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Optimizing Concentration and Timing of a Phage Spray Application To Reduce *Listeria monocytogenes* on Honeydew Melon Tissue

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ABSTRACT

A phage cocktail was applied to honeydew melon pieces 1, 0.5, and 0 h before contamination with *Listeria monocytogenes* strain LCDC 81-861 and 0.5, 1, 2, and 4 h after contamination. The phage application was most effective when applied 1, 0.5, or 0 h before contamination with *L. monocytogenes*, reducing pathogen populations by up to 6.8 log units after 7 days of storage. This indicates that under commercial conditions, if contamination occurs at the time of cutting, phage would have to be applied as soon as possible after cutting the produce. However, all phage applications from 1 h before to 4 h after contamination and all phage concentrations ranging from 10^4 to 10^8 PFU/ml reduced bacterial populations on honeydew melon pieces. Higher phage concentrations were more effective in reducing pathogen populations. A phage concentration of approximately 10^8 PFU/ml was necessary to reduce the pathogen populations to nondetectable levels immediately after treatment, and pathogen growth was suppressed by phage concentrations of 10^6 through 10^8 throughout the storage period of 7 days at 10°C. In an attempt to enhance the effectiveness of the phage cocktail on low pH fruit, such as apples, the phage was applied in combination with $MnCl_2$. This combination, however, did not enhance the effectiveness of the phage on apple tissue. The results from this study indicate that the effectiveness of the phage application on honeydew melon pieces can be optimized by using a phage concentration of at least 10^8 PFU/ml applied up to 1 h after processing of the honeydew melons.

Listeria monocytogenes, a foodborne human pathogen, has been associated with serious outbreaks of foodborne diseases and recalls of fresh produce *(3, 4)*. In recent years, there were recalls of halved red bell peppers (February 2001), romaine lettuce (July 2003), sprouts (August 2002), apple slices (March 2001), and processed and mixed fruits and vegetables (June 2002) (http://www.safetyalerts.com/).

Phages are ubiquitous and can be found in fresh water in high numbers *(2)*. They are also a natural part of fermentation processes such as the production of sauerkraut *(1, 14)* or pickles *(13)*. Phages have been used experimentally against a variety of pathogenic bacteria on plants, animals, and food items. They are used, for example, against xanthomonads on peach trees *(22)*, pseudomonads in fish cultures *(15)*, or as aerosol sprays against *Escherichia coli* infection in broiler chickens *(9)*. Recently, phage cocktails were shown to reduce populations of foodborne human pathogens on honeydew melons and apples alone and in combination with a bacteriocin *(11, 12)*. Homologous phages and bacteriophage pools have been used against spoilage bacteria on meat *(5–7)*, but concerns have been raised about bacteria developing resistance to these phages *(5–7)*. In our present work, we selected a *L. monocytogenes* strain that has caused outbreaks on food. To reduce the potential for the development of phage resistant mutants on fresh-cut fruit, we applied a cocktail of different lytic bacteriophages. Since these phages are specific for the pathogen strains, they leave the nonpathogenic microflora intact, which can then function as an additional hurdle for pathogens. The phage treatment may be optimized by adjusting the timing and concentration of the phage application.

Although some phages have been isolated from low-acid environments, such as sauerkraut and pickles *(1, 13, 14, 18)*, the effectiveness of many phages seems to decline at acidic pH *(11, 12)*. The recovery of acid sensitive phages at a low pH could be improved by the addition of $MnCl_2$ *(18)*, and their effectiveness in reducing bacterial infections was improved by increasing the environmental pH to neutral *(19)*.

The objectives of this study were to determine (i) the quantitative relationship between the concentrations of *L. monocytogenes* and the lytic phages needed for the control of the bacterium with a phage spray application, (ii) the effectiveness of the spray application of specific lytic phages in relation to the time of contamination of honeydew melon pieces with *L. monocytogenes*, and (iii) whether combining the phages with $MnCl_2$ would enhance the effectiveness of the phages on low pH (approximately 4) apple tissue.

MATERIALS AND METHODS

Fruit. Honeydew melons and Golden Delicious apples were obtained from the local market. The honeydew melons were cut into 10-mm-thick rings with a deli slicer (model 827, Berkel Inc.,

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La Porte, Ind.). The 10-mm-thick melon rings were cut into equally sized pieces (30 mm^2). For experiments on the pH tolerance of the phages in the presence of $MnCl_2$, apple tissue plugs (10 mm thick and 10 mm in diameter) were removed from Golden Delicious apples using a cork borer. The fruit surfaces, the cork borer, and the deli meat slicer were disinfected with 70% ethanol immediately before use.

Phage. The phage mixture, LMP-102, contained six distinct lytic phages specific for *L. monocytogenes*, including serotypes 1/2a, 1/2b, and 4b, which have been predominantly associated with human listeriosis. The mixture was provided by Intralytix, Inc. (Baltimore, Md.). The phage concentration was approximately 10^9 PFU/ml in 1 M phosphate-buffered saline (pH 7.4). The mixture was diluted with sterile distilled water (pH 6.5 to 7.0) to approximately 5×10^7 PFU/ml immediately before application to the fruit pieces, unless otherwise indicated.

Bacterial inoculum. The *L. monocytogenes* culture, strain LCDC 81-861 serotype 4b, implicated in an outbreak from processed cabbage (cole slaw), was obtained from Robert Brackett, Department of Food Science and Technology, University of Georgia, Agricultural Experiment Station, Griffin, Ga., and stored at −80°C in Luria-Bertani (LB) broth (BD Diagnostic Systems, Sparks, Md.) and 15% glycerol (Difco, Becton Dickinson, Sparks, Md.). The strain was naturally resistant to nalidixic acid (Sigma, St. Louis, Mo.). For inoculation of the fruit pieces, *L. monocytogenes* was grown overnight on tryptic soy agar (TSA; BD Diagnostic Systems) plates with 100 μg/ml of nalidixic acid at 30°C and then transferred to 30 ml of LB broth for 6 h. The cells were harvested by centrifugation at 10,000 × *g* for 15 min and washed once with sterile saline solution (0.85% [wt/vol] NaCl). The pellet was resuspended in saline solution and adjusted to a concentration of 5×10^8 CFU/ml at an optical density of 420 nm using a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Richmond, Calif.). The suspension was further diluted to a concentration of 5×10^5 CFU/ml and used to inoculate fruit pieces. The exact cell concentration was determined by plating the inoculum with a spiral plater (DW Scientific, Shipley, West Yorkshire, England) onto TSA medium with nalidixic acid followed by incubation at 37°C for 1 day.

Treatment application. The honeydew melon fruit pieces were placed in commercial, 530-ml, dome fruit plastic bowls (no. 518, Rock-Tenn. Co., Chicago Plastics, Franklin Park, Ill.). The fruit pieces were then each pipette inoculated with 25 μl of the *L. monocytogenes* suspension. The procedure for inoculating the fruit pieces took approximately 10 minutes.

For the experiments with different phage concentrations, a spray gun (Model 350-3, Badger Air-Brush Co., Franklin Park, Ill.) was used for phage application. The phage cocktail was applied as a spray to runoff to the entire piece (approximately 250 μl per piece) after contamination with the bacteria. The phages were applied at concentrations of 10^4, 10^5, 10^6, 10^7, and 10^8 PFU/ml. There were two containers per treatment at each recovery time, each containing two pieces of fruit. The covers on the plastic bowls allowed air exchange, which ensured maintenance of an unmodified air atmosphere. For the timing experiments, 25 μl of the phage cocktail was pipetted onto a depression on the fruit pieces at 1, 0.5, and 0 h before contamination with *L. monocytogenes* and at 0.5, 1, 2, and 4 h after contamination.

Manganese chloride solution. Manganese chloride (tetrahydrate) (Sigma) was dissolved in sterile distilled water at 0.05, 0.1, and 0.5 M and adjusted to pH 3.8 (pH of the apple tissue) with 0.01 N HCl. The solutions were then used to dilute the phage mixture to a concentration of approximately 10^8 PFU/ml before application to the apple plugs. The manganese chloride solutions containing the phages were then pipetted (25 μl) onto the apple plugs that had been placed in sterile, capped, glass tubes. Following contamination of the apple plugs with 25 μl of *L. monocytogenes* at approximately 10^5 CFU/ml, they were stored at 10 and 25°C. Samples were taken immediately after inoculation and after 3 days.

Recovery of bacteria. Recovery and quantification of the *L. monocytogenes* populations were performed after 0, 2, 5, and 7 days of storage at 10°C as described previously *(5)*. The day 0 recovery was done immediately following the treatments. Briefly, 1-cm^3 tissue plugs were removed with a cork borer from the stored honeydew pieces. Apple or honeydew tissue plugs were each placed into a sterile plastic bag containing 4.5 ml of buffered peptone water (8.5 g/liter of NaCl, 1 g/liter of Bacto Peptone, pH 7.4; BD Biosciences) and homogenized in a stomacher blender for 120 s at a speed setting of 8 (Bagmixer 100 Minimix, Interscience, Weymouth, Mass.). Aliquots (50 μl) of the homogenized mixtures or appropriate dilutions thereof were plated in duplicate on TSA medium using a spiral plater. The agar contained 100 μg/ml of nalidixic acid (Sigma). The plates were incubated overnight at 30°C. Colony counts were conducted using an automated plate counter (ProtoCol, Synoptics, Cambridge, England), and the data were plotted as CFU per sample. All experiments were repeated at least once.

Phage titer. Samples from the phage treatments were homogenized and then filtered through a 0.45-μm pore size membrane (Acrodisk, Pall Gelman, Ann Arbor, Mich.). The phage titer in the filtrates was determined using a soft agar overlay *(1)* using *L. monocytogenes* strain LCDC 81-861 serotype 4b as the host. The resulting plaques were counted with the ProtoCol plate counter.

Statistical analyses. The CFU data were transformed to log units ($x + 1$). One was added to the values to allow use of zero values in the analysis. Because most of the values were large, this should have a negligible effect on the results of the analysis. Any treatments where all the values were zero or had the same value (no variability) were omitted from the analysis.

The data were analyzed using Proc Mixed *(17)* as the highest-order linear model, although not all treatments could be included in these models. The data were also analyzed as the linear model with the most factors to include all the treatments. The assumptions of the general linear model were tested. When necessary to correct for variance heterogeneity, the variance grouping technique was used.

When effects were statistically significant, mean comparisons were done with Sidak-adjusted *P* values with an experiment-wise error of 0.05. The means and standard errors of the means are presented for the main effects, and interaction effects are presented as tables accompanying the figures to which they refer.

RESULTS

Effect of phage concentration. The *L. monocytogenes* population increased on honeydew melons over 7 days at 10°C, but this increase was reduced by the phage application (Table 1). At a concentration of 10^6 PFU/ml and higher, the higher the phage concentrations applied, the greater the decrease in the *L. monocytogenes* populations on honeydew melons at the time of treatment (day 0). Phage concentrations of 10^4 and 10^5 PFU/ml were not different from the

TABLE 1. *Effect of phage concentration and concentration × time interaction on* Listeria monocytogenes *populations on honeydew melon pieces stored up to 7 days at 10°C*

Storage time (days)	Counts (log CFU/sample) at each phage concentration (log PFU/ml)[a]					
	0	10^4	10^5	10^6	10^7	10^8
0	1.5 AZ	1.3 AZ	1.2 AZ	0.7 ABZ	1.1 AY	0.0 B
2	2.7 AY	2.7 ABY	2.6 AY	2.2 BY	1.1 CY	0.0 D
5	5.3 AX	5.2 AX	5.0 AX	4.4 BX	3.1 CW	0.4
7	6.1 AW	6.1 AW	6.3 AW	5.3 BW	4.0 CW	1.8 C

[a] Treatment means within rows (time) with different letters (A through D) are different at the 0.05 significance level. Means within columns (phage concentration) with different letters (W through Z) are different at the 0.05 significance level. Because treatment with the phage concentration of 10^8 PFU/ml resulted in little or no variation, phage values at 0, 2, and 5 days were not included in the statistical analysis. However, all *L. monocytogenes* populations treated with phage concentrations of 10^4 to 10^7 PFU/ml were significantly different from zero, except 10^6 at 0 days. The phage was applied immediately after contamination with *L. monocytogenes.*

control, but the trend indicated lower *L. monocytogenes* populations on the fruit tissue at higher phage concentrations. Although there was no difference in effect between the phage concentrations at the time of application, except for 10^8 PFU/ml, which resulted in lower *L. monocytogenes* populations, there were differences between the concentrations of 10^5, 10^6, and 10^7 PFU/ml at 2, 5, and 7 days. A phage concentration of 10^8 was necessary to reduce the initial *L. monocytogenes* load at day 0 to nondetectable levels by standard plating procedures. For the *L. monocytogenes* control and the treatments with phage concentrations of 10^4 to 10^7 PFU/ml, the log (CFU) values were analyzed as a three-factor general linear mixed model, with concentration and time as the fixed factors and experiment as the random block factor. Variance grouping was used to correct for variance heterogeneity. Since the concentration × time interaction was statistically significant, mean comparisons were made (Table 1).

TABLE 2. *Populations of* Listeria monocytogenes *recovered from honeydew melon pieces treated with phage before, at, and after contamination with the bacterium and stored for up to 7 days at 10°C*[a]

Time of phage application	Counts (log CFU/sample) on storage day[b]:			
	0	2	5	7
−1-h phage	0.9 A	0.3 B	0.8 B	2.3 B
−30-min phage	0[c]	0	0.8 B	0.4 B
0-h phage	0	0	0	0
0-h no phage	0.8 A	2.9 A	5.7 A	7.2 A
30-min phage	0	0	3.2 B	4.6 B
30-min no phage	1.4	2.7	5.7 A	7.0 A
1-h phage	1.3 A	0.3 B	2.7 B	3.1 B
1-h no phage	1.0 A	3.0 A	5.8 A	7.0 A
2-h phage	1.3 A	0.7 B	3.7 B	4.1 B
2-h no phage	1.6 A	3.1 A	6.2 A	7.0 A
4-h phage	0.3 B	1.7 B	4.0 B	5.6 B
4-h no phage	1.6 A	3.5 A	6.6 A	7.1 A

[a] The *L. monocytogenes* concentration used to contaminate the fruit was 10^5 CFU/ml and the phage concentration was 10^8 PFU/ml.

[b] Within storage time (columns), the treatment means at each phage application time compared with the corresponding no-phage treatments that have different letters are different at the 0.05 significance level.

[c] Zero values were omitted from the analysis.

Timing of phage application. All phage applications reduced *L. monocytogenes* populations on fresh-cut honeydew melon pieces (Table 2). The application of the phage mixture to the fruit pieces at the time of contamination and up to 1 h before contamination with *L. monocytogenes* resulted in either nondetectable or very low levels of the bacterium. The bacterial populations increased during 7 days of storage at 10°C. However, the increases were less on fruit pieces treated with the phages before or at the time of contamination with *L. monocytogenes* than on fruit pieces that had been contaminated with the bacterium for 0.5 h or longer at the time of the phage application. When the phage mixture was applied 0.5, 1, or 2 h after contamination, the populations were still low (0 to below 1 log of CFU per sample) after 2 days of storage but then increased more rapidly by the fifth day of storage.

At the different phage application times in relation to the contamination with *L. monocytogenes,* the initial reduction of *L. monocytogenes* populations by the phage was not significant, except when the phage mixture was applied 4 h after inoculation (Table 2). The bacterial populations had already increased on the fruit tissue during the 4 h at room temperature, accounting for the greater reduction. The trend indicates that when the phage mixture was applied to the fruit pieces immediately after contamination with *L. monocytogenes,* bacterial populations were reduced to the lowest level (Table 3). This was followed in efficacy by the application at 0.5 and 1 h before contamination. Table 3 further indicates that the phage application before or at the time of contamination with *L. monocytogenes* reduces the pathogen growth more than at the other application times after 2, 5, and 7 days of storage.

The log (CFU) values were analyzed as a one-factor general linear model with one code for treatment and time as the factor. Variance grouping was used to correct for variance heterogeneity. The means were not all equal (F value = 283.38, $P < 0.0001$, df = 39). Selected mean comparisons are given in Table 2. All the means of the no-

TABLE 3. *Level of reduction of the* Listeria monocytogenes *populations on honeydew melon pieces by application of the phage at different times*[a]

Application time (h)[b]	Reduction values (log CFU/treatment) on storage day: 0	2	5	7
−1	0.01	2.5*	4.9*	4.8*
−0.5	[0.009]	[2.7]	4.9*	6.8*
0	[0.009]	[2.7]	[5.6]	[6.9]
0.5	[1.009]	[2.6]	2.6*	2.4*
1	0.2	2.6*	3.1*	4.0*
2	0.3	2.4*	2.5*	2.9*
4	1.3*	1.8*	2.6*	1.5*

[a] Reduction values with an asterisk are estimates based on the difference between the control treatment mean and the corresponding noncontrol treatment means. Reduction values in brackets are estimates based on the difference between a lower 95% confidence level for the control treatment mean and the constant zero. This was done to estimate the reduction for those matching noncontrol treatments where all values were zero. All reduction estimates are statistically significant at the 0.05 level.

[b] Phage application time is given in hours after inoculation with bacteria.

phage treatments (controls) are statistically different from zero at the 0.05 significance level.

Phage titer. The titration of the phages recovered from the inoculum was approximately 7 log PFU/ml. This was not dependent on the $MnCl_2$ concentration or the pH. The pH of the inoculum had been adjusted to the pH of the apples, which was pH 3.8. In all the treatments that included phages recovered from the apple tissue, the titer was below detectable levels. On honeydew melons, the phage titer stayed at the level of inoculation.

Effect of $MnCl_2$ on phage effectiveness.. At 10°C, there was no significant difference between the treatments applied to *L. monocytogenes* populations on fresh-cut apple plugs (Table 4). However, at 25°C, although there was no difference due to $MnCl_2$, there was a strong effect of storage time. The addition of $MnCl_2$ to the apple plugs contaminated with *L. monocytogenes* did not increase phage effectiveness at 10 or 25°C.

DISCUSSION

The phage application was most effective on honeydew pieces when applied between 1 to 0 h before contamination with *L. monocytogenes*, which suggests that it would have to be applied at the time of cutting or as soon as possible after cutting the produce to be effective against potential contaminations occurring between the time of cutting and packaging. The correct timing of the phage application increases its effectiveness at the longer storage times as well. The growth of the pathogen is inhibited even after 7 days of storage at 10°C, if the phage is applied up to 0.5 h before or at the time of contamination. This effect is smaller the later the phage is applied after contamination, which may be partly explained by the increasing bacterial populations at later times.

TABLE 4. *Effect of several combinations of phage and different $MnCl_2$ concentrations on* Listeria monocytogenes *populations on Golden Delicious apple tissue plugs stored at 10 or 25°C for 3 days*[a]

	CFU/sample[b]			
	10°C		25°C	
Treatment	Day 0	Day 3	Day 0	Day 3
L	3.18	3.33	3.19 AX	5.59 BX
LP	3.22	3.66	3.24 BX	3.24 BX
LP-Mn005	3.22	2.56	3.22 BX	6.29 AX
LP-Mn01	3.22	2.87	3.21 BX	6.23 AX
LP-Mn05	2.4	2.85	2.39 BX	5.96 AX
L-Mn05	3.21	2.78	3.25 BX	6.22 AX

[a] Statistical analysis of all nonzero treatments ($F = 404.86$, $P < .0001$, df = 11). At 10°C the treatments were not different from each other. L, *Listeria monocytogenes* control; LP, *L. monocytogenes* + phage; LP-Mn005, *L. monocytogenes* + phage + 0.05 M $MnCl_2$; LP-Mn01, *L. monocytogenes* + phage + 0.1 M $MnCl_2$; LP-Mn05, *L. monocytogenes* + phage + 0.5 M $MnCl_2$; L-Mn05, *L. monocytogenes* + 0.5 M $MnCl_2$.

[b] Time means within treatment (rows) with different letters (A or B) are different at the 0.05 significance level. Treatment means within time (columns) with different letters (X or Y) are different at the 0.05 significance level.

Increasing concentrations of the phage mixture increased its effectiveness. A similar trend was reported for the reduction of viable cell numbers in biofilms of *Pseudomonas aeruginosa* after phage treatment *(8).* Although *L. monocytogenes* populations were reduced by all phage concentrations (10^5 to 10^8 PFU/ml), higher phage concentrations resulted in a greater reduction of pathogen populations at the time of application. In addition, for maximum effectiveness in reducing populations of *L. monocytogenes* throughout the entire storage period of 7 days at 10°C, there seems to be the necessity for the phage concentration to be 10^8 PFU/ml at the time of application. Similar thresholds (i.e., the multiplicity of infection threshold, which is the average number of virus particles infecting each cell) have been reported for an infection of 99.99% of the bacteria or for the inhibition of subsequent bacterial growth in a model system using *Escherichia coli (10, 21).* However, the optimal phage/bacteria ratio may also be specific to the organisms used. To be successful in eradicating the bacterial host, in some cases, the phage has to eliminate the bacteria at the time of inoculation (first generation), whereas in other cases the phages can actively multiply (second generation) and infect more bacteria *(16).* It appears that in our system it is important to reduce the bacterial levels initially as much as possible with a high phage concentration. However, there is an additional effect of the timing of the application that influences the reduction level over several days of storage and there may be second phage generations.

Previously, we reported on the reduced effectiveness of phages on low pH fruit such as apples *(12).* Manganese chloride, which may be used as a dietary supplement, has generally recognized as safe status according to the Food and Drug Administration in its list referred to as Everything

Added to Food in the United States *(20)*. Adding $MnCl_2$ has proved successful in the recovery of phages from water using negatively charged microporous filters that require a low pH solution *(18)*. In our experiments, the phages survived the low pH of 3.8 of the inoculum in the presence of $MnCl_2$, since the titer recovered was approximately 7 log PFU/ml. However, combining $MnCl_2$ with the phages increased neither phage survival nor effectiveness against *L. monocytogenes* on apple plugs. In addition, the phage titer on apples had dropped below the level of detection at 0 days, approximately 0.5 h after application. It may be that the phages are prevented from attaching or infecting their bacterial hosts by a specific acid or some other compound present in the apples rather than the reduction in effectiveness being solely a pH effect. In our experiments, the growth of the *L. monocytogenes* pathogen far outweighed the numbers of phages that survived or were active on apples.

Although further experiments are necessary to improve the effectiveness of the phage application on apples, the phage treatment is very effective in reducing *L. monocytogenes* on honeydew melons. The current results indicate that phage application on honeydew melon pieces is most successful when using a high concentration of approximately 10^8 PFU/ml and by applying the phage cocktail up to 1 h before contamination of the fruit. Commercially, this implies that the phage would have to be applied as soon as possible after the time of cutting.

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