



Efficacy of bacteriophage LISTEX™ P100 combined with chemical antimicrobials in reducing *Listeria monocytogenes* in cooked turkey and roast beef

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ABSTRACT

The aim of this study was to verify the effectiveness of the commercially available anti-*Listeria* phage preparation LISTEX™ P100 in reducing *Listeria monocytogenes* on ready-to-eat (RTE) roast beef and cooked turkey in the presence or absence of the chemical antimicrobials potassium lactate (PL) and sodium diacetate (SD). Sliced RTE meat cores at 4 and 10 °C were inoculated with cold-adapted *L. monocytogenes* to result in a surface contamination level of 10³ CFU/cm². LISTEX™ P100 was applied at 10⁷ PFU/cm² and samples taken at regular time intervals during the RTE product's shelf life to enumerate viable *L. monocytogenes*. LISTEX™ P100 was effective during incubation at 4 °C with initial reductions of *L. monocytogenes* of 2.1 log₁₀ CFU/cm² and 1.7 log₁₀ CFU/cm², respectively, for cooked turkey and roast beef without chemical antimicrobials (there was no significant difference to the initial *L. monocytogenes* reductions in the presence of LISTEX™ P100 for cooked turkey containing PL and roast beef containing SD-PL). In the samples containing no chemical antimicrobials, the presence of phage resulted in lower *L. monocytogenes* numbers, relative to the untreated control, of about 2 log CFU/cm² over a 28-day storage period at 4 °C. An initial *L. monocytogenes* cell reduction of 1.5 log₁₀ CFU/cm² and 1.7 log₁₀ CFU/cm², respectively, for cooked turkey and roast beef containing no chemical antimicrobials was achieved by the phage at 10 °C (abusive temperature). At this temperature, the *L. monocytogenes* cell numbers of samples treated with LISTEX™ P100 remained below those of the untreated control only during the first 14 days of the experiment for roast beef samples with and without antimicrobials. On day 28, the *L. monocytogenes* numbers on samples containing chemical antimicrobials and treated with LISTEX™ P100 stored at 4 and 10 °C were 4.5 log₁₀ CFU/cm² and 7.5 log₁₀ CFU/cm², respectively, for cooked turkey, and 1.2 log₁₀ CFU/cm² and 7.2 log₁₀ CFU/cm², respectively, for roast beef. In both cooked turkey samples with and without chemical antimicrobials stored at 10 °C, the phage-treated samples had significantly lower numbers of *L. monocytogenes* when compared to the untreated controls throughout the 28-day storage period ($P < 0.0001$). For roast beef and cooked turkey containing chemical antimicrobials treated with LISTEX™ P100 and stored at 4 °C, no more than a 2 log CFU/cm² increase of *L. monocytogenes* was observed throughout the stated shelf life of the product. This study shows that LISTEX™ P100 causes an initial reduction of *L. monocytogenes* numbers and can serve as an additional hurdle to enhance the safety of RTE meats when used in combination with chemical antimicrobials.

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1. Introduction

Listeria monocytogenes is a major food-borne pathogen of public health concern with a high mortality rate in at risk individuals such as pregnant women, neonates, immunocompromised individuals, and the elderly (Farber and Peterkin, 1991). *L. monocytogenes* has the ability to grow at a wide range of temperatures (0.4 °C to 45 °C) and pH values (4 to 9.6) enabling it to persist within food processing environments for

long periods (Walker et al., 1990; Gandhi and Chikindas, 2007). Human listeriosis cases have been traced to consumption of contaminated dairy products, unwashed raw vegetables, cantaloupes, under-cooked meat, delicatessen (deli) meats, seafood, and poultry products (Painter and Slutsker, 2007). Of the 24 listeriosis outbreaks in the United States of America between 1998 and 2008, deli meats were implicated in six incidents (Catwright et al., 2013). The Canadian listeriosis outbreak of 2008 was linked to consumption of contaminated ready-to-eat (RTE) deli meats (Gilmour et al., 2010).

RTE foods are classified according to consumer risk. Category 1 RTE foods are those foods which can support the growth of *L. monocytogenes*

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throughout the stated shelf life. These foods include deli-meats, soft cheeses, hot dogs, and pâté. Category 2 is subdivided into 2A and 2B. Category 2A is composed of RTE food products in which limited growth of *L. monocytogenes* to levels not greater than 100 CFU/g can occur throughout the stated shelf life. These foods include refrigerated gravlax/cold-smoked rainbow trout and salmon, fresh-cut produce, etc. For these foods, processors are required to validate and verify their processes to ensure that the levels of *L. monocytogenes* are consistently equal to or less than 100 CFU/g throughout the stated shelf life. Category 2B is composed of RTE food products in which the growth of *L. monocytogenes* cannot occur throughout the expected shelf life of that food. Category 2B foods include ice cream, hard cheese, dry salami, dried-salted fish, and varieties of prosciutto ham (Health Canada, 2011). In Canada and the United States of America, food processors are advised to employ antimicrobial agents/processes and/or post-lethality procedures to suppress or limit growth of *L. monocytogenes* in RTE meat and poultry products. An antimicrobial agent is considered acceptable if it allows no more than 2 log CFU/g increase of *L. monocytogenes* throughout the stated shelf life of the product. RTE products produced using this criterion may qualify to a lower relative risk level for sampling purposes within their respective food risk category but cannot be moved to a lower food risk category (Canadian Food Inspection Agency, 2011; Food Safety and Inspection Service, FSIS, 2012).

Bacteriophages have been found to be effective in the control of *L. monocytogenes* on food (Leverentz et al., 2003; Carlton et al., 2005; Schellekens et al., 2007; Guenther, 2009; Soni et al., 2010; Soni and Nannapaneni, 2010). In 2006, the U.S. Food and Drug Administration approved two bacteriophage preparations (LISTEX™P100 and LMP-102) for use in a select number of foods to control *L. monocytogenes* contamination (U.S. Food and Drug Administration, 2006a, 2006b, 2007). In 2010, a letter of no objection was issued by Health Canada for application of LISTEX™P100 as a processing aid against *L. monocytogenes* in deli meat and poultry products, cold-smoked fish, vegetable prepared dishes, soft cheeses, and/or other dairy products (Microcos Food Safety, 2010). The Food Standards Australia/New Zealand (FSANZ) also approved LISTEX™P100 as a processing aid to reduce contamination of *L. monocytogenes* in a variety of foods (Food Standards Australia/New Zealand, 2012).

Previous studies have shown that successful phage-based pathogen intervention in RTE products greatly depends on the phage dose applied, the chemical composition of the food and its specific matrix (Guenther et al., 2009; Spricigo et al., 2013). It has thus been suggested that there is a need to individually optimize protocols for the application of phages with respect to the phages and the target bacteria as well as considering the food matrix (Guenther et al., 2009). Phages remain stable on solid food surface and their initial concentrations can be recovered throughout the storage period of the food where they have been applied. However, the phages are immobilized soon after addition and due to limited diffusion cannot infect bacteria (Guenther et al., 2009). This results in growth of bacteria that have survived phage treatment. It has thus been suggested that additional hurdles be present in the product to further inhibit the outgrowth of *L. monocytogenes* (Holck and Berg, 2009).

The objective of this study was to determine the efficacy of LISTEX™P100 in reducing *L. monocytogenes* on RTE roast beef and cooked turkey while taking into account real-world scenarios and avoiding common errors reported in previous phage decontamination assays. Some of the key features of our assay that are lacking in previous phage studies are as follows: (a) a *L. monocytogenes* four strain cocktail was added at low and realistic concentrations that occur in real-life scenarios (Wong et al., 2005), (b) bacterial viable counts were determined following removal of unbound phages from stomached rinses prior to direct plating for *L. monocytogenes* in order to avoid overestimation of phage killing effect, (c) the rate of phage and host application are presented per unit area, and (d) the phage decontamination study was performed at recommended storage temperature

of the cooked turkey and roast beef (4 °C) in comparison to abusive temperature (10 °C).

Chemical antimicrobials such as lactates and diacetates act as bacteriostatic agents against *L. monocytogenes* in meat and poultry products (Qvist et al., 1994; Blom et al., 1997). In this study, we also evaluated the efficacy of LISTEX™P100 in reducing *L. monocytogenes* on RTE cooked turkey and roast beef meat slices containing these growth inhibitors. The cooked turkey contained potassium lactate (PL), whereas the roast beef contained sodium diacetate (SD) and PL. Lactates inhibit *L. monocytogenes* growth through reduction of water activity (a_w) of the product (Miller and Acuff, 1994) and intracellular acidification (Hunter and Segal, 1973), whereas SD is bactericidal by lowering the intracellular pH (Shelef and Addala, 1994). Health Canada and the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) permit 0.25% SD as a flavor enhancer and as an inhibitor of pathogen. PL-SD in fully cooked meat, meat-food products, poultry, and poultry-food products, are permitted at levels of up to 2–3% of total product formulation (Food Safety and Inspection Service, FSIS, 2012; Health Canada, 2008, 2012b).

Soni et al. (2012) evaluated the single and combined effects of phage preparation LISTEX™P100, lauric arginate, and potassium lactate-sodium diacetate mixture (PL-SD) against *L. monocytogenes* cold growth in queso fresco cheese (QFC). LISTEX™P100 was found to be stable in the presence of lauric arginate and PL-SD. The combined treatment of LISTEX™P100 with PL-SD reduced the initial *L. monocytogenes* counts by 2–4 log₁₀ CFU/cm² and also kept the *L. monocytogenes* counts at that reduced level in QFC for 28 days at 4 °C.

In this study, these growth inhibitors would serve as an additional hurdle to prevent the outgrowth of *L. monocytogenes* that have escaped killing by the LISTEX™P100. Thus, our aim was to observe whether an additive effect exists between the phage and growth inhibitors in reducing *L. monocytogenes* on the RTE turkey and beef.

2. Materials and methods

2.1. *L. monocytogenes* strains and phages

Four *L. monocytogenes* strains were used in this study. These were the Canadian 2008 outbreak strain 08-5578 (serotype 1/2a) obtained from The National Microbiology Laboratory, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba (Gilmour et al., 2010) and strains Li0512 (serotype 1/2b), Li0529 (serotype 1/2a), and ATCC19115 (serotype 4b) from the University of Guelph Laboratory Services Division, Guelph, ON, Canada. The inoculum included a representative strain of serotypes 1/2a, 1/2b, and 4b and a strain isolated from an outbreak or sporadic case as recommended by the Health Canada stipulated method of assessing the efficacy of antimicrobial treatments for *L. monocytogenes* in RTE refrigerated foods (Health Canada, 2012a).

Bacteriophage LISTEX™P100 (MICREOS Food safety, Inc., Wageningen, The Netherlands) was serially diluted in sterile SM buffer (10 mM NaCl, 10 mM MgSO₄, 50 mM Tris · HCl, pH 7.5) to a working stock of 1×10^9 PFU/ml.

2.2. Deli meats

Sliced roast beef and cooked turkey that contained no chemical antimicrobials were obtained directly from a processing facility and stored at 4 °C overnight prior to use in experiments. Sliced roast beef containing SD and PL and cooked turkey containing sodium nitrite and PL (as stated on the packaging labels) from the same lot number were purchased from a local supermarket in Guelph, ON. The concentrations of the chemical antimicrobials were 2.8% for PL in cooked turkey and 0.2% SD and 2.8% PL + 0.2% SD for the PL-SD combination in roast beef. This is within the recommended levels by Health Canada (Health Canada, 2008, 2012b). All experiments were conducted in triplicate with a single batch of product of each deli meat used for tests.

2.3. Sample preparation

To prepare inoculum for spiking RTE meat samples, each strain was cultured in 5 ml Trypticase Soy Broth (TSB; BD Biosciences, San Jose, CA, USA) at 37 °C for 24 h. This resulted in cell concentrations of 10^9 CFU/ml (equivalent to an optical density at 600 nm [OD₆₀₀ ~ 1.2]). The *L. monocytogenes* cell cultures were then centrifuged at 4420 g for 10 min, the supernatant discarded and the pellet resuspended in 5 ml phosphate-buffered saline (PBS; 100 mM NaCl, 20 mM Na₂HPO₄, pH 7.4). Serial dilutions of the *L. monocytogenes* cell suspensions in PBS were then performed to obtain desired cell numbers. The target viable count in spiked RTE meat was 10^3 CFU/cm². This inoculation level mimics the inoculum concentration likely to occur in commercial RTE product (Wong et al., 2005) and is also within the recommended inoculation level of between 10^2 and 10^5 CFU/cm² by Health Canada to be used when evaluating antimicrobial agent or post-processing lethality tests (Scott et al., 2005; Health Canada, 2012a). The four *L. monocytogenes* strain cultures with target viable count of approximately 10^3 CFU/cm² were then mixed to form a four-strain cocktail.

The RTE meat samples were placed on sterile cutting boards and using sterilized stainless steel core cutters, uniform cores of RTE meat with 10 cm² top-surface areas and 5 mm thick were excised from the RTE meat slices. To prepare the spiked samples, three cores were placed individually on a Styrofoam tray (Dyne-A-Pak Inc., Laval, QC Canada) and were inoculated with 100 µl volume of serially diluted four-strain cocktail of *L. monocytogenes* to yield an inoculation level of approximately $3 \log_{10}$ CFU/cm². The bacteria were distributed using a sterile spreader and were allowed to adhere for 15 min. The trays with the inoculated (positive control) or uninoculated (negative control) cores were then transferred to 8 × 6-inch commercial barrier bags (oxygen transmission rate of 40–50 cc m⁻² 24 h⁻¹; Winpak Ltd., Winnipeg, MB Canada) and vacuum packaged (Multivac AGI, Knud Simonsen Industries Ltd, Rexdale, ON Canada). After vacuum sealing, the samples were incubated for 30 min, 1, 2, 3, 7, 10, 14, 20, and 28 days at 4 or 10 °C and then subjected to *L. monocytogenes* enumeration.

2.4. Phage treatment

Phage-treated samples were prepared the same way as the spiked samples except that a phage treatment step was incorporated before vacuum sealing and incubation of the samples at the required temperatures. The concentration of Listex™P100 in the preparation was determined by standard soft agar overlay method and then appropriately diluted to achieve 2.0×10^7 PFU/cm² when 100 µl of the diluted phage was added to each spiked sample. The diluted phage preparation was added on the same surface side that the *L. monocytogenes* cells were inoculated. For the “no phage” control, the RTE meat samples were surface treated with 100 µl PBS. These were left in a biosafety level-2 laminar flow hood for 15 min before being put in vacuum system bags (Winpak) and vacuum sealed (Multivac). The samples were then incubated for 30 min, 1, 2, 3, 7, 10, 14, 20, and 28 days at 4 or 10 °C then subjected to *L. monocytogenes* enumeration.

2.5. Enumeration of *L. monocytogenes*

Three roast beef or cooked turkey samples per treatment were observed at each sampling period (30 min, 1, 2, 3, 7, 10, 14, 20, and 28 days) to determine the inhibitory activity of Listex™P100 alone or in combination with PL and SD against the growth of *L. monocytogenes*.

A virucidal solution was prepared as described by Jassim (1998). Briefly, 330 µl of tea infusion [7.5% w/v black loose-leaf tea (Kenyan Tinderet; Davids Tea, Montreal, QC, Canada) boiled in milliQ water for 10 minutes and filtered using Whatman paper (Grade 4: 20–25 µm); Whatman International Ltd., Ipswich, UK] was mixed with 700 µl of freshly prepared 4.3 mM ferrous sulphate.

RTE meat samples inoculated with bacteria only and those inoculated with bacteria and treated with phage were aseptically transferred into stomacher bags fitted with filters (BagPage, BagSystem, Interscience, St-Nom-la-Breteche, France) containing 15 ml PBS and 2 ml virucidal solution to inactivate the remaining phage on the samples. This was homogenized for 2 min in a stomacher (model 80, Seward Medical, London, UK) at 230 rpm. After homogenization, the virucide was inactivated by addition of 2% Tween 80 (Fisher BioReagents, Fairlawn, NJ, USA). The solution was then centrifuged at 4420g for 10 min and the supernatant discarded except for 1 ml, which was used to resuspend the pellet, and 100 µl of the homogenate was then plated in duplicate on 90 mm Oxford agar plates (EMD Chemicals Inc., Gibbstown, NJ, USA). This was determined to allow a mean 89.05% ($\pm 1.6\%$ Standard deviation) recovery of *L. monocytogenes*. The same procedure, except for the addition of the virucide, was repeated with the control samples included on each sampling day, which had not been inoculated with bacteria and also had no phage added to them. This was to serve as a negative control for confirming the lack of any background *L. monocytogenes* in the samples.

When required, a serial dilution step was performed after resuspending the pellet to yield a countable plate (30–300 CFU/plate of Oxford agar with selective supplement) for *L. monocytogenes*. When the recovered *L. monocytogenes* numbers were below 30 CFU/plate, the entire pellet in the 1 ml was spread plated using 250 µl per plate for four Oxford agar plates. After 48 h of incubation at 37 °C, *L. monocytogenes* CFU were counted in Oxford agar plates and resulting numbers multiplied with the dilution factor for determination of CFU/cm².

2.6. Test for phage resistance by regrown *L. monocytogenes* cells after phage Listex™P100 treatment

Ten *L. monocytogenes* colonies per phage treatment condition (Listex™P100 + cooked turkey; Listex™P100 + roast beef; Listex™P100 + cooked turkey + PL; Listex™P100 + roast beef + PL-SD) were randomly picked from Oxford agar plates (total 40 colonies) and streaked onto non selective Trypticase Soy Agar (TSA) plates. Single independent colonies were isolated from three streaking stages and were used to prepare *L. monocytogenes* liquid cultures in TSB media. For phage assay, 200 µl of the liquid cultures of bacterial isolates was mixed with 3 ml of molten 0.5% soft agar and serially diluted phage Listex™P100, and then poured onto TSA plates. After solidifying and drying, the plates were incubated overnight at 30 °C. The number of plaques formed on these isolates was compared to the number for the wild-type *L. monocytogenes* strain. Isolates on which Listex™P100 did not form plaques were subjected to a further test to confirm that they are truly resistant and no longer amenable to phage treatment. In this test, approximately 3×10^2 CFU of the isolate was plated on Oxford agar plate and the plate subsequently spread with 1×10^7 PFU/cm² Listex™P100. The plates were then incubated at 37 °C for 48 h. The number of colonies in the Listex™P100-treated plate were compared to those on the un-treated plate. A comparison was also made between the reduction of colonies due to Listex™P100 treatment in the phage-sensitive strain and the strains which did not support plaque formation.

2.7. Determining infective phage remaining on RTE meat after phage treatment

Phage Listex™P100 stability on roast beef and cooked turkey was determined at both 4 °C and 10 °C during the 28-day storage period. Phage Listex™P100 (100µl) was used to surface treat the 10 cm² cores of either roast beef or cooked turkey to yield a final concentration of 10^7 PFU/cm². After phage treatment, Styrofoam trays containing the triplicate samples were vacuum sealed and stored at 4 and 10 °C for up to 28 days. Phage Listex™P100 was enumerated at 30 min, 1, 2, 3, 7, 10, 14, 20, and 28 days using the standard plaque-forming assay (Adams, 1959) with *L. monocytogenes* strain ATCC 19115 used as

indicator strain. After each incubation period, the roast beef or cooked turkey sample was homogenized in 5 ml PBS buffer by stomaching and the entire homogenate filter sterilized with 0.22- μm filter syringe. The filtered homogenate was serially diluted and used in the plaque-forming assay to determine the titre of the phage after each incubation period.

2.8. Effectiveness of virucide in inactivating LISTEX™P100

Virucidal solution was prepared as outlined in Section 2.5. Approximately 3×10^2 CFU of the four-strain *L. monocytogenes* cocktail was prepared as in Section 2.3. Eighty microliters of the virucide was mixed with 100 μl LISTEX™P100 (Concentration 1×10^9 PFU/cm²) and 100 μl of the 3×10^2 CFU *L. monocytogenes* cocktail. The mixture was then spread on Oxford agar plate and the plates incubated at 37 °C for 48 h. A control without the virucide comprising of a mixture of 80 μl of the PBS mixed with 100 μl LISTEX™P100 (Concentration 1×10^9 PFU/cm²) and 100 μl of the 3×10^2 CFU *L. monocytogenes* cocktail was also plated on Oxford agar and incubated at 37 °C for 48 h. The experiment was done in triplicate and the average percentage number of *L. monocytogenes* colonies recovered in the LISTEX™P100 treated with virucide plate were compared to those on the no-virucide plate.

2.9. Statistical analysis

All the test results of the negative control samples yielded no recovery of *L. monocytogenes* throughout the 28 days. Therefore, the recovered *L. monocytogenes* were from the artificial inoculation only. The recovered *L. monocytogenes* numbers were transformed into log₁₀ in order to normalize the data. Analysis of variance (ANOVA) was conducted to examine the effect of phage and chemical antimicrobials as well as their interaction, for cooked turkey and roast beef stored at 4 and 10 °C separately using the general linear model in SAS 9.3 (SAS Institute Inc., Cary, NC). Significance was based on a level of 5.0% ($P < 0.05$).

3. Results

3.1. Effectiveness of virucide in inactivating LISTEX™P100

The virucidal solution, which comprised of black tea extract and 4.3 mM ferrous sulphate, was prepared and its efficacy in inactivating LISTEX™P100 determined as described in the Materials and methods. An average of 89% of the initial number of *L. monocytogenes* recovered on Oxford agar plates in samples treated with virucide and LISTEX™P100 compared to 2% recovery of the initial number of *L. monocytogenes* in the samples containing LISTEX™P100 without virucide.

3.2. Growth of *L. monocytogenes* inoculated on sliced cooked turkey and roast beef not containing any chemical antimicrobials and treated with phage LISTEX™P100

In cooked turkey positive controls (slices inoculated with *L. monocytogenes* only), the *L. monocytogenes* population increased from initial concentration of 3.4 log₁₀ CFU/cm² to 8.5 log₁₀ CFU/cm² during the 28-day storage at both 4 °C (Fig. 1A) and 10 °C (Fig. 1B). The maximum *L. monocytogenes* counts were reached after 28 days incubation in the case of the 4 °C incubation. In contrast, these numbers were realized after only 7 days incubation at 10 °C. In both cooked turkey and roast beef, the *L. monocytogenes* numbers exceeded the 2 log₁₀ CFU/cm² after 10 days and 3 days at 4 and 10 °C, respectively. Treatment of the cooked turkey with phage LISTEX™P100 reduced the *L. monocytogenes* counts by 2.1 log₁₀ CFU/cm² to a minimum level of 1.3 log₁₀ CFU/cm² after 2 days storage at 4 °C. At 10 °C, phage LISTEX™P100 reduced the *L. monocytogenes* on cooked turkey to a minimum of 2.0 log₁₀ CFU/cm² (1.4 log₁₀ CFU/cm² reduction) after

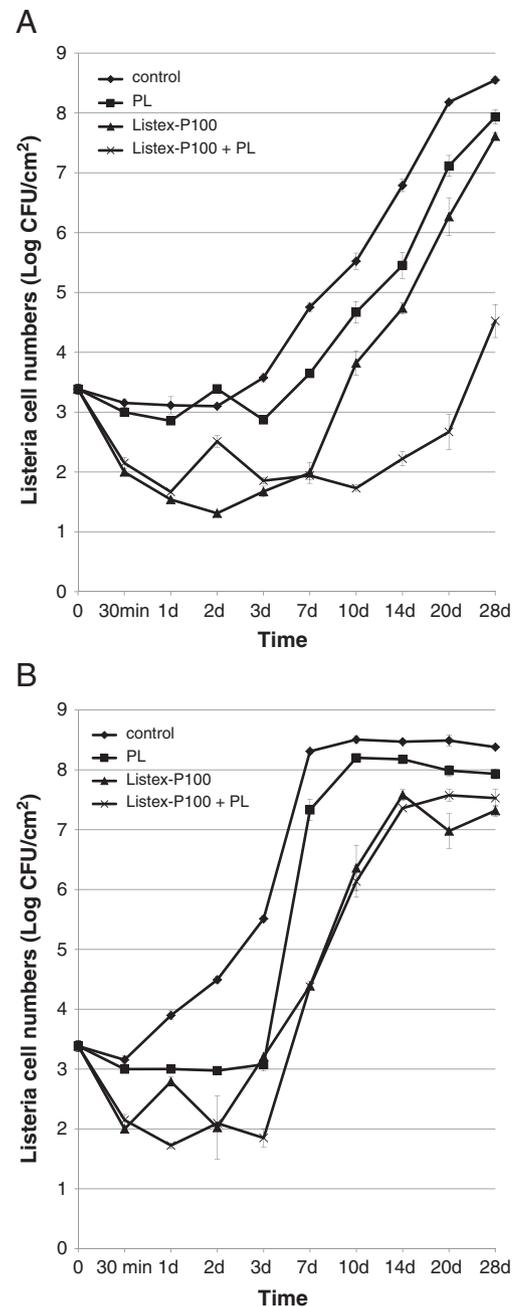


Fig. 1. Effect of LISTEX™P100 on the growth of four-strain cocktail of *L. monocytogenes* when applied alone (LISTEX-P100) or on cooked turkey sample containing potassium lactate (LISTEX-P100 + PL) and compared to no phage treatment (control) growth and on cooked turkey containing potassium lactate alone (PL) at 4 °C (Fig. 1A) and 10 °C (Fig. 1B). Bars represent SE.

2 days storage. There was regrowth of *L. monocytogenes* during subsequent storage at 4 °C and at 10 °C, with cells growing back to 7.6 log₁₀ CFU/cm² at 4 °C and 7.3 log₁₀ CFU/cm² at 10 °C at the end of 28 days (Fig. 1A and B). During the regrowth, the *L. monocytogenes* numbers exceeded the 2 log₁₀ CFU/cm² after 14 days and 3 days at 4 and 10 °C, respectively. However, at both 4 and 10 °C, the *L. monocytogenes* numbers in the phage-treated samples remained significantly lower than the untreated control samples ($P < 0.0001$) throughout the 28-day incubation period (Fig. 1A and B).

The *L. monocytogenes* population in roast beef increased from an initial concentration of 3.4 log₁₀ CFU/cm² to a maximum of between 8.1 and 8.5 log₁₀ CFU/cm² during the 28-day storage at both 4 °C (Fig. 2A) and 10 °C (Fig. 2B). At 4 °C, the maximum cell counts were achieved

after storage for 28 days, while this was achieved after 7 days incubation at 10 °C. LISTEX™P100 reduced the *L. monocytogenes* counts to a minimum level of 1.7 log₁₀ CFU/cm² after a 1-day storage period at both 4 °C and at 10 °C. There was regrowth of *L. monocytogenes* to 6.1 log₁₀ CFU/cm² at 4 °C and 8.8 log₁₀ CFU/cm² at 10 °C at the end of 28 days (Fig. 2A and B). For the roast beef samples stored at 4 °C, the *L. monocytogenes* numbers were significantly lower in the phage-treated samples as compared to the untreated control samples ($P < 0.0001$) throughout the 28-day incubation period (Fig. 2A). At 10 °C, the *L. monocytogenes* numbers in the phage-treated samples remained significantly lower than the untreated control samples

($P < 0.0001$) for only 14 days, after which the numbers increased and surpassed those of the untreated control samples (Fig. 2B). During the regrowth, the *L. monocytogenes* numbers exceeded the 2 log₁₀ CFU/cm² after 20 days and 7 days at 4 and 10 °C, respectively.

3.3. Growth of *L. monocytogenes* on sliced cooked turkey containing PL and roast beef containing PL-SD and treated with LISTEX™P100

L. monocytogenes increased from 3.4 log₁₀ CFU/cm² to a maximum of 7.9 log₁₀ CFU/cm² at 4 °C (Fig. 1A) and 8.2 log₁₀ CFU/cm² at 10 °C (Fig. 1B) during the 28-day storage on cooked turkey containing PL. Maximum cell counts were achieved after the 28-day storage period at 4 °C (Fig. 1A) and after only 10 days incubation at 10 °C (Fig. 1B). Treatment of the cooked turkey containing PL with LISTEX™P100 reduced the *L. monocytogenes* counts to a minimum level of 1.7 log₁₀ CFU/cm² (1.7 log₁₀ CFU/cm² reduction) after 1 day storage at both 4 and 10 °C. During subsequent storage at 4 °C after treatment of cooked turkey containing PL with phage LISTEX™P100, there was regrowth of *L. monocytogenes* to a maximum 4.5 log₁₀ CFU/cm² after 28 days (Fig. 1A). In the samples treated with LISTEX™P100 and incubated at 10 °C, there was regrowth of *L. monocytogenes* to a maximum of 7.5 log₁₀ CFU/cm² after 28 days (Fig. 1B). During the regrowth, the *L. monocytogenes* numbers exceeded the 2 log₁₀ CFU/cm² after 7 days for samples stored at 10 °C. At both 4 and 10 °C, a significant additive effect was found between LISTEX™P100 and PL as detected by their interaction in ANOVA ($P < 0.0001$, ANOVA results not shown). The interaction was significant at both 4 °C and 10 °C but the additive effect was only observed for samples stored at 4 °C (Fig. 1A and B).

There was a 1.5-log₁₀ CFU/cm² reduction in *L. monocytogenes* numbers on roast beef containing PL-SD after 1 day storage 4 °C (Fig. 2A). No further reductions in *L. monocytogenes* counts were observed during the subsequent storage of the PL-SD containing roast beef samples. The *L. monocytogenes* numbers remained below the day 0 inoculation level of 3.4 log₁₀ CFU/cm² throughout the storage period of 28 days at 4 °C. An initial reduction of 1.5 log₁₀ CFU/cm² in *L. monocytogenes* numbers was realized upon treatment of the roast beef containing PL-SD with phage LISTEX™P100 after a 1-day storage period at both 4 °C (Fig. 2A). There was no subsequent increase in *L. monocytogenes* numbers beyond 2.2 log₁₀ CFU/cm² (1.2 log₁₀ CFU/cm² below day 0 inoculation level of 3.4 log₁₀ CFU/cm²) for samples stored at 4 °C for 28 days (Fig. 2A). The *L. monocytogenes* numbers in the phage-treated roast beef samples containing PL-SD at 4 °C remained significantly lower than the untreated control samples ($P = 0.002$) throughout the 28-day incubation period (Fig. 2A). At 10 °C, untreated roast beef containing PL-SD had a reduction in *L. monocytogenes* numbers of 1.3 log₁₀ CFU/cm² after 1 day storage (Fig. 2B). During subsequent storage at 10 °C for 28 days, the *L. monocytogenes* numbers increased to a maximum of 7.2 log₁₀ CFU/cm² (Fig. 2B). An initial reduction of 1.7 log₁₀ CFU/cm² in *L. monocytogenes* counts was realized upon treatment of roast beef containing PL-SD with phage LISTEX™P100 after 1 day storage at 10 °C (Fig. 2B). There was no subsequent increase in *L. monocytogenes* numbers beyond 1.8 log₁₀ CFU/cm² (1.6 log₁₀ CFU/cm² below day 0 inoculation level of 3.4 log₁₀ CFU/cm²) for samples stored at 10 °C for 10 days (Fig. 2B). During this period, the *L. monocytogenes* numbers in the phage-treated roast beef samples containing PL-SD remained significantly lower than the untreated control samples ($P < 0.0001$). After 10 days there was an increase in *L. monocytogenes* numbers in the phage-treated samples stored at 10 °C, from a minimum 1.6 log₁₀ CFU/cm² to a maximum of 6.3 log₁₀ CFU/cm² after a 28-d storage (Fig. 2B). Similar to the cooked turkey containing PL, in the roast beef containing PL-SD, a significant additive effect were found between phage and antimicrobials as detected by their interaction in ANOVA ($P < 0.0001$, ANOVA results shown, Fig. 2A and B).

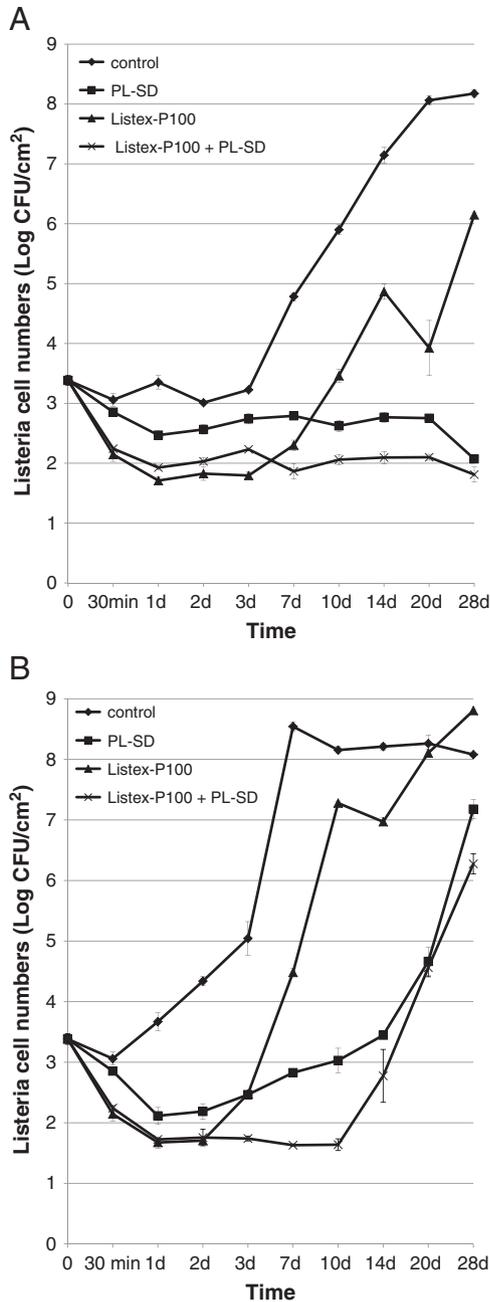


Fig. 2. Effect of LISTEX™P100 on the growth of four-strain cocktail of *L. monocytogenes* when applied alone (LISTEX-P100) or on roast beef sample containing potassium lactate-sodium diacetate (LISTEX™P100 + PL-SD) and compared to no phage treatment (control) growth and on roast beef containing potassium lactate-sodium diacetate alone (PL-SD) at 4 °C (Fig. 2A) and 10 °C (Fig. 2B). Bars represent SE.

3.4. Regrowing *L. monocytogenes* are lysed by LISTEX™P100

A total of 40 *Listeria* isolates were recovered by plating of phage-treated roast beef and cooked turkey on randomly selected sampling day points. Phage LISTEX™P100 was able to form plaques on 36 of the isolates while four isolates did not form plaques. The number of plaques formed in each of the plaque-forming isolates was equivalent to that in the wild-type *L. monocytogenes* outbreak strain 08-5578.

The four non-plaque forming isolates were subjected to further tests to confirm that they are truly resistant and no longer amenable to phage treatment as described in the Materials and Methods. All four isolates displayed the same level of CFU reduction as the wild-type *L. monocytogenes* outbreak strain 08-5578 (results not shown). From the results, all the isolates tested were sensitive to Listex™P100.

3.5. Infective phage isolated from food samples

The concentration of phage LISTEX™P100 on the RTE cooked turkey and roast beef slices was determined at different time points over the incubation period of 28 days at both 4 and 10 °C. This was in order to monitor the infectivity of phage LISTEX™P100 when present in the RTE meat food matrix over the 28-day storage period at either 4 or 10 °C. The concentration of the phages remained between 10⁷ and 10⁸ PFU/cm² throughout the 28-day storage (results not shown). This is comparable to the initial inoculum level of 10⁷ PFU/cm².

4. Discussion

This is the first study that has tested the efficacy of phage LISTEX™P100 in reducing *L. monocytogenes* contamination in RTE roast beef and cooked turkey. It has been found that the effectiveness of this phage varies with the type of food product in which it is applied (Guenther et al., 2009). Successful phage killing of *L. monocytogenes* was achieved in both cooked turkey and roast beef samples. Initial reductions of *L. monocytogenes* by 1.5–2.1 log₁₀ CFU/cm² were realized in all RTE meat samples treated with LISTEX™P100. This had an impact on the total bacterial counts during the 28-day storage period of the RTE meats at either 4 or 10 °C. Our findings show that when RTE meat is treated with phage LISTEX™P100 and stored at the usually recommended temperature of 4 °C, the initial phage lysis of inoculated bacteria enabled the *L. monocytogenes* numbers to remain significantly lower than in the case of an untreated control by about 2 log CFU/cm² over the 28-day storage period. A noteworthy fact is that progeny phages are not a factor in efficacy of phage treatment of low-level contaminations. Inactivation of *L. monocytogenes* is caused by inundation by the initial infecting phage population even if the infection is non-productive.

During the entire 28-day storage period of the cooked turkey and roast beef samples, it was possible to recover infective phage LISTEX™P100 particles at a concentration similar to that initially inoculated on the sample on day 0. This was proof that the phage remained stable in these food matrices during the entire storage period. It has been suggested that the re-growth of bacterial cells in phage-treated foods can be attributed to inability of phage particles to reach the bacterial targets in the food matrix leading to the bacteria multiplying in protected niches (Guenther et al., 2009; Guenther and Loessner, 2011; Guenther et al., 2012). An additional barrier in the form of chemical antimicrobials PL and SD was meant to inhibit *L. monocytogenes* outgrowth after the phage treatment. There was regrowth of *L. monocytogenes* in all phage-treated samples except the roast beef containing SD-PL stored at 4 °C. For these phage-treated samples, the *L. monocytogenes* counts remained 1.2 log₁₀ CFU/cm² below day 0 inoculation level of 3.4 log₁₀ CFU/cm². In cooked turkey containing PL treated with phage LISTEX™P100, the regrowth of *L. monocytogenes* achieved a maximum increase of 1.1

log₁₀ CFU/cm². As a general conclusion, we found that for roast beef and cooked turkey stored at 4 °C, LISTEX™P100 combined with chemical antimicrobials allows no more than 2 log₁₀ CFU/cm² increase of *L. monocytogenes* throughout the stated shelf life of the product. This is the criterion used by the Canadian Food Inspection Agency (CFIA) that may allow a food product containing these antimicrobials to qualify to a lower relative risk level for sampling purposes within their respective food risk category but cannot be moved to a lower food risk category (Canadian Food Inspection Agency, 2011).

An additive effect between phage and chemical antimicrobials was more pronounced in cooked turkey containing PL and incubated at 4 °C as compared to in roast beef containing SD-PL stored at the same temperature. The chemical antimicrobials SD-PL role in keeping the *L. monocytogenes* numbers low in roast beef was more pronounced as compared to LISTEX™P100 (Fig. 2A).

The emergence of phage-resistant cells has often been cited that a potential drawback of using phages as decontaminants in foods. In addition to the use of phage cocktails or phage rotation schemes, the treatment of products with phages immediately prior to packing has been recommended as a viable method of overcoming development of phage resistance (Hagens and Loessner, 2007; Guenther et al., 2009). In this study, forty randomly selected re-grown *L. monocytogenes* colonies after phage treatment on the RTE meat surface were subjected to tests to check if they were resistant LISTEX™P100 and not amenable to phage treatment. LISTEX™P100 was able to kill all the isolates selected. A possible explanation for re-growth of the bacteria on the RTE meat after phage treatment is that some of the bacteria were not phage-resistant but had escaped lysis by the phage because they had not come into contact with phage particles. It has previously been suggested that low bacterial numbers (as is the case during most real-life scenario contamination of foods) are unlikely to be affected by low numbers of phages because the phages and bacteria are unlikely to meet (Hagens and Loessner, 2010).

To the best of our knowledge, the phenomenon of transient phage resistance through phase-variable surface antigenic modifications has only been observed in a number of Gram-negative bacteriophage systems (Fischer et al., 2004; Zaleski et al., 2005; Kocharunchitt et al., 2009; Sorensen et al., 2011; Kim and Ryu, 2012). If proven that this phenomenon is also prevalent among Gram positive bacteria, then the re-growth of *L. monocytogenes* on the RTE after Listex™P100 can be attributed to it.

As expected, the re-grown *L. monocytogenes* cell counts achieved higher numbers with samples stored at 10 °C than those at 4 °C for 28 days. However, in the presence of chemical antimicrobials PL (in cooked turkey) and SD-PL (in roast beef), it took a longer time for the cell numbers to plateau.

This is the only study in which the surviving bacteria were enumerated after adding a virucide (Jassim et al., 1998) to the stomacher bags when homogenizing the samples in order to inactivate unbound phages. The efficacy of the virucide comprised of black tea extract and ferrous sulfate in inactivating LISTEX™P100 was verified in this study. Thus, the inclusion of the virucide in the stomacher bags when homogenizing samples prevented the over-estimation of the phage killing effect due to previously unbound phage particles coming into contact with surviving bacteria during the homogenization process. The samples were incubated at the recommended storage temperatures of RTE deli meats of 4 °C as well as at abusive temperatures of 10 °C. As mentioned earlier in this discussion, it was found that phage LISTEX™P100 in combination with the chemical antimicrobials PL and SD were most effective in retarding the growth of *L. monocytogenes* on RTE roast beef and cooked turkey when stored at 4 °C.

In conclusion, this study has shown that phages such as LISTEX™P100 in the presence of chemical inhibitors PL and SD provide an effective hurdle which can be used to enhance safety in RTE roast beef and cooked turkey contaminated with *L. monocytogenes*.

Author's contribution

AC contributed in the project design, inoculum preparation, inoculation, sampling, microbial enumeration, data analysis and interpretation, and preparation of manuscript; LA contributed in the inoculum preparation, sampling, and microbial enumeration; AG contributed in the statistical analysis and interpretation of data; PMS contributed in the design and oversight of the project; AMK: contributed in the design and oversight of project; SB: contributed in the project design, oversight, coordination, analysis, and interpretation of data.

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