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Effectiveness of a phage cocktail as a biocontrol agent against *L. monocytogenes* biofilms

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ABSTRACT

Listeria monocytogenes can persist and form biofilms in a food environment which are difficult to eradicate because biofilms are inherently resistant to a variety of antimicrobial treatments. Therefore, alternative approaches such as bacteriophages have been suggested as a promising biocontrol agent against biofilms. The aim of this study was to evaluate the efficacy of a cocktail bacteriophage product (ListShield™) against *L. monocytogenes* biofilms. These biofilms were established on lettuce, stainless steel, rubber, and a MBEC biofilm device and exposed to the ListShield™ phage preparation (1×10^8 PFU/mL) for 2 h. ListShield™ had sufficient potency to significantly reduce the biofilm ($P < 0.05$) in all cases. Biofilm reduction achieved after ListShield™ treatment on the stainless steel coupon was 1.9–2.4 log CFU/cm^2 and on the rubber surface approximately 1.0 log CFU/cm^2. Phage application on lettuce inactivated biofilm bacteria up to 0.7 log CFU/cm^2. These results suggest that bacteriophage preparation ListShield™ is an effective tool for the inactivation of *L. monocytogenes* biofilms in the food industry.

1. Introduction

Listeriosis caused by *Listeria monocytogenes* is one of the deadliest foodborne disease owing to its high mortality rate (up to 40%). A zero-tolerance policy in the USA and a 100 CFU/gram limit in Ready-to-Eat (RTE) foods have been proposed as a contingency measure for listeriosis (Sillankorva, Oliveira, & Azeredo, 2012). Listeriosis is usually related to contaminated, minimally processed foods or associated with cross contamination after an inactivation process followed by storage at low temperatures (Chan & Wiedmann, 2009; Tompkin, 2002). The organism can survive and grow in different adverse environmental conditions such as temperature (0.4–45 °C), pH values (4–9.6), high salt content (10–20%), and low oxygen levels, which enables it to persist within food and food-processing environments for long periods (Gandhi & Chikindas, 2007; Seeliger & Jones, 1986; Walker, Archer, & Banks, 1990). *L. monocytogenes* has the ability to attach and develop biofilms on most of the surfaces that are widely used in food processing, including stainless steel, rubber gaskets, and polymers (Borucki, Peppin, White, Loge, & Call, 2003; Lundén, Autio, & Korkeala, 2002; Rieu et al., 2008). During the processing step, biofilm organisms can be easily transmitted to the final food product.

Despite several decontamination efforts adopted by the food industry, massive foodborne listeriosis outbreaks continue to emerge. In 2008, a luncheon meat outbreak caused 56 human foodborne illness and 22 deaths (Weatherill, 2009). Recent outbreaks related to consumption of fresh cuts such as lettuce, onions, and tomatoes have been linked to surface colonization by biofilm-associated pathogenic microorganisms.

Additionally, the cantaloupe outbreak in 2011 (USA) caused 146 human cases with foodborne illnesses and 30 deaths (CDC, 2011). *L. monocytogenes* strains that persist in food environments produce stronger biofilms than sporadically isolated strains (Lunden, Misttinen, Autio, & Korkeala, 2000), indicating that biofilm production is a survival strategy of *L. monocytogenes* in the food-processing environment and could be a possible source of foodborne listeriosis. Biofilms are recognized as a dominant lifestyle of microorganisms attached to a biotic or abiotic surface and embedded within self-produced extracellular polymeric substances (EPS). Bacterial biofilms are an alarming issue in the food industry owing to their increased resistance to antimicrobials,

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desiccation, heat, and disinfectants. Major foodborne pathogens such as *Listeria, Salmonella, Escherichia coli, Campylobacter*, among others, have the ability to form biofilms and pose a significant safety challenge within the food industry (Brandl, 2006; Gandhi & Chikindas, 2007; Murphy, Carroll, & Jordan, 2006; Wood, 2009). Numerous physical, chemical, and biological strategies such as natural substances, quorum sensing inhibition, ultrasound, UV-C irradiation, cold oxygen plasma, bactericidal coating, and bacteriophages have been suggested to combat biofilms. However, these approaches have limited efficacy, are less cost-effective, and most of the techniques are not practically suitable to implement in the food industry to control biofilms. Nevertheless, among these, new intervention bacteriophages seem a promising approach to eradicate biofilms from food and food contact surfaces.

Bacteriophages (commonly known as phages) are natural predators of bacteria and are highly specific to their host. Therefore, they are unlike other traditional antimicrobial approaches widely employed by the food industry that indiscriminately attack other normal and beneficial bacteria. Bacteriophage preparation has gained the Generally Recognized As Safe (GRAS) status from the FDA. Recently, numerous commercial phage-based preparations like ListShield™, Salmo Fresh™, EcoShield™ (Intralytix Inc, USA), and Listex P100™ (micreos Food Safety) have gained FDA approval for direct food applications. Current food growers, producers, and industry insiders have begun to apply a novel and promising approach for improving food safety using bacteriophages. ListShield™ was the first commercial phage preparation approved by the FDA as a food additive consisting of a cocktail of six natural and safe bacteriophages. Phage cocktails are better than monophage preparations because they can kill more strains, and resistance against them develops slower. Several studies have reported the effectiveness of bacteriophage preparations such as ListShield™ against *L. monocytogenes* present in food (Hong, Choi, Lee, & Conway, 2015; Leverentz, Conway, et al., 2003). However, very few studies have examined the efficacy of phage preparations against biofilms present in food and food contact surfaces.

Therefore, the aim of our study was to evaluate a bacteriophage preparation (ListShield™) against biofilms of *L. monocytogenes* on fresh produce (lettuce) and food-processing surfaces (stainless steel and rubber).

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three *L. monocytogenes* strains (ATCC 19113, ATCC 19115, and ATCC 13932) were used in this study. Bacterial strains were stored at −70 °C in tryptic soy broth (TSB; BD Diagnostics, Franklin Lakes, NJ, USA) containing 15% (v/v) glycerol as a cryoprotectant. Individually, each strain was twice consecutively subcultured aerobically at 30 °C for 24 h. Cultured cells were then centrifuged (4000×*g* at 4 °C for 20 min) and washed three times with sterile phosphate-buffered saline (PBS; pH 7.4). The pellets were resuspended in peptone water (PW; BD diagnostics, Franklin Lakes, NJ, USA) and the exact bacterial concentration was determined by plating the inoculum onto PALCAM agar (Oxoid, Basingstoke, England) with a PALCAM-selective supplement, followed by incubation at 30 °C for 48 h.

2.2. Bacteriophage preparation

For this study, we used the bacteriophage product ListShield™, which consists of six individually purified phages in equal proportions: List-36 (ATCC# PTA-5376), LMSP-25 (ATCC# PTA-8353), LMTA-57 (ATCC# PTA8355), LMTA-94 (ATCC# PTA-8356), and LMTA-148 (ATCC# PTAPTA-8357). Each of these monophages is specifically effective against a specific *L. monocytogenes* serotype. The phage product was obtained from Intralytix, Inc, Baltimore, MD, USA. The supplied ListShield™ is a liquid preparation that has a minimum phage concentration of 10.0 ± 0.3 log PFU/mL. Immediately before application, the mixture was diluted with sterile distilled water (pH 6.5 to 7.0) to approximately 1×10^8 PFU/mL.

2.3. Transmission electron microscopy (TEM) for ListShield™ phages

To visualize the phage appearance in ListShield™, a suspension of the bacteriophage preparation was centrifuged at 25,000×*g* for 2 h. Then, the supernatant of the suspension was removed and the pellet was resuspended in sterile distilled water. The phage suspension was dripped onto a carbon-coated 300 mesh copper grid and fixed for 1 min. The extra liquid was removed and the grid was air-dried for 30 s at room temperature (23 ± 2 °C). After air-drying, the grid was negatively stained with 2% uranyl acetate for 10 s and washed twice in sterile distilled water. Energy-Filtering Transmission Electron Microscope (EF-TEM, LIBRA 120; Carl Zeiss, Oberkochen, Germany) images were taken at 135,000× with 120 kv accelerating voltage (Fig. 5).

2.4. Sensitivity of L. monocytogenes strains to ListShield™

The sensitivity of the phages in ListShield against three different *L. monocytogenes* strains was examined by a plaque-forming assay. The bacteriophage preparation was serially diluted in a sterile SM buffer (100 mM NaCl, 10 MmM $MgSO_4$, 50 mM Tris HCl, pH 7.5), and 100 μL of the diluted phage suspension was mixed with 100 μL of the different bacterial strains (optical density at 600 nm−0.4) in 5 mL of sterile soft agar (TSB containing 0.4% agar) at 48 °C. The soft agar mixture was gently vortexed, overlaid onto a substrate of the TSA agar plate and distributed evenly by gentle rotation of the plate. The agar plates were allowed to solidify for 30 min at room temperature, and subsequently incubated in an inverted position for 18–24 h at 37 °C. Following the incubation period, the ability of the phage to form plaques on different bacterial strains was recorded.

2.5. Biofilm formation and effect of phages against biofilm cells on stainless steel and rubber surfaces

ListShield™ was tested for its ability to remove biofilm cells from stainless steel and rubber surfaces inoculated with *L. monocytogenes* strains. Stainless steel (2 × 2 × 0.1 cm, type: 302) coupons were processed as previously described (Shen, Luo, Nou, Bauchan, Zhou, & Wang, 2012). Rubber (Latex, natural rubber, 0.06 thickness) was cut into pieces (2 × 2 cm) with sterile scissors. For sterilization, the rubber pieces were dipped into 70% ethanol for 10 min followed by switching on UV light for 1 h and air-drying in a biosafety cabinet overnight. The bacterial culture was diluted (1:50) and inoculated into TSB in 50 mL Falcon tubes; stainless steel and rubber coupons were placed in each Falcon tube containing 10 mL TSB for biofilm formation. The tubes were incubated without shaking at 30 °C for 72 h with a medium change every 24 h, and at 10 °C for 144 h without a medium change to develop mature biofilms on the stainless steel and rubber coupons. After the incubation period, coupons were removed from the tubes and washed three times with PBS to remove non-biofilm cells. Bacteriophage treatment on biofilms was performed as previously described (Sillankorva, Neubauer & Azeredo, 2008), with minor changes. Briefly, the coupons were submerged in a tube containing 5 mL TSB and 5 mL ListShield™ solution (final concentration of 10^8 PFU/mL)