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Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods

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ABSTRACT

ListShield™, a commercially available bacteriophage cocktail that specifically targets *Listeria monocytogenes*, was evaluated as a bio-control agent for *L. monocytogenes* in various Ready-To-Eat foods. ListShield™ treatment of experimentally contaminated lettuce, cheese, smoked salmon, and frozen entrées significantly reduced ($p < 0.05$) *L. monocytogenes* contamination by 91% (1.1 log), 82% (0.7 log), 90% (1.0 log), and 99% (2.2 log), respectively. ListShield™ application, alone or combined with an antioxidant/anti-browning solution, resulted in a statistically significant ($p < 0.001$) 93% (1.1 log) reduction of *L. monocytogenes* contamination on apple slices after 24 h at 4 °C. Treatment of smoked salmon from a commercial processing facility with ListShield™ eliminated *L. monocytogenes* (no detectable *L. monocytogenes*) in both the naturally contaminated and experimentally contaminated salmon fillets. The organoleptic quality of foods was not affected by application of ListShield™, as no differences in the color, taste, or appearance were detectable. Bio-control of *L. monocytogenes* with lytic bacteriophage preparations such as ListShield™ can offer an environmentally-friendly, green approach for reducing the risk of listeriosis associated with the consumption of various foods that may be contaminated with *L. monocytogenes*.

1. Introduction

Listeriosis, caused by the Gram-positive bacterial pathogen *Listeria monocytogenes*, is a major foodborne illness in the United States. Listeriosis in adults usually occurs after consumption of contaminated food, with Ready-To-Eat (RTE) foods being responsible for nearly all of the 1600 listeriosis infections reported annually in the United States (Centers for Disease and Prevention, 2013; Scallan et al., 2011). The disease is most frequently clinically manifested as septicemia, central nervous system infections, and feto-maternal infections. Persons over the age of 65, pregnant women, and immunocompromised individuals account for approximately 75% of these infections (Centers for Disease and Prevention, 2013). Of particular concern are both the high mortality rate and the ability of *L. monocytogenes* to cross the placenta and infect the fetus. In susceptible populations, nearly 30% (Mead et al., 1999) of those who have listeriosis will die (as compared to, for example, 0.04% and 0.33% for diseases caused by nontyphoidal *Salmonella* and *Toxoplasma gondii*, respectively (Mead et al., 1999; Scallan et al., 2011)). In addition, colonization or infection of pregnant women, who may be asymptomatic carriers, can cause prenatal or perinatal disease, which often results in loss of the fetus (Endersen et al., 2014).

L. monocytogenes is a particularly problematic foodborne pathogen because it can thrive under conditions that normally inhibit the growth of other types of bacteria, including low temperatures, high salt, and acidic conditions (Fernandes et al., 1998; Gandhi and Chikindas, 2007). Post-processing contamination of RTE foods by *L. monocytogenes* can also occur through environmental contact and poor sanitation (Carpentier and Cerf, 2011; Lianou and Sofos, 2007). In recent years, the largest multi-state outbreaks of listeriosis have been due to contaminated RTE foods, including outbreaks from Mexican style cheese (142 cases), frankfurters (108 cases), and cantaloupe (147 cases) (Centers for Disease and Prevention, 2013). Thus, there are a number of treatments currently employed using

Abbreviations: AS, antibrowning solution; PBS, phosphate buffered saline; PFU, plaque forming unit; CFU, colony forming unit; RTE, ready-to-eat; LB, Luria–Bertani.

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either physical or chemical means of decontamination (Aymerich et al., 2008; Beuchat and Brackett, 1990; Gunes et al., 2012; Islam et al., 2002; Yoon et al., 2004). These methods vary in their efficacy and also tend to be fairly broad–spectrum; i.e., they kill all bacteria present in the foods, a majority of which are beneficial – which reduces the nutritional/health value of the foods. Moreover, some of these methods, such as thermal processing or ionizing irradiation, can result in changes to the organoleptic properties of the food (Ferrini et al., 2014; Millar et al., 2000a, c; Wheeler et al., 1999). Bacteriophages, or phages, may offer natural means to specifically reduce or eliminate specific foodborne bacteria in foods, without many of the above-mentioned shortcomings (Endersen et al., 2014).

Bacteriophages are viruses that kill bacteria. They are the most ubiquitous organisms on Earth, with the numbers estimated to be 10^{30}–10^{32} (Ackermann and Wegrzyn, 2014). Phages can be isolated from the environment, water, and the fresh foods we eat, and are common components of the microflora of the human gut (Armon and Kott, 1993; Bergh et al., 1989; Breitbart et al., 2003; Gautier et al., 1995; Hsu et al., 2002; Kennedy et al., 1986; Whitman and Marshall, 1971a, b). Virulent (lytic) bacteriophages infect bacteria, resulting in lysis of the bacteria (i.e., their action is bactericidal) while replicating themselves. Importantly, bacteriophages are highly specific to the subgroup of strains (usually within the same species or across closely related species) of bacteria they kill. Therefore, and unlike traditional, non-specific chemical antimicrobials and other interventions currently employed by the food industry, bacteriophage-based bio-control may enable targeted elimination of a specific foodborne bacteria in foods without destroying normal and often beneficial microflora of the foods (Bueno et al., 2012; Guenther and Loessner, 2011).

A number of bacteriophage based preparations have been approved for direct food applications in the United States and Europe, including (in chronological order of receiving Food and Drug Administration (FDA) clearances) ListShield™ (formerly LMP-102), Listex P-100™, EcoShield™, SalmoFresh™, and Salmonelex™. This study evaluated the ability of one of these commercial preparations (ListShield™) to reduce or eliminate the *L. monocytogenes* contamination in various foods (including apples, lettuce, cheese, and smoked salmon), and its impact on the organoleptic properties (including taste, sight, and smell) of various RTE foods.

2. Materials and methods

2.1. Bacteriophage preparation

The bacteriophage product used in our studies is ListShield™, a cocktail of six lytic bacteriophages: LIST-36 (ATCC # PTA-5376), LMSP-25 (ATCC # PTA-8353). LMTA-34 (ATCC # PTA-8354), LMTA-57 (ATCC # PTA-8355), LMTA-94 (ATCC # PTA-8356), LMTA-148 (ATCC # PTA-8357). The preparation complies with FDA food additive regulations, for direct application to meat and poultry products that meet the ready-to-eat definition (21 CFR § 172.785). ListShield™ lots 0111E040222, 0112B240183, 0113H150142, 0108A040159, 0109B060109, and 10022003A (all ca. 1×10^{10} plaque forming units (PFU)/mL) were used during this study. The titers of these lots were determined using the agar layer method (Adams, 1959). The lots were diluted appropriately, as indicated in relevant sections below, before applying onto foods.

2.2. Media and reagents

L. monocytogenes were grown in Luria–Bertani (LB), Miller agar and broth (Neogen). Oxford agar and Oxford *Listeria* Selective Supplement (Fluka Biochemica) were used to plate and count *L. monocytogenes* colonies. Peptone water (Becton, Dickinson and Co.) was used to suspend food samples except for the experiments at the salmon production facility where samples were suspended in Buffered *Listeria* Enrichment Broth (BLEB) (Acumedia Manufacturers, Inc.). For experiments with apples, an antioxidant/anti-browning solution (AS) (80 g/L), a proprietary blend that included vitamin c and calcium, was used (supplied from a fresh cut apple producer).

2.3. Bacterial strains and inoculum preparation

A single strain culture of *Lm*320 (ATCC 19115, serotype 4b) was used in studies on lettuce, and *Lm*376, an environmental strain obtained from a Scottish smoked salmon facility, was used in the studies at the same smoked salmon production facility. To test the efficacy of ListShield™ when challenged with a cocktail of bacteria, a 1:1:1 mixture of three *L. monocytogenes* strains, *Lm*68 (serotype 1/2b), *Lm*82 (serotype 1/2a) and *Lm*320 (ATCC 19115, serotype 4b) was used in the apple, cheese, smoked salmon, and frozen entrées studies. The strains were grown in LB media at 37 ± 2 °C for up to 24 h to a target concentration of ca. 10^8 colony forming units (CFU)/mL. Challenge cultures were diluted in LB for each experiment as necessary to achieve a specific contaminating dose specified in the respective sections below and unless otherwise stated, the volume of bacteria added was between 0.5 mL and 1 mL.

2.4. ListShield™ application

All foods were treated by application of either phosphate buffered saline (PBS) or sterile water, for control samples, or ListShield™ for test samples. ListShield™ was diluted with sterile water or PBS, according to the control treatment used in the respective experiment. In all instances, application was via spraying, using a spray gun (Badger Air-Brush Co., Franklin Park, IL; Basic Spray Gun, model #250-2).

2.5. Effect of ListShield™ on experimentally contaminated lettuce

Long-leaf green lettuce was obtained from a local grocery store in Baltimore, MD. Nine 100 g portions of lettuce were measured. The *L. monocytogenes* innocula was applied onto the lettuce surfaces at ca. 2×10^3 CFU/g of lettuce. The samples were covered loosely and the bacteria were allowed to colonize the samples' surfaces at room temperature for 60 min. All samples were treated at the rate of 1 mL/100 g lettuce; the control group (three 100 g samples) was treated with sterile water and the test groups (three 100 g samples each) were treated with ListShield™ at 1×10^7 PFU/g or 1×10^8 PFU/g of lettuce. Following a 5 min incubation at room temperature, three 25 g portions were cut from each group and placed into sterile bags to which 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached (Stomacher 400, Seward) for a approximately 30 s. The number of viable *L. monocytogenes* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached lettuce/peptone water mixture onto separate Oxford plates, containing Oxford *Listeria* Selective Supplement, and incubated at 35 ± 2 °C for 24 ± 2 h. The resulting colonies were counted and the CFU/g was calculated taking sample size and dilution into account.

Residual free phages were not removed from the homogenized samples prior to bacterial enumeration; however, we believe that the reductions observed were not due to residual phage. In that context, the duration of our studies, from the time the food is sampled, stomached and plated, was <7 min. In contrast, a phage lytic cycle takes 20–40 min to complete; therefore there would not be sufficient time for phage replication during our study (i.e. free