coupons) to keep the surface moist during storage and to maintain humidity in the incubator to simulate a food manufacturing environment.

2.4 | 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 hr and typical colonies counted and recorded.

2.5 | 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 hr. 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.

2.6 | 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).

TABLE 1 *Listeria* levels on stainless steel coupons treated with bacteriophage

Phage concentration (PFU/ml)		0	2×10^7	1×10^8
Storage temperature ($^\circ\text{C}$)	Dwell time (hr)	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

^aMean of six 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 ($^\circ\text{C}$)	Dwell time (hr)	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

^aMean of six 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 (hr)	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

^aMean of six replicates (duplicate samples and three independent trials).

TABLE 4 Interaction of coupon material versus storage temperature on reduction of *Listeria* (log CFU/cm²)

Material	Storage temperature (°C)	Log CFU/cm ² reduction
Polyurethane thermoplastic belting	4	1.59 ^c
Polyurethane thermoplastic belting	20	1.97 ^b
Epoxy flooring	4	1.56 ^c
Epoxy flooring	20	1.46 ^c
Stainless steel	4	1.62 ^c
Stainless steel	20	2.32 ^a

Note: Means within the same column with different superscripts were significantly different ($p \leq .05$).

3 | RESULTS

Log reductions of *Listeria* from bacteriophage on three coupon surfaces are summarized in Tables 1–3. Overall reductions ranged from 1.27 to 3.33 log₁₀ CFU/cm² on stainless steel, 1.17–2.76 log₁₀ CFU/cm² on polyurethane thermoplastic belting, and 1.19–1.76 log₁₀ CFU/cm² 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 ($p \leq .05$) were observed on stainless steel and thermoplastic belting when held at ambient temperature compared to refrigeration (Table 4).

Two phage concentrations, 1 and 5%, were evaluated on all three coupon types. The 5% phage concentration resulted in significantly greater reductions ($p \leq .05$) in *Listeria* levels on the stainless steel and belting materials. The level of reduction on the epoxy flooring material at the two phage concentrations were not significantly different (Figure 1). Initial inoculum levels of coupons were targeted at $\sim 1 \times 10^6$ log₁₀ CFU/cm². Reductions from water spray and through the treatment process without bacteriophage (control) are shown in Table 1.

Two treatment times, one and 3 hr, 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 hr resulting in higher log-level reductions (Figure 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 5% phage concentration for 3 hr 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 oversight and by 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 (a) sanitary standard operational procedures, (b) the implementation of regulatory HACCP in the mid-1990's, (c) sanitary design principles for equipment and facilities circa 2004, (d) advancement of antimicrobials in the early part of this century, and (e) 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.

FIGURE 1 Phage concentration (log PFU/cm²) and log CFU/cm² reduction *Listeria* on different materials. Means with different letters were significantly different ($p < .05$)

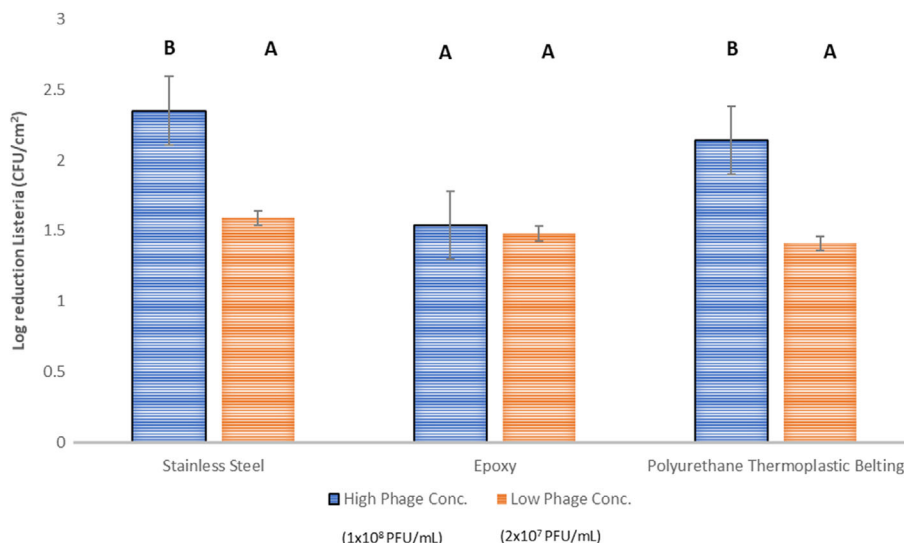
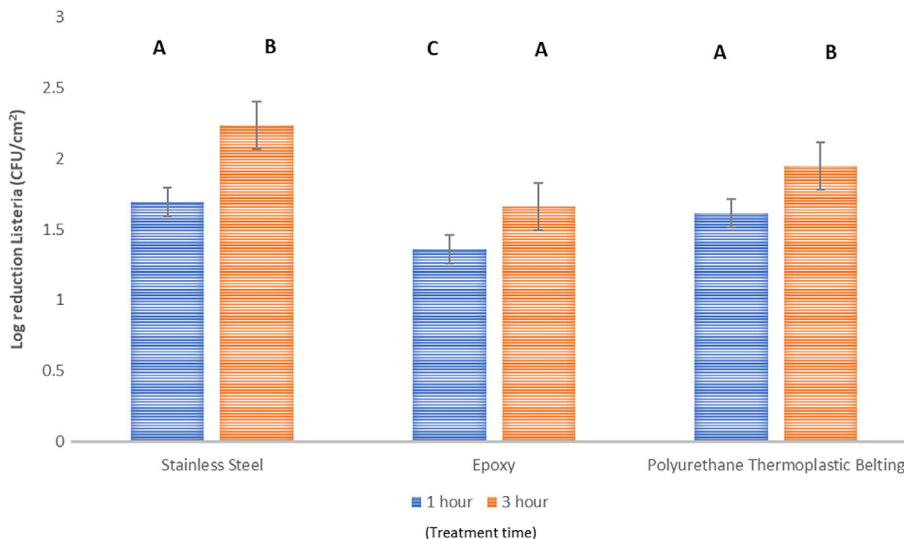


FIGURE 2 Treatment time and log CFU/cm² reduction *Listeria* on different materials. Means with different letters were significantly different ($p < .05$)



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 1 and 5% phage concentrations for both 1 and 3-hr 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 noncontact 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 & 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. While MilliQ™ water was used in these lab trials, it would not be practical in a commercial food production plant. The direct use of potable water is not recommended, due to the sensitivity of the bacteriophage to the low level of chlorine present in these municipal water sources. Therefore, in-plant treatment should use either spring water, or have an inline chlorine filter of the incoming potable water source for the facility.

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 RTE 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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ORCID

Robert G. Reinhard  <https://orcid.org/0000-0002-3388-0521>

Joseph D. Eifert  <https://orcid.org/0000-0002-2542-2459>

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