

Effect of a bacteriophage cocktail in combination with modified atmosphere packaging in controlling *Listeria monocytogenes* on fresh-cut spinach

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Abstract

A *Listeria monocytogenes*-specific bacteriophage cocktail was evaluated for its activity against a nalidixic acid-resistant *L. monocytogenes* (*Lm-Nal*^R) isolate on fresh-cut spinach stored under modified atmosphere packaging at various temperatures. Pieces (~2 × 2 cm²) of fresh spinach inoculated with 4.5 log CFU/cm² *Lm-Nal*^R were sprayed with the phage cocktail (6.5 log plaque-forming units [PFU]/cm²) or a control. The samples were stored at 4°C or 10°C for up to 14 d in sealed packages filled with either atmospheric air (AA) or modified atmosphere (MA). At 4°C under AA, the phages significantly ($P \leq 0.05$) lowered the *Lm-Nal*^R populations on spinach, compared to control-treated inoculated samples, by 1.12 and 1.51 log CFU/cm² after 1 and 14 d, respectively. At 4°C under MA, *Lm-Nal*^R was significantly reduced by 1.95 log CFU/cm² compared to control leaves after both 1 and 14 d. At 10°C under AA, the phages significantly reduced *Lm-Nal*^R by 1.50 and 2.51 log CFU/cm² after 1 and 14 d compared to the control. Again at 10°C under MA, the phages significantly reduced *Lm-Nal*^R by 1.71 and 3.24 log CFU/cm² compared to control after 1 and 14 d, respectively. The results support the potential of lytic bacteriophages in effectively reducing populations of *L. monocytogenes* on fresh-cut leafy produce, under both AA and MA conditions.

Keywords

bacteriophage • biocontrol • *L. monocytogenes* • modified atmosphere packaging • spinach

Abbreviations

Lm-Nal^R: nalidixic acid-resistant *L. monocytogenes*; Nal: nalidixic acid; TSBN250: Tryptic soy broth with 250 mg/mL Nal; TSAN200: Tryptic soy agar with 200 mg/mL Nal

Introduction

Consumption of fresh fruits and vegetables contaminated with enteric bacterial pathogens has caused several high-profile food-borne disease outbreaks in the United States and Europe over the past decade (Martínez-Vaz *et al.*, 2014). Traditionally, leafy greens are not consumed without a somewhat effective decontamination process such as washing with watered-down vinegar. However, the 'Ready-to-Eat' claim on the packages of pre-cut spinach and lettuce misleads the young generation of adults who have not learnt safe food preparation habits. Due to the increasing concern from both the consumers and the industry, the US Food and Drug Administration (FDA) has allowed the use of ionising

radiation for the control of food-borne pathogens (FDA, 2008). This is not a mandatory routine treatment as consumers' perception against radiation poses a great constraint (Dickson, 2012). Once contamination occurs, pathogens may find favourable conditions to grow on the damaged surfaces of the fruits and vegetables (Leverentz *et al.*, 2003). Cases of produce-related food-borne illnesses are estimated to cost the US \$39 billion per year (Scharff, 2010). In recent years, one food-borne human pathogen, *Listeria monocytogenes*, has been associated with a number of serious food-borne disease outbreaks and recalls (Centers for Disease Control and Prevention [CDC], 2013). *L. monocytogenes* survives at temperatures <4°C and creates a serious health risk by contaminating refrigerated food items (Ukuku and Fett, 2002). However, *L. monocytogenes* is not limited only to refrigerated food items. During August and September of 2011, a multi-State *L. monocytogenes* outbreak in the US was traced back to the consumption of contaminated fresh cantaloupes grown in a farm in Colorado, leading to 33

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deaths and 114 cases of listeriosis (CDC, 2011). In many cases, a partially cut cantaloupe was stored in the refrigerator when the whole fruit was not consumed, potentially allowing growth of the pathogen. Furthermore, five deaths in Texas, US, were linked to the consumption of fresh-cut celery contaminated with *L. monocytogenes* in chicken salads (Gaul *et al.*, 2013). These outbreaks show that *L. monocytogenes* should be considered as a food-borne disease-causing agent in fruits and vegetables, which may subsequently be stored at refrigeration temperatures.

Hypochlorite is the most widely used chemical for the treatment of wash water for fresh and fresh-cut produce to prevent bacterial cross-contamination of produce (Takeuchi and Frank, 2001). However, in most cases, the hypochlorite concentrations used to prevent cross-contamination are insufficient to completely kill 100% of the bacteria present on these produce commodities (Behrsing *et al.*, 2000). The oxidative effect of the free chlorine in hypochlorite solutions is inactivated by the organic substances released from the damaged or cut surfaces of the produce, potentially limiting the effectiveness of the free chlorine in the hypochlorite formulations (Marriott, 1999). Therefore, novel, environmentally friendly and effective interventional approaches are urgently needed to specifically target and reduce populations of pathogenic bacteria on fresh-cut produce.

One such novel approach is the use of lytic bacteriophages (phages) to selectively control bacterial pathogens on fresh produce. Phages are viruses that can be extremely specific for bacterial strains (Elbreki *et al.*, 2014) and interact with and infect their respective host strains. They are extremely diverse and abundant; thus, lytic phages present a promising natural approach in controlling enteric pathogenic bacteria on produce. In nature, phages are responsible for killing almost half of the bacterial population generated daily (Suttle, 2005). Considering the short generation time of the bacteria under optimal growth conditions, phages represent a natural mechanism to keep bacterial populations under control. In the past decade, scientists have researched new phage-based solutions to control pathogenic bacteria in or on foods. Currently, there are four commercial bacteriophage products (SalmoFresh™, Listex™ P100, ListShield™ and EcoShield™) on the market, which have received FDA and/or Environmental Protection Agency regulatory approvals for use on food items or food contact surfaces to decontaminate food-processing facilities.

Under current practices, fresh-cut produce may be transported long distances to reach the consumers. Modified atmosphere packaging (MAP) offers advantages to preserve the freshness of the produce during transportation by modifying the ambient atmosphere conditions to help reduce the rate of physiological deterioration of the fresh-cut produce commodity and extend its shelf life. In this study, the ability of ListShield™, a phage cocktail composed of lytic bacteriophages specific for

L. monocytogenes, was evaluated on fresh-cut spinach stored under MAP conditions (low oxygen/high carbon dioxide) commonly used in the industry. Thus, the objective of this study was to test and compare the effectiveness of the phage cocktail against *L. monocytogenes* on fresh-cut spinach packaged under atmospheric air and modified atmosphere conditions and stored at two different storage temperatures.

Materials and methods

L. monocytogenes strains and bacteriophage cocktail

L. monocytogenes ATCC® strains 7644™, 19115™ and 19117™ were obtained from the Agricultural Research Services of the US Department of Agriculture (Albany, CA, USA). The strains were plated on tryptic soy agar (TSA) (Becton Dickinson, Franklin Lakes, NJ, USA), and then a single colony from the TSA was inoculated and cultured in tryptic soy broth (TSB) (Becton Dickinson) at 37°C.

ListShield™, a bacteriophage cocktail (10¹⁰ plaque-forming units [PFU]/mL in 100 mM saline, pH 7.4) specific against *L. monocytogenes*, was provided by Intralytix, Inc. (Baltimore, MD, USA). The cocktail was stored at 4°C and diluted in 1.5% peptone water (PW) (Becton Dickinson) immediately before application on spinach.

Effect of the phage cocktail against *L. monocytogenes* strains in TSB

The effect of the ListShield™ phage cocktail was measured against *L. monocytogenes* strains 7644, 19115 and 19117 in TSB stored at 4°C for 0.5, 2 and 24 h. *L. monocytogenes* strains at about 5 log CFU/mL were treated independently with the bacteriophage cocktail at 7 log PFU/mL.

Preparation of fresh-cut spinach leaves

Commercially pre-washed, packaged and ready-to-eat fresh spinach was purchased from a local grocery store and stored in the laboratory at 4°C for up to 1 d until use. All spinach was handled with nitrile gloves that were washed in 70% ethanol before handling. Damaged spinach leaves and leaves that were not healthy were discarded. Using a sterile knife and surface-disinfected glass cutting board, spinach leaves were cut into 2 × 2 cm² pieces. The pieces of the leaves were immediately placed into a sterile Stomacher® bag to reduce wilting due to water loss and kept at room temperature during preparation steps.

Effect of the phage cocktail against *L. monocytogenes* on spinach at 4 and 10°C under AA or MAP conditions

L. monocytogenes 19115 was used in the following experiments to evaluate the efficiency of the phage cocktail on fresh-cut spinach. The strain was made resistant to 400 mg/

mL nalidixic acid (Nal) (Sigma Aldrich, St. Louis, MO, USA) through a combination of spontaneous mutation and repeated culture methods, in order to distinguish it from the background microflora indigenous to spinach leaves. Similar to the previous reports by other researchers (Leverentz *et al.*, 2003, 2006), *L. monocytogenes* was found to be resistant to 250 mg/mL Nal. *L. monocytogenes* grown overnight in TSB supplemented with 250 mg/mL Nal (TSBN250) reached 8 log CFU/mL. To enumerate the *L. monocytogenes* Nal^R (*Lm-Nal*^R) population, Nal was added to the TSA plates at a concentration of 200 mg/mL (TSAN200), which was found to be effective in preventing the growth of all indigenous microorganisms on spinach for 2 d. The leaf pieces were placed as a grid on aluminium foil sheet (50 leaf pieces per ~600 cm²) for bacteria and phage applications. An overnight culture of *Lm-Nal*^R was diluted in PW to achieve a population of ~7 log CFU/mL. An aliquot (10 µL) of this diluted *Lm-Nal*^R culture was inoculated gently on the top side of each 2 × 2 cm² piece of spinach. Extra care was taken to distribute the total volume evenly in 15–20 small droplets on the entire surface by gently touching the micropipette tip to the surfaces of the leaf pieces without disturbing or damaging the surface. The inoculated droplets did not contact the cut edges of the leaves. Contaminated leaf pieces were air-dried for 20 min to allow for bacterial attachment and then sprayed with either ListShield™ phage cocktail adjusted to 7 log PFU/mL with PW or PW alone (control). A small fingertip sprayer (Bottle Crew, West Bloomfield, MI, USA) that delivers 100 µL in a single spray volume was used to deliver the phage cocktail at a concentration of ~6.0 log PFU/cm² of leaf (Boyacioglu *et al.*, 2013). The phage was sprayed from 25–30 cm above the surface, with one stroke (100 µL) per leaf, plus one extra stroke for the leaves around the edges of the leaf grid on the foil. Final phage titres (in PFUs per square centimetre) recovered from spinach leaf pieces that were not inoculated with *Lm-Nal*^R were ~6.5 log PFU/cm², determined using a soft agar overlay assay to verify the repeatability of the phage delivery/spray method (data not shown) (Boyacioglu *et al.*, 2013). The spinach pieces were incubated for up to 14 d at 4 or 10°C on sterile, wet filter papers inside sterile Petri dishes placed in vacuum pouches (Prime Source Vacuum Pouches, Kansas City, MO, USA), which were filled with the desired gas mixture (atmospheric air [AA] or modified atmosphere [MA]) by using a table-top vacuum packaging machine (Supervac, Wien, Austria). The MA was composed of 5% O₂, 35% CO₂ and 60% N₂. After storage in pouches for 1, 4, 7, 10 or 14 d, the surviving *Lm-Nal*^R populations on spinach leaves were determined as previously stated with some modifications (Boyacioglu *et al.*, 2013). Briefly, the leaf pieces were removed from the pouches using a sterile forceps and homogenised in 9 mL of 1.5% PW using a handheld laboratory blender (Polytron PT 1200 E; Kinematica AG, Lucerne, Switzerland). Between the homogenisation of two leaf samples, the probe

of the blender was rinsed in a three-step process that included tap water, 100% denatured ethanol and sterile water to prevent cross-contamination of *Lm-Nal*^R. The homogenised leaves were diluted in 1.5% PW and surface-plated on TSAN200. Recovered *Lm-Nal*^R colonies were enumerated after overnight incubation at 37°C.

Statistical analysis

All experiments were performed three times with at least two replicates in each trial. Populations (in CFUs per square centimetre) of *Lm-Nal*^R recovered from spinach homogenates treated with either the control or ListShield™ phage cocktail were log-transformed and subjected to analysis of variance, multiple regression analysis and Fisher's LSD test using SAS, version 9.2 (Cary, NC, USA). The level of statistical significance was kept at $P \leq 0.05$.

Results and discussion

While the two strains, 7644 and 19115, showed reduction of >2.5 log CFU/mL during 24 h storage in the presence of the phage cocktail, strain 19117 showed only 1.55 log CFU/mL (Figure 1). Strain 19115 was chosen for the spinach experiments as it was one of the two strains that were most susceptible to the phage cocktail.

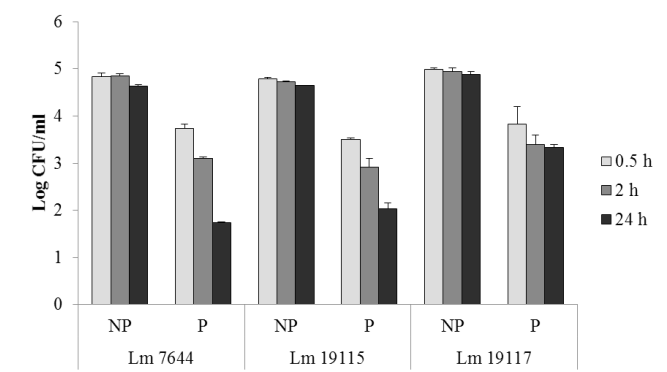


Figure 1. Effectiveness of ListShield™ bacteriophage cocktail against three different *L. monocytogenes* strains in tryptic soy broth at 4°C. The experiments were performed three times in duplicates. NP: no phage (control); P: phage treatment.

Use of *Lm-Nal*^R 19115

ListShield™ was effective in reducing populations of *Lm-Nal*^R at levels comparable to the parental *Lm* 19115 in preliminary tests (data not shown), indicating that *Lm-Nal*^R was suitable for evaluating the effectiveness of the phage mixture on fresh-cut spinach leaves. Preliminary studies also indicated that *Lm-Nal*^R could be recovered on TSAN200 from fresh-cut spinach samples that were stored for as long as 14 d (Figure 2).

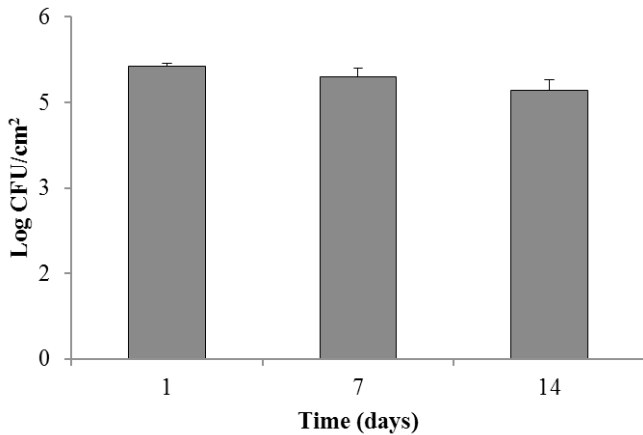


Figure 2. Recovery of nalidixic acid-resistant *L. monocytogenes* strain 19115 (*Lm-Nal^R*) from fresh-cut spinach leaves stored at 4°C for up to 14 d.

Effectiveness of lytic bacteriophage cocktail against *Lm-Nal^R* on fresh-cut spinach leaves stored under MAP

Application of the ListShield™ phage cocktail to fresh-cut spinach leaves stored at 4°C for 1 d significantly ($P \leq 0.05$) lowered the populations of *Lm-Nal^R* by 1.12 log CFU/cm² when compared to the control-treated, inoculated leaf pieces stored under AA (Figure 3a). Populations of *Lm-Nal^R* were reduced by 1.95 log CFU/cm² on phage-treated spinach compared to control-treated spinach when packaged under MA (5% O₂, 35% CO₂ and 60% N₂) and stored at 4°C for 1 d (Figure 3a). The reduction in *Lm-Nal^R* populations due to phage treatment under MA storage was not significantly different ($P > 0.05$) from the population reduction observed under AA storage for 1 d at 4°C. The population of *Lm-Nal^R* stayed significantly ($P \leq 0.05$) lower for 14 d on spinach leaves treated with the phage cocktail compared to the

control-treated inoculated leaf pieces when stored at 4°C under both AA and MA storage conditions. At the end of the 14-d storage period at 4°C, *Lm-Nal^R* populations on phage-treated spinach leaves were lower by 1.51 and 1.95 CFU/cm² than those of control-treated leaves packaged under AA and MA conditions, respectively. On average, the populations of *Lm-Nal^R* in all treatments (control- and phage-treated) at 4°C grew by 1.05 log CFU/cm², consistent with the psychrotrophic nature of *L. monocytogenes* (Ukuku and Fett, 2002).

Application of the ListShield™ phage cocktail to fresh-cut spinach leaves stored at 10°C for 1 d significantly ($P \leq 0.05$) lowered the populations of *Lm-Nal^R* by 1.50 log CFU/cm² when compared to control-treated inoculated leaf pieces stored under AA (Figure 3b). When packaged under MA, the phage mixture reduced the *Lm-Nal^R* populations on fresh-cut spinach pieces by 1.71 log CFU/cm² when compared to those on control-treated spinach after 1 d (Figure 3b). The reduction in *Lm-Nal^R* populations on phage-treated spinach leaves under MA storage was not significantly different from the population reduction observed under AA storage after 1 d. Under both AA and MAP, populations of *Lm-Nal^R* on spinach leaves treated with the phage cocktail stayed significantly ($P \leq 0.05$) lower after 14 d compared to the control-treated leaves stored at 10°C. After 14 d of storage at 10°C, the populations of *Lm-Nal^R* on phage-treated spinach pieces stored under both AA and MA were 2.51 and 3.24 CFU/cm² lower than their respective control-treated inoculated samples.

Multiple regression analysis on the entire data set showed that the phage treatment significantly ($P \leq 0.05$) reduced the *Lm-Nal^R* populations on spinach pieces by 1.79 log CFU/cm² on average compared to control-treated inoculated samples. Overall, *Lm-Nal^R* populations on the phage-treated spinach pieces were consistently lower than those on the control-treated spinach leaves throughout the study. Not surprisingly, *Lm-Nal^R* populations on spinach leaves stored at 10°C

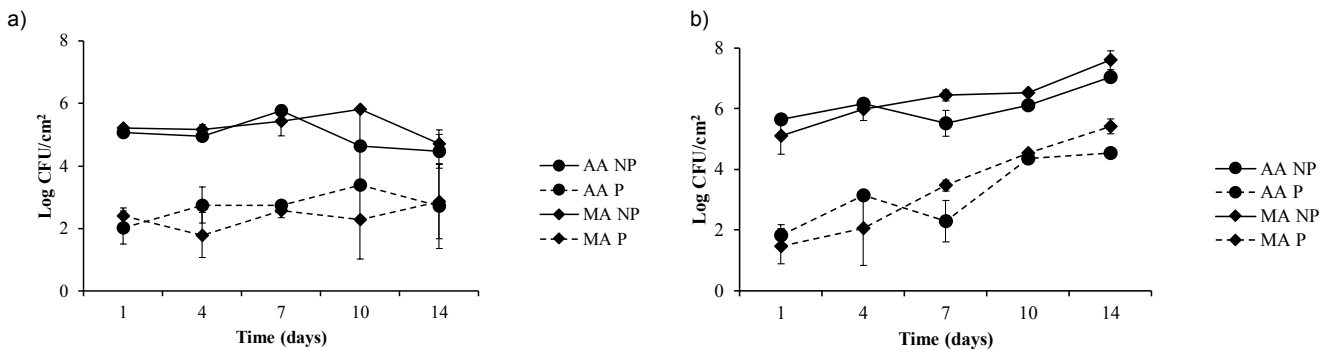


Figure 3. Counts of bacteria recovered from fresh-cut spinach leaves contaminated with *Lm-Nal^R* and treated with control or ListShield™ bacteriophage cocktail and stored under MAP with ambient atmosphere (AA) or modified atmosphere (MA); a) storage at 4°C; b) storage at 10°C. The experiments were performed three times in duplicates. NP: no phage (control); P: phage treatment.

significantly ($P \leq 0.05$) increased by 0.26 log CFU/cm², on average, compared to populations recovered from spinach leaves stored at 4°C. On average, throughout the course of the study, *Lm-Nal*^R populations on spinach pieces stored under MA and AA were roughly equivalent.

The observed reductions in *Lm-Nal*^R populations on fresh-cut spinach treated with ListShield™ are in agreement with results of previous studies (Leverentz *et al.*, 2004, 2006). Supporting the results of this experiment, a recent study concluded that the same MA conditions used in this study in combination with an *Escherichia coli* O157:H7-specific lytic phage mixture resulted in lower *E. coli* O157:H7 populations on fresh-cut leafy greens compared to populations on phage-treated, inoculated samples packaged with atmospheric air (Boyacioglu *et al.*, 2013). In a 2009 report, application of bacteriophage A511 to leafy greens reduced *L. monocytogenes* populations by ~2.5 log CFU/g on lettuce and cabbage samples during a 6-d storage period at 6°C (Guenther *et al.*, 2009). Bacteriophage A511 was shown to be stable and active on lettuce and cabbage samples, showing only one log reduction during 6 d of storage at 6°C (Guenther *et al.*, 2009). Bacteriophage A511 reduced *L. monocytogenes* populations in cheese by levels ranging between 3 and 6 log CFU/cm² (depending on the initial *L. monocytogenes* populations) during the ripening process (11 d at 12°C, followed by another 11 d at 6°C) (Guenther and Loessner, 2011).

The partial oxygen and carbon dioxide concentrations were measured for the entire duration of the MAP studies (data not shown). During the 14-d storage period, the O₂ concentration in the packages filled with AA dropped by 1.4% and 3.8%, whereas the CO₂ levels increased by 0.6% and 2.2% at 4°C and 10°C, respectively. Under MA storage for 14 d, the O₂ concentration increased by 1.3% and 0.2%, while the CO₂ concentration decreased by 10.8% and 1.9% at 4°C and 10°C, respectively. The different O₂ and CO₂ concentrations used in this study did not significantly alter the reduction level observed in *Lm-Nal*^R populations on fresh-cut spinach due to the lytic activity of the phage cocktail. Studies concluded that there was no change in *E. coli* O157:H7 (Abdul-Raouf *et al.*, 1993) and *L. monocytogenes* (Beuchat and Brackett, 1990) populations when stored under atmosphere that was composed of 3% O₂ and 97% N₂. Another study reported that *E. coli* growth on shredded iceberg lettuce was not altered by any of the four different MAP conditions under 13°C and 22°C (Diaz and Hotchkiss, 1996). Several recent studies noted that the various O₂ and CO₂ levels developed inside the different

packaging film materials incubated at either 4°C, 5°C and 25°C for 10 d did not change the survival or growth of *E. coli* O157:H7 on shredded iceberg or romaine lettuce (Oliveira *et al.*, 2010; Sharma *et al.*, 2011). These studies seem to conclude that MAP did not affect the survival of food-borne pathogens as much as storage temperature. In this study, the effectiveness of the lytic phages specific for *L. monocytogenes* on fresh-cut spinach was not inhibited by the MAP conditions tested. The results of this study indicate the potential use of lytic phages as an intervention against *L. monocytogenes* on fresh-cut leafy greens.

Conclusion

In this study, the effect of a *L. monocytogenes*-specific phage cocktail (ListShield™) against a nalidixic acid-resistant *L. monocytogenes* strain (*Lm-Nal*^R) on fresh-cut spinach leaves stored under different atmospheric conditions at 4 and 10°C was investigated. ListShield™ was effective in significantly ($P \leq 0.05$) reducing *Lm-Nal*^R populations on spinach under both AA and MA conditions at both refrigeration (4°C) and potentially abusive (10°C) storage conditions encountered during transportation of fresh produce commodities. These results indicate that the application of lytic bacteriophage mixtures specific for *L. monocytogenes* can be effective in reducing target bacterial populations on fresh-cut leafy greens under commercial packaging conditions. Therefore, lytic bacteriophages specific for *L. monocytogenes* are a potential intervention against the pathogen on commercially packaged fresh-cut leafy greens and, potentially, other fresh-cut produce commodities.

Disclosure of potential conflicts of interest

AS holds an equity stake in Intralytix, Inc., a corporation based in Baltimore, MD, USA, involved with the development of phage preparations (including ListShield™) for various practical applications.

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