COMBINING OF BACTERIOPHAGE AND G. ASAII APPLICATION TO REDUCE L. MONOCYTOGENES ON HONEYDEW MELON PIECES

Yoonpyo Hong[1], Sun Tay Choi[2], Bong Hoon Lee[3], William S. Conway[4*]

Postharvest Technology Division[1]; International Technology Cooperation Center[2]; UNDP-RDA Cooperative Project Secretariat, Rural Development Administration, 250 Seodun-dong, Suwon 441-707, Korea(Republic of)[3]; Produce Quality and Safety Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, USA[4]

Abstract

Gluconobacter asaii (a bacterial antagonist naturally occurring on apple fruit) and bacteriophage were tested as biocontrol agents of *Listeria monocytogenes* on fresh-cut honeydew melon pieces. *Gluconobacter asaii* and bacteriophage were effective against of *L. monocytogenes*, but combining the two treatment was even more effective. G. asaii alone reduced populations approximately 3 to 4 logs and phage alone reduced populations by one log compared to the L. monocytogenes control. In comparison *Listeria* control treatment combining treatments reduced populations up to 6 logs by day 7. The results of our study suggest that *G. asaii* and phage combined can be very effective in reducing *L. monocytogenes* contamination of fresh-cut honeydew melon.

1. Introduction

Listeria monocytogenes is a Gram-positive, motile organism capable of growth between –0.4 and 50°C (Faber and Peterkin 1991). It is ubiquitous, highly virulent and tolerant to environmental stress (Doyle 1999). It also grows at refrigeration (Van 2000) temperature which is of great concern especially with the emergence of the new generation of minimally processed foods relying only on refrigeration and intrinsic parameters as proliferation of undesirable microorganisms. The presence of *L. monocytogenes* on decaying vegetation (Welshimer 1968), agronomic crops (Weiss 1975) and silage (Skovgaard 1988) is well documented.
This organism has been associated with a number of serious foodborne outbreaks and recalls of fresh produce (Beuchat 2002, Farber and Peterkin 1991). Usually occur at pathogen populations greater than 10^3 CFU per g or per ml (Tompkin 2002). Because of the high case fatality rate, which is account for up to 96% of human listeriosis, associated with *L. monocytogenes* infections, the U.S. Food and Drug Administration have established a zero-tolerance for *L. monocytogenes* in ready-to-eat foods, including processed fresh-cut fruits and vegetables. Recently, there were recalls of cut honeydew and cut cantaloupe melons processed and mixed fruits and vegetables and

apple slices. Some products were prepared in store such as a bulk salad sold at a store in New York that was recalled due to *L. monocytogenes* contamination.

Bacteriophages (phages) are ubiquitous and can be found in fresh water in high numbers (Bergh 1989). They are also a natural part of fermentation processes such as the production of sauerkraut (Barrangou 2002, Lu 2003) or pickles (Lu 2003). Phages have been used experimentally against a variety of pathogenic bacteria on plants, animals, and food items. Recently, phage cocktails were shown to reduce populations of foodborne human pathogens on honeydew melons and apples alone and in combination with a bacteriocin (Leverentz 2003, Leverentz 2003).

Gluconobacter strains are gram-negative acetic acid bacteria. While they are non-pathogenic towards humans, they may cause browning of some apple. The bacteria are able to grow at a low pH of 3.5 in highly concentrated sugar solutions and on fruit such as apples, pears and grapes as well as in ciders (Deppenmeier 2002, Gupta 2001, Van 1981). *Gluconobacter* species are found and utilized in fermentation processes and production of wine, vinegar and vitamin C (Deppenmeier 2002, Macauley 2001, Yamada 1999).

Gluconobacter asaii proved effective in preventing the growth or survival of *L. monocytogenes* on fresh cut apple tissue (2006 in press).

The objectives of this study were to determine (i) the effectiveness of phage and *Gluconobacter asaii* as antagonists of *L. monocytogenes* on honeydew melon pieces and (ii) whether combining the phages with *Gluconobacter* would enhance the effectiveness against *L. monocytogenes*.

2. Materials and Methods

Fruit. Honeydew melons obtained from the local market were cut into 10-mm-thick rings with a deli slicer (model 827, Berkel Inc., La Porte, Ind.). The 10-mm-thick melon rings were cut into equally sized squares about 25 mm^2. The fruit surfaces as well as the deli meat slicer were disinfected with 70% ethanol immediately before slicing. A cork borer was used to cut tissue plugs of honeydew that was 10 mm thick and 10mm in diameter, resulting in the tissue plugs of 0.785cm^3. The pH of the melon slices was monitored using a Semi-Micro pH combination electrode (81-03 Ross, Orion Research, Inc., Beverly, Mass).

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 peptone water (pH 7.4) to approximately 10^6 PFU/ml immediately before application to the fruit pieces.

***Glucanobacter*.** The *Glucanobacter asaii* originally isolated from apple surfaces, and that was grown on nutrient yeast dextrose agar (NYDA) plates overnight at 25°C. One colonys were scraped from the agar plates and suspended in NYDB+grown 6hr at 25°C on a shake (150 rpm). The solution was in peption water (pH 7.4). The cell concentration was adjusted to 10^5 – 10^6 CFU/ml using a SmartsSpec 3000

spectrophotometer (Bio-Rad Laboratories, Richmond, Calif.) at 600nm according to standard curves. The exact cell concentration was determined by plating the inoculum with a spiral plater (DW Scientific, Shipley, West Yorkshire, England) into NYDA medium followed by incubation at 20°C for 1 day.

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, Depatment of Food Science and Technology, University of Georgia, Agricultural Experiment Station, Griffin, Ga., and stored at -80°C in Luria-Bertani (LB) broth (BD Dianostic 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 ug/ml of nalidixic acid at 30°C and then transferred to 10ml of TSB broth for 6 h. The cell were harvested by centrifugation at 10,000 x g for 15min. The pellet was resuspended in peptone water (pH 7.4) and adjusted to a concentration of 10^4 or 10^5 CFU/ml at an optical density of 600nm using a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Rechmond, Calif.). The exact cell concentration was determined by plating the inoculum with a spiral plater (DW Scientific, Shipley, West Yorkshire, England) into 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, III.). The fruit tissue was then inoculated with 25 ul of the *L. monocytogenes* suspension containing approximately 1 × 104 CFU/ml. The procedure for inoculating the pieces of fruit took approximately 10 min. Then, the phage and/or *Glucanobacter* treatments were applied by pipette to a 5-by 5-mm area in 25-ul aliuots. There were three or four fruit samples per treatment at each recovery time. The covers on the plastic bowls allowed air exchange, which ensured that the environmental conditions did not change and therefore did not create a modified atmosphere.

Recovery of pathogen and antagonist. The pathogen and antagonist populations were recovered from the honeydew melon plugs after 0, 2, 5, and 7 days of storage at 10°C as described previously (Conway 2000). Briefly, the melon tissue plugs were each placed into a sterile plastic bag containing 4.5 ml of peptone water and homogenized in a stomacher blender for 120 s at a high speed set at 8 (Bagmixer 100 Minimix;Intersceence, Weymouth, Mass). Aliquots (50ul) of the homogenized mixtures or dilutions thereof were plated in duplicate on TSA containing 100 ug per ml of NAL for *L. monocytogenes* or nutrient yeast dextrose agar (NYDA; put this under gluconobacter when NYDA is 1st mentioned) using a spiral plater. The TSA + NAL plates were incubated overnight at 37°, the NYDA plates were incubated overnight 20°C. Colony counts were determined using an automated plate counter (ProtoCol;Synoptics, Cambridge, United Kingdom), and the data were plotted as CFU per sample. All experiments were repeated.

Phage titration. Smaples from phage treatments in each experiment were homogenized and then filtered through a 0.45-um-pore-size membrane (Acrodisk; Pall

Gelman, Ann Arbor, Mich.). The phage titer in the filtrates was determined using a soft agar overlay (Adams 1959). The resulting plaques were counted with the ProtoCol plate counter (Synoptics), and the data were plotted as PFU per sample.

Statistical analyses. The bacterial recovery data (CFU per sample) were analyzed for each experiment as two-factor general linear models using PROC MIXED (SAS Institute) with Treatment and Day as the factors. The assumptions of the linear model were tested. To correct for variance heterogeneity the treatments were grouped into similar variance groups for the analysis. When effect(s) were statistically significant, mean comparisons were done with Sidak adjusted p-values so that the experiment-wise error was 0.05.

3. Results

Recovery of *Glucanobacter* and phage. *Gluconobacter asaii* was originally isolated for its biocontrol activity against postharvest fungal decay pathogen on apple. In a preliminary experiment we found it was also able to grow or survive on fresh-cut honeydew melon pieces over time (data not shown). When co-inoculated with *L. monocytogenes* and phage, *Gluconobacter* was increased over time from day 0 to day 7 (Fig 1). The titration of the phages recovered from the inoculum was between 3 and 5 log PFU/ml. Phage recovered well by itself over 5 days but there was no recoverable phage by 7 days when combined with *Gluconobacter asaii* (Fig 2).

Control of *L. monocytogenes* . When fruit were inoculated on *L. monocytogenes* at 1 x 10^4 CFU/ml the biocontrol agents were effective at controlling and even reducing populations compared to the *Listeria* alone treatment (Fig. 3).
The phage alone treatment reduced *L. monocytogenes* populations approximately 1 log from 2 to 7 days. *Gluconobacter asaii* alone was more effective than phage at 5. and 7 days, reducing *L. monocytogenes* populations 3 and 4 logs respectively. Combining phage and *Gluconobacter asaii* was the most effective at controlling the listeria populations. In comparison with control, combining of the phage and *Gluconobacter asaii* reduced the bacterial populations by 4.5 to 5.8 log units. There was a phage x *Gluconobacter asaii* interaction on honeydew melons (Fig 3). Even when the concetration of *L. monocytogenes* inoculated was increased from $1x10^4$ to $1x10^6$ the same rate as biocontrol agent, by day 5, all treatments significant reduced the bacterial populations on fresh cut honeydew melon.

4. Discussion

We found that combining phage and *Gluconobacter asaii* treatments is an effective method for reducing *L. monocytogenes* contamination on fresh-cut honeydew melon. Because of previous successes with respect to lytic bacteriophages on honeydew melon (Leverentz 2003) and *Gluconobacter asaii* on apples (Leverentz 2006), we hoped to see an added effect to the combination. This was, however, the first test of *Gluconobacter asaii* on honeydew melon as a biocontrol agent. The recovery of *Gluconobacter asaii* from honeydew melon pieces stored 10°C was increased over 7 days. This result is similar to those of our previous experiments with *Listeria* and *Salmonella* growing on fresh-cut apples. The more growth of *Gluconobacter asaii* at

10°C with *L. monocytogenes* compared to *Gluconobacter asaii* on fresh-cut apples stored at 25°C may be due to the greater cold tolerance of *Gluconobacter asaii* (Leverentz 2006).

Phage was known as effective biocontrol agent about reducing *L. monocytogenes* and *Salmonella* on fresh-cut fruits through our previous reports (Leverentz 2004, Leverentz 2003, and Leverentz 2001). Phage recovered well when co-inoculated with *L. monocytogenes* but no phage was recovered by 7 days when combined with *Gluconobacter asaii*. There are several possibilities to explain the low recovery of phage at 7 days. One of the most reasonable is the low concentration of *L. monocytogenes* remaining and the phage could not "find" the necessary host.

The combining of phage and *Gluconobacter asaii* was the most effective against controlling *L. monocytogenes* populations on honeydew melon pieces. In addition, for maximum effectiveness in controlling populations of *L. monocytogenes* throughout the entire storage period of 7 days at 10°C, there seems to be synergy effect between phage and *Gluconobacter asaii*. In past experiments we had observed that phage decreased in biocontrol activity over time (Leverentz 2003) and that the *Gluconobacter asaii* was increased in biocontrol activity over time effective later (Leverentz 2006). We hypothesized that the combination would more effectively control *L. monocytogenes* populations over the course of the trial.

This is reversed result compared to previous experiment for combining phage cocktail/nisin on fresh-cut honeydew melon (Leverentz 2003). The phage may be different reaction depend on its application method that were inoculation or spray to fresh-cut produce.

In conclusion, the results of our study suggest that combining *G. asaii* and phage can be very effective in reducing *L. monocytogenes* contamination of fresh-cut honeydew melon. They can be used, alone or in combination with at least some of the approaches currently used in the produce industry, to reduce or prevent the contamination of whole or fresh-cut fruits and vegetables with foodborne pathogens.

Acknowledgements

We would like to thank Amy Blodgett and Michelle Orton for technical and logistical support.

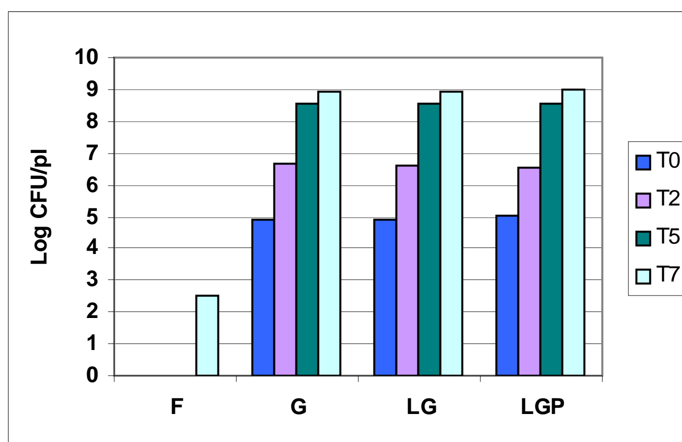

Fig. 1. Populations of *G. asaii* alone or in the presence of *L. monocytogenes* alone or combined with phage when wedges were stored at 10°C over 7 days

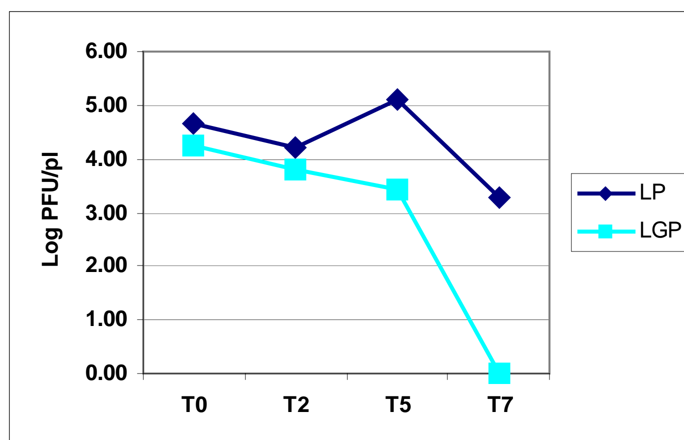

Fig. 2. Populations of phage in the presence *L. monocytogenes* alone or combined with *G. asaii* when wedges were stored at 10°C over 7 days

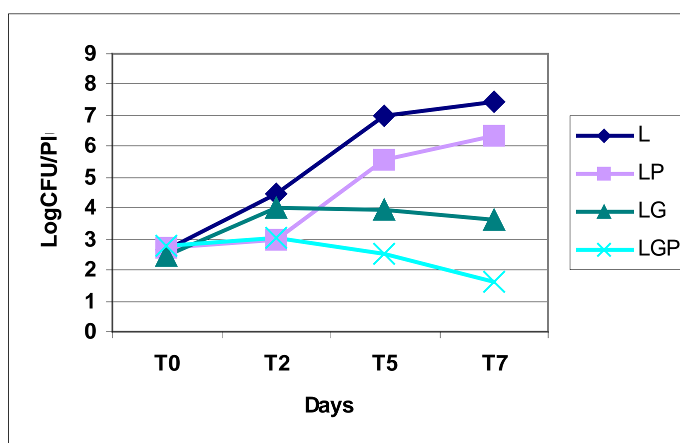

Fig. 3. Effect of *G. asaii* (G) and phage (P) on the recovery of *L. monocytogenes* (L, 10^4) from honeydew wedges stored at 10°C over 7 days.

	Day			
Treatment	0	2	5	7
L	2.637a[1]γ[2]	4.452aβ	7.061aα	7.474aα
LP	2.737aγ	3.006bγ	5.536bβ	6.377bα
LG	2.491aβ	4.007aα	3.951cα	3.651cα
LGP	2.785aα	3.069bα	2.496dαβ	1.618dβ

[1] Treatment means within Day with different a, b, c, d letters are different at the 0.05 significance level.

[2] Day means within Treatment with different α, β, γ letters are different at the 0.05 significance level.

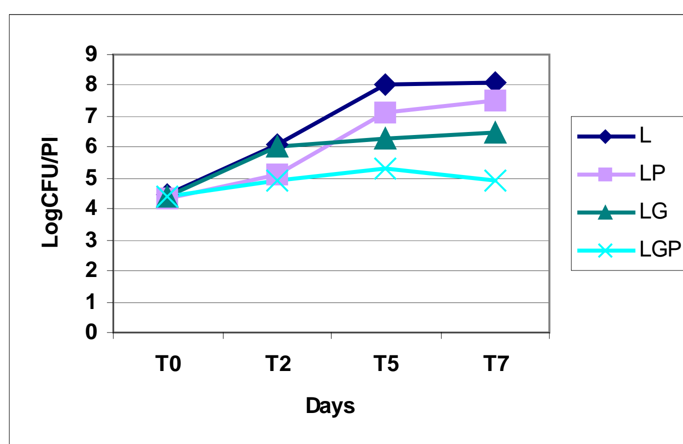

Fig. 4. Effect of *G. asaii* (G) and phage (P) on the recovery of *L. monocytogenes* (L, 10^4) from honeydew wedges stored at 10°C over 7 days.

	Day			
Treatment	0	2	5	7
L	4.463a[1]γ[2]	6.152aβ	8.038aα	8.169aα
LP	4.313aγ	4.995bβ	7.305bα	7.365bα
LG	4.406aβ	6.104aα	6.526bcα	6.883abα
LGP	4.397aβ	5.107bα	5.440cα	5.219cα

[1] Treatment means within Day with different a, b, c, d letters are different at the 0.05 significance level.
[2] Day means within Treatment with different α, β, γ letters are different at the 0.05 significance level.

References

1. Adams, M. H. 1959. Bacteriophages. Interscience publishers, New York, N. Y.
2. Barrangou, R., S. S. Yoon, F. Breidt, H. P. Fleming, and T. R. Klaenhammer. 2002. Characterization of six Leuconostoc fallax bacteriophages isolated from an industrial sauerkraut fermentation. Appl. Environ. Microbiol. 68:5422-5458.
3. Bergh, O., K. Y. Borsheim, G. Bratbak, and M. Heldal. 1989. High abundance of viruses found in aquatic environments. Microbes Infect. 4:413-423
4. Beuchat, L. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes infect. 4:413-423.
5. Conway, W. S., B. Leveentz, R. A. Saftner, W. J. Janisiewicz, C. E. Sams, and E. Leblanc. 2000. Survival and growth of Listeria monocytogenes on fresh-cut apple slices and its interaction with Glomerella cingulata and Penicillium expansum. Plant Dis. 84:177-181.
6. Deppenmeier, U., M. Hoffmeister, and C. Prust. 2002. Biochemistry and biotechnological applications of Gluconobacter strains. Appl. Microbiol. Biotechnol. 60:233-242.
7. Doyle M P (1988) Effect of environmental and processing conditions on Listeria monocytogenes. Food Technology 42. 169-171.

8. Faber, J.M. and Peterkin, P.I. (1991) Listeria monocytogenes, a food-borne pathogen. Microbiological Reviews 55, 476-511
9. Gupta, A., V. K. Singh, G. N. Qazi, and A. Kumar. 2001. Gluconobacter oxydans:Its biotechonogical applications. Journal of Molecular Microbiology and Technology 3:445-456.
10. Leverentz, B., W. S. Conway, Z. Alavidze, W. J. Janisiewicz, Y. Fuchs, M. J. Camp, E. Chighladze, and A. Sulakvelidze. 2001. Examination of bacteriophage as a biocontrol method for Salmonella on fresh-cut fruit – a model study. J. Food Prot. 64:1116-1121.
11. Leverentz, B., W. S. Conway. M. J. Camp, W. J. Janisiewicz, T. Abuladze, M. Yang, R. Saftner, and A. Sulakvelidze. 2003. Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Appl. Environ. Microbiol. 69:4519-4526.
12. Leverentz, B., W. S. Conway. W. J. Janisiewicz, and M. J. Camp. 2004. Optimizing concentration and timing of a phage spray application to reduce Listeria monocytogenes on honeydew melon tissue. J. of Food Protection, 67:1682-1686
13. Lu, Z., f. Breidt, V. Plengvidhya, and H. P. Fleming. 2003. Bacteriophage ecology in commercial sauerkraut fermentations. Appl. Environ. Microbiol. 69:3192.
14. Lu, Z., F. Breidt, H. P. Fleming, E. Altermann. And T. R. Klaenhammer. 2003. Isolation and characterization of a Lactobacillus plantarum bacteriophage. Phi JL-1, from a cucumber fermentation. Int. J. Food Microbiol. 84:225-235.
15. Macauley, S., B. McNeil, and L. M. Harvey. 2001. The genus Gluconobacter and its applications in biotechnology. Crit. Rev. Biotechnol. 21:1-25.
16. SAS Institute Inc. 1999. *SAS/STATⓡ User's Guide, Version 8*. SAS Institute, Cary, N. C.
17. Skovgaard, N., and C.-A. Morgen. 1988. Detection of Listeria spp. In faeces from animals, in feeds, and in raw foods of animal origin. Int. J. Food Microbiol. 6:229-242.
18. Tompkin, R. B. 2002. Control of Listeria monocytogenes in the food-processing environment. J. Food Prot. 65:709-725.
19. Van de Venter T (2000) Emerging food-borne diseases: a global responsibility. Food nutrition and Agriculture (FAO) 26, 4-13
20. Van Keer, C., P. Vanden Abeele, J. Swings, F. Gossele, and J. De Lay. 1981. Acetic acid bacteria as causasl agents of apples and pears. Zbl. Bakt. Hyg., I. Abt. Orig. C:197-204.
21. Weiss, J., and H. P. R. Seeliger. 1975. Incidence of Listeria monocytogenes in nature. Appl. Environ. Microbiol. 30:29-32.
22. Welshimer, H. J. 1968. Isolation of Listeria monocytogenes from vegetation. J. Bacteriol. 95:300-320.
23. Yamada, Y., R. Hosono, P. Lisdyanti, Y. Widyastuti, S. Saono, T. Uchimura, and K. Komagata. 1999. Identification of acetic acid bacteria isolated from Indonesian souces, especially of isolates classified in the genus Gluconobacter. J. Gen. Appl. Microbiol. 45:23-28.