NATIONAL WATERMELON PROMOTION BOARD
Final Project Report 2012

PROJECT TITLE: Comparative survival of *Listeria innocua*, *Listeria monocytogenes* and *Salmonella enterica* on watermelon surfaces

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Project description

The survival of pathogenic and nonpathogenic strains of *Listeria spp.* on watermelon surfaces, in comparison with *Salmonella enterica*, was evaluated under different conditions of temperature exposure during storage for up to 14 days. In this study we evaluated;

a. The survival of a cocktail of *Listeria monocytogenes* in comparison with the nonpathogenic indicator *Listeria innocua* and a cocktail of pathogenic strains of *Salmonella enterica.* Comparison with *L. innocua* was performed in order to evaluate and validate whether *L. innocua* could be used as a safe surrogate for further studies in laboratory or field environment evaluations not allowed with infectious human pathogens. Additionally, with previous knowledge of the survivability traits of *S. enterica* on watermelon surfaces (funded by the NWPB) this pathogen of concern was utilized as a control for the reproducibility from prior studies in our lab and as a benchmarking threshold to evaluate *Listeria* survival with a pathogen known to survive in harsh conditions.
b. The survival of *Listeria* spp. and *Salmonella* was studied on the upper fruit surface green-rind as well as on the yellow-rind ground spot. This was performed in order to evaluate whether differences in surface traits, including structure and wax cuticle formation, would impact the survival
c. The effect of holding-temperature exposure on the survival of *Listeria spp.* and *S. enterica* during simulated distribution storage was evaluated.

d. The relative survival under different initial inoculation doses and strains of *Listeria spp.*
e. Preliminary assessment of postharvest disinfection of *Listeria spp.*was conducted

Major Key outcomes of this study

a. *Listeria spp.* survival on watermelon surfaces is highly dependent on postharvest holding temperature and inoculation dose.
b. Overall, after inoculation *Listeria* populations tend to drop significantly within the first 24 hours, the remaining population seems to be stable and persistent for up to 10 days when temperatures of storage are below 20 ^{o}C (68°F) and tend to become non-viable/non-recoverable after this time.
c. No differences in survival were observed when the bacteria was inoculated on green surfaces or onto the yellow ground spot
d. Although further studies are recommended, the specific *Listeria* strain could impact the fate of these bacteria on watermelon surfaces. This may be of greater significance in the preharvest environment.
e. Although establishment of GAPs and preventive controls must be established during the production of any fruit and vegetable, surface disinfection of watermelon appears to be a post-harvest intervention operation that can be implemented for successful risk reduction and mitigation of enteric bacterial pathogens.

Materials and methods

Bacterial strains and inoculum preparation.

The bacterial strains utilized in this study are described in Table 1. A cocktail of PTVS 333, PTVS 334 and PTVS 335 was prepared for *L. monocytogenes*. A cocktail with PTVS 042, PTVS 045 and PTVS 043 was prepared for *S. enterica.* All strains were first re-streaked on selective media; Oxford and XLT-4 for *Listeria spp.* and *Salmonella,* respectively, in order to evaluate the purity of the isolates. An isolated colony was then re-streaked on Tryptic Soy Agar supplemented with 50 mg/L of rifampicin (TSA+rif), plates were incubated at 37 ^{o}C for 24 and 48 h for *Salmonella* and *Listeria*, respectively. A single colony was resuspended in 100 μL of Butterfield's buffer and spread onto TSA+rif and incubated until confluent growth was observed (approximately 24 and 48 h for *Salmonella* and *Listeria*, respectively). The lawns were resuspended in Butterfield's buffer until the bacterial suspension achieved an optical density of 0.750 at 600 nm, which corresponded to approximately log 9 CFU/mL. The inoculum was plated after being serially diluted to determine the exact concentration.

Cocktails of *L. monocytogenes* and *Salmonella* as well as *L. innocua* inoculum were prepared by mixing an equal amount of cells required to have a total of log 7, 5 or 3 CFU/mL (10 million, 100,000, or 1000 cells per ml).

Table 1. Bacterial strains utilized in this study

Strain ID	Microorganism	Description
PTVS 333	*L. monocytogenes*	Selected for spontaneous mutation to rifampicin from strain PTVS 287 obtained from a produce farm
PTVS334	*L. monocytogenes*	Selected for spontaneous mutation to rifampicin from strain PTVS 290 obtained from irrigation water
PTVS335	*L. monocytogenes*	Spontaneous mutation to rifampicin from strain PTVS 308 obtained from cantaloupe
TVS 451	*L. innocua*	Selected for spontaneous mutation to rifampicin from strain TVS450, ATTC 33090
PTVS 042	*S. enterica* sv. Michigan	Selected for spontaneous mutation to rifampicin, from environmental strain
PTVS 045	S. enterica sv. Montevideo	Selected for spontaneous mutation to rifampicin, from environmental strain
PTVS 043	S. enteriva sv. Agona	Selected for spontaneous mutation to rifampicin, from environmental strain

Watermelon inoculation

The watermelons were obtained from either from a retail or wholesale source, or directly from a grower/shipper. As much as possible we aimed to work with untreated recently harvested watermelons to ensure that the rind was minimally altered from preharvest field conditions. After reception, all watermelons were stored at 10 ^{o}C (50°F) for up to 3 days until they were inoculated. Prior to inoculation, fruit was allowed to equilibrate to ambient temperature for about 2 hours. All fruit was marked with a circle of about 16 cm^2 or 45 cm^2 for regular and mini watermelons, respectively, using indelible ink. For regular size fruit, up to 20 circles were marked on each fruit in either the green rind or on the yellow ground spot. In contrast, for mini-watermelons a single circle was marked on each fruit rind surface.

The inoculum was deposited within each circle through spot inoculation, a total of 60 or 150 μL of the inoculum were deposited on the surface of regular and mini watermelons, respectively, by discharging droplets of 3-5 μL that were distributed within the marked area (Figure 1). The inoculum was allowed to dry on the surface at room temperature and fruits were stored in corrugated fiberboard cartons, making sure inoculated areas were not touching either the carton sidewalls or other fruit. Due to lab biosecurity restrictions and the pathogenicity of the utilized strains, boxes were covered with plastic wrap to restrict aerosols and accidental human contact. Storage was done at 15, 25 or 37 ^{o}C (59, 77, 98.6 °F) for up to 14 days. Temperature and relative humidity were recorded with a data logger.

Figure 1. Inoculation of watermelons

Watermelon processing.

The inoculated area of the watermelon was aseptically removed with a knife that was surface disinfected with 96% ethanol. The inoculated portion was transferred to a sterile bag and either 10 or 5 mL of potassium phosphate buffer supplemented with 1% Tween 20 (a wetting agent surfactant) were added (5 mL were added after 5 days of storage to increase sensitivity of the detection), samples were vigorously, manually massaged to detach inoculated bacteria. The bacterial suspension was plated onto TSA+rif supplemented with PCNB to inhibit fungal growth, in order to recover and quantify viable bacterial cells. Recovery plates with *Listeria* were incubated for up to 48 h at 37 ^{o}C while plates with *Salmonella* were incubated for 24 h. When plates had no visible colonies, the bacterial suspension was enriched with 10 mL of double strength Buffered peptone water or Listeria enrichment broth, for *Salmonella* and *Listeria* samples respectively; enrichments broths were supplemented with rifampicin to have a final concentration of 50 mg/mL. Enrichments were incubated under the same conditions described for TSA plates. After enrichment, two-spots of 25 μL each were placed on either XLT-4 or Oxford-Listeria media supplemented with rifampicin, for *Salmonella* and *Listeria* respectively. Typical colony phenotypes on these media were considered as positive sample outcomes.

Watermelon disinfection

Mini-watermelons were inoculated with 150 μL of log 9 CFU/mL of a cocktail of *L. monocytogenes* as previously described, the inoculum was allowed to dry overnight. Inoculated areas were aseptically removed and treated with either peracetic Acid (Bio Safe Systems LCC), Prosan ® (Microcid Inc.), sodium hypochlorite at 50 and 100 mg/L at pH=7 and with tap water. The disinfection consisted of total immersion of the inoculated section following manufacturer instructions; the contact time was 5 min for peracetic acids and 2 min for the remainder of the treatments. After submersion in the respective disinfection solution, inoculated rinds were rinsed with water for an additional two minutes; a non-treated control was included. Sections were placed in a sterile bag and recovery of inoculated bacteria was performed as previously described. Log reduction was determined among the different treatments.

An additional trial was performed utilizing peracetic acid and Prosan. In this trial, the disinfectant solutions were prepared following manufacturer instructions, after solution preparation paper towels were submerged in the antimicrobial wash. The paper towels were utilized to cover the inoculated area for a period of 1 min, after the end of the contact time of the saturated towel with the watermelon surface, samples were rinsed for a minute in water and recovery

of the bacteria was performed as previously described. Log reduction was also determined among the different treatments.

Results

Survival of L. monocytogenes, L. innocua and S. enterica on watermelon surfaces during storage at 20 ^{o}C

Initial inoculation dose of about log 5 CFU/cm^2 of a cocktail of *L. monocytogenes, L. innocua* and *Salmonella,* resulted in an approximate reduction of 2.5-log (> 200%) after only 48 h of storage. Thereafter the population of all bacteria tested, remained largely unchanged for up to 10 days after which a drop in the recoverable population was observed to almost below the limit of detection (log 0.65 CFU/cm^2) (Fig 2A-B). Overall, no significant differences were determined among the three bacteria within each day of storage ($p<0.05$). Also, comparison in survival between the yellow ground spot and the green area was not significant ($p>0.05$) except for *Salmonella* whose population on yellow surfaces was significantly greater than the population on the green surface (Fig 2B).

The behavior observed for the three microorganisms, suggests that inoculation and subsequent drying produce a drop in the population which likely is associated with rapid desiccation, however the remaining population is stable for approximately 8 days until cells start to die off. This could be the result of bacteria remaining in a limited area in which any source of nutrient is exhausted or the metabolism required to survive under these conditions, is no longer feasible to maintain viable cells, or cells enter a condition that prevents resuscitation using standard recovery techniques. Only in the case of *S. enterica* inoculated on the green surface an increase in the population was determined (Fig. 2A), however it followed the same fate of the other bacteria over time. It is unlikely that the conditions in which bacteria are maintained and the watermelon surface itself promote bacterial growth, so it could be mostly likely associated to sample variation rather than actual microbial growth.

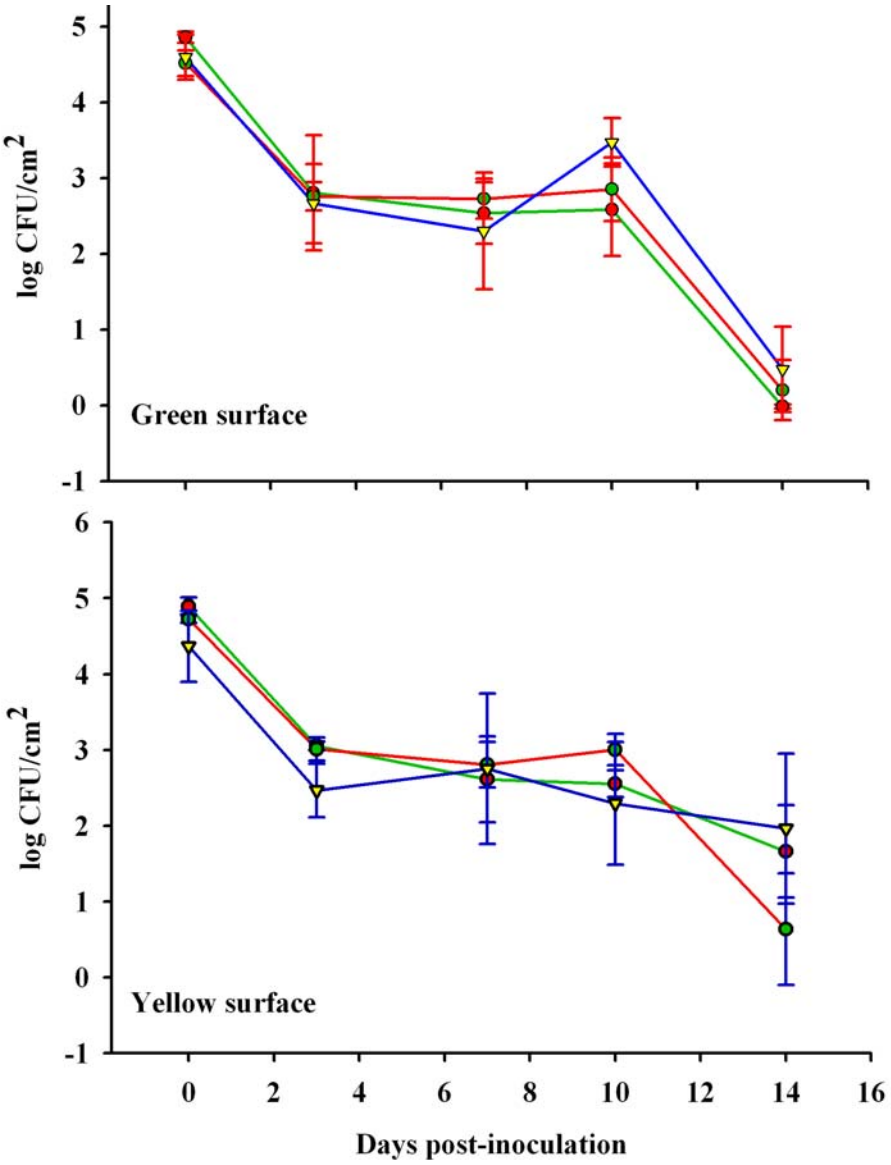


Figure 2 A-B. Survival of *Listeria* and *Salmonella* on watermelon surfaces. Each point represent the mean and standard deviation (bars) of n=4 samples of 15 cm^2 of inoculated surface.

Effect of temperature of storage on the survival of Listeria on watermelon surfaces

Watermelons were stored at 15, 25 and 37 ^oC, results showed that survival of *Listeria spp.* was temperature dependent (Fig. 3); greater survival was inversely related with temperature. As occurred before, rapid die- off is observed within the first 24 hours and then the surviving population maintains stable persistence, however for those watermelons stored at 15 ^oC, the population drop was about 2.5-log after 14 days of storage while populations where below the limit of detection (≥ 4 log) after 3 and 7 days for those stored at 25 and 37 ^oC respectively (Fig. 3), thus only qualitative results where obtained after sample

enrichment (Table 2). Results indicate that if contamination occurs, it is likely that Listeria cells have better survival rate when stored at lower temperatures than at temperatures above 20 ^{o}C, similar results have been also observed with other enteric pathogens in leafy greens, stone fruit, and tomatoes stored at lower temperatures.

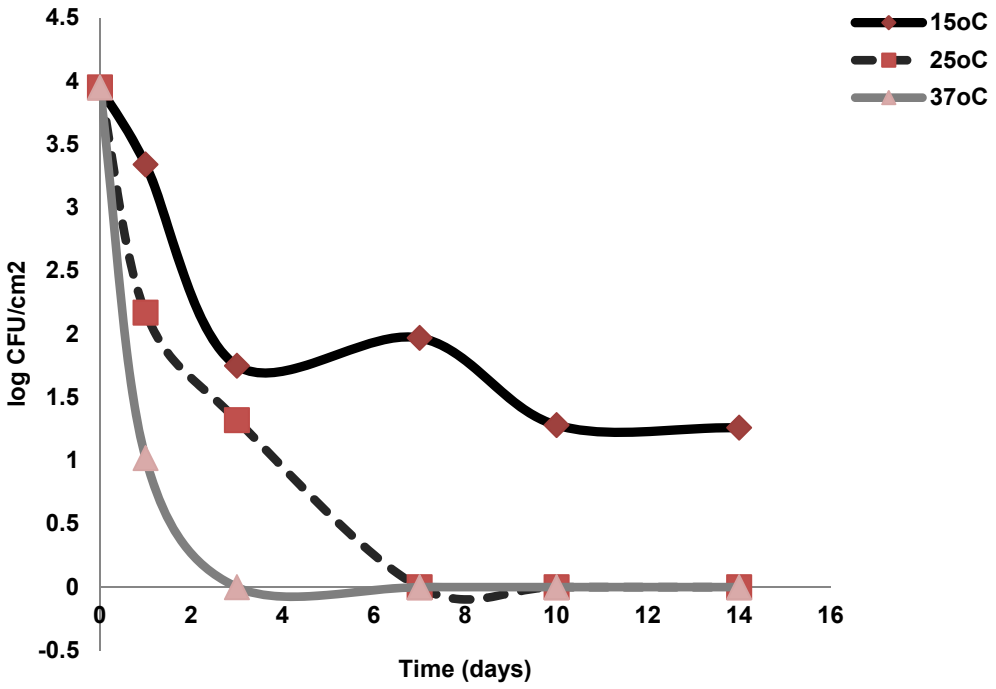


Figure 3. Effect of storage temperature on the survival of *Listeria spp*. (No significant difference was found between *L.monocytogenes* and *L. innocua* as well as survival on yellow ground spot or green surfaces, thus results were averaged. Each bacteria isolate and surface-type was done with a replication of n=6. Recovery of bacteria below log 0.5 CFU/cm^2 was only possible after enrichment and therefore plotted data is just a representation of those samples (See Table 2)

Additionally, results obtained after sample enrichment, in which the population of bacteria is below the limit of detection, showed that after 10 days of storage the majority of the samples held at 25 or 37 ^{o}C are no longer viable and thus recovery or detection after enrichment is not feasible, which also indicate that survival of *Listeria spp.* on watermelon under these conditions is likely limited (Table 2). In this study we did not address whether cells were in a viable but non culturable state (VBNC) but it is an assessment that would be of interest as several bacterial species tend to enter in this state under stressful conditions.

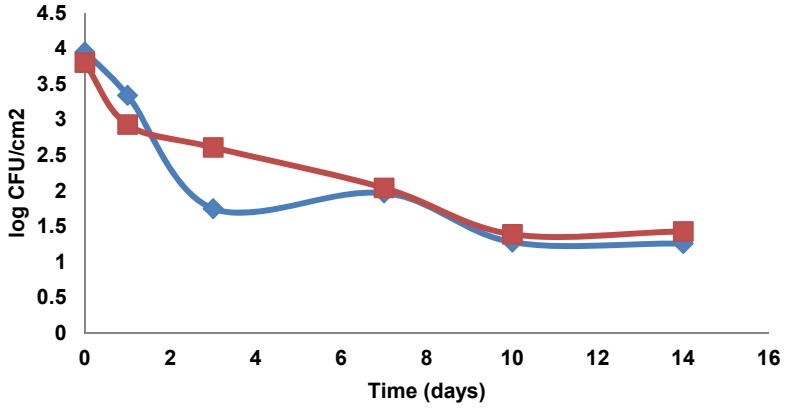
Table 2. Comparison of *Listeria* survival on different watermelon surfaces at various temperatures of storage.

	Listeria innocua		**Listeria monocytogenes**	
	(Number of positive samples after enrichment/total samples analyzed)			
Time of storage at 15°C (days)[a]	**Green**	**Yellow spot**	**Green**	**Yellow spot**
0	6/6	6/6	6/6	6/6
1	6/6	6/6	6/6	6/6
3	5/6	6/6	6/6	6/6
7	6/6	6/6	6/6	6/6
10	6/6	5/6	6/6	6/6
14	4/6	6/6	4/6	6/6
Time of storage at 25°C (days)[b]				
0	6/6	6/6	6/6	6/6
1	6/6	6/6	6/6	6/6
3	2/6	6/6	6/6	6/6
7	0/6	6/6	1/6	4/6
10	0/6	3/6	0/6	3/6
14	1/6	0/6	0/6	0/6
Time of storage at 37°C (days)[b]				
0	6/6	6/6	6/6	6/6
1	6/6	6/6	6/6	6/6
3	0/6	2/6	6/6	6/6
7	0/6	2/6	0/6	2/6
10	1/6	2/6	1/6	0/6
14	0/6	0/6	0/6	1/6

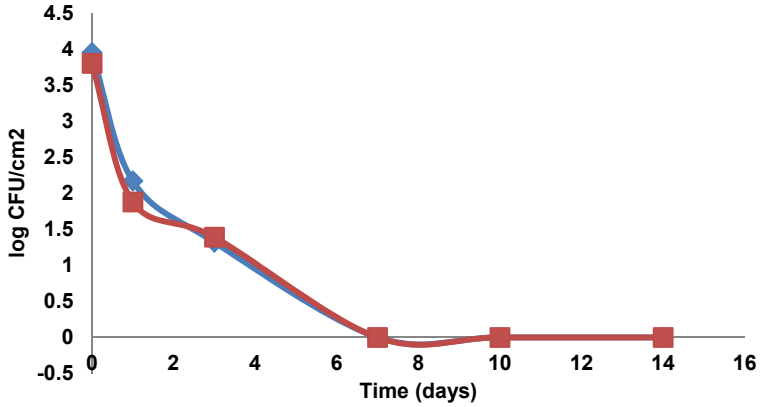
(a) Quantification of the population was obtained by direct plating and it corresponds to the data presented in figure 3.

(b) Quantification of the population was only feasible after 3 and 1 day of storage for watermelons stored at 25 and 37°C respectively, after these times, survival was only assessed by sample enrichment

Comparison of survival of Listeria on yellow ground spot and green surfaces showed that there was no significant difference ($p>0.05$) in the survival among *L. monocytogenes* cocktail and *L. innocua*. Additionally, the behavior of both species showed no significant difference in their survival when inoculated on watermelon surfaces, which indicates that *L. innocua* could be a defensible surrogate for further studies (Fig. 4 and Table 2).



(A)



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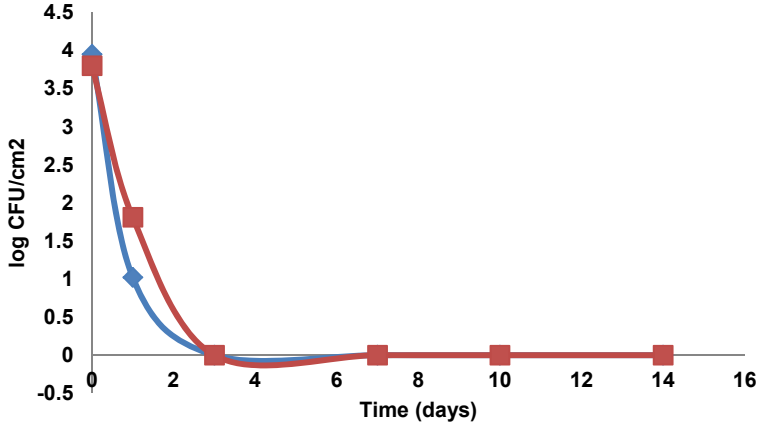

(C)

Figure 4. Comparison of survival of *Listeria monocytogenes* (pink line) and *Listeria innocua* (blue line) on watermelon surfaces at (A) 15 ^{o}C, (B) 25 ^{o}C and (C) 37 ^{o}C. Results between yellow and green surfaces showed no significant difference ($p>0.05$) thus results were averaged. Each point represents a replication of n=12 inoculated surfaces. Recovery of bacteria below log 0.5 CFU/cm^2

was only possible after enrichment therefore plotted data is just a representation of those samples (See Table 2)

Effect of inoculation dose and Listeria strain on survival during storage on watermelon surfaces.

Watermelons were inoculated to target a population of log 6, 4 and 2 CFU/cm^2 within the inoculated area, in addition to inoculation as a cocktail individual strains were evaluated for their ability to survive on watermelon surfaces at a temperature of storage of 25 ^{o}C for up to 10 days.

Recovery and quantification of the individual strains, the mixed-strain cocktail, as well as *L. innocua* was only possible on those watermelons inoculated with the highest inoculum dose (log 6 CFU/cm^2). In contrast, evaluation of their survival with lower doses was only possible after enrichment (Table 4).

Results obtained after recovery from watermelons inoculated with the highest dose showed a similar trend to that observed in experiments described above; a rapid drop in the population within 24 h followed by a stable period in which the population is maintained and a further die off after 5 days (Table 3). Upon analyzing strains individually, it was determined that no significant difference was observed between the cocktail and PTVS333 ($p>0.05$), however strains PTVS 334 and 335, originally isolated from a irrigation source and cantaloupe, respectively, actually maintained their population during the length of the experiment and where comparable to the population of *L. innocua* (TVS451). Although sample variation could be a major factor, variations in survival depending of strain are not uncommon as minimal phenotypic and genotypic variations can influence the survival and fitness in various environments, including interactions with produce surfaces.

Table 3. Survival of *Listeria spp*. on watermelon surfaces stored at 25 ^{o}C

Strain	**PTVS333**	**PTVS334**	**PTVS335**	**Cocktail**	**TVS451**
Time of storage (days)	(log CFU/cm^2) *Initial inoculation dose log 5.77 CFU*				
1	1.53 ± 0.70	1.60 ± 0.24	1.24 ± 0.53	1.48 ± 0.56	1.11 ± 0.33
5	1.52 ± 0.49	1.56 ± 0.36	1.79 ± 0.48	1.01 ± 0.92	0.24 ± 0.78
10	0.06 ± 0.23	1.49 ± 0.45	0.74 ± 1.01	0.20 ± 0.00	1.43 ± 0.48

Detection of Listeria spp. after sample enrichment from watermelons inoculated with lower doses provide evidence that a dose lower than log 2 CFU/cm^2 (100 cells) resulted in an absence of detection after just 24 hours, even with enrichment, which suggest that at this inoculum level the desiccation

process is sufficiently acute to produce cells that are not viable for cultivation or has resulted in cell death. Inoculation with doses lower than log 4 CUF/cm^2 showed a reduction in the number of positive samples after approximately 5 days of storage. In these cases, there is no evidence that there is a major difference among strains, however larger sample sizes and replication could allow for a statistical comparison utilizing qualitative data.

Overall, this experiment indicates that survival is also dependent on the initial inoculum dose and low doses of the bacterium (not associated with a protective organic matrix or carrier) result in a rapid cell death or loss of viability.

Table 4. Influence of inoculation dose and strain in the survival of *Listeria spp*. on watermelon surfaces (Detection after sample enrichment).

	Strain				
	PTVS333	**PTVS334**	**PTVS335**	**Cocktail**	**TVS451**
	Total number of positive detection after enrichment/Total samples analyzed				
Time of storage (days)	***Initial inoculation dose: log 5.77 CFU***				
0	5/5	5/5	5/5	5/5	5/5
1	5/5	5/5	5/5	5/5	5/5
5	5/5	5/5	5/5	5/5	5/5
10	5/5	5/5	5/5	5/5	**5/5**
	Initial inoculation dose: log 3.65 CFU				
0	5/5	5/5	5/5	5/5	5/5
1	4/5	3/5	4/5	5/5	3/5
5	2/5	1/5	0/5	2/5	0/5
10	0/5	0/5	2/5	1/5	0/5
	Initial inoculation dose: log 1.75 CFU				
0	5/5	5/5	5/5	5/5	5/5
1	0/5	0/5	0/5	0/5	0/5
5	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5

Effect of disinfection strategies to reduce the population of Listeria spp. on watermelon surfaces.

Inoculated areas from watermelons were removed and treated with various disinfection agents, including sodium hypochlorite, peroxyacetic acid (PAA) and another commercial product, Prosan ®. All disinfectant solutions were prepared a few minutes before application. Two types of application were performed; one in which the watermelon rind was completely submerged in the disinfectant solution for up to 2 minutes except for PAA in which 5 min were

utilized as contact time following label recommendations. In the second application strategy, antimicrobial saturated paper towels were first soaked in the disinfectant solution and then towels were utilized to cover the watermelon surface. The first method could be adopted for large-scale minimally-processed fruit, while the second strategy could be recommended for consumers.

For fully submerged watermelons, the greatest log reduction was achieved utilizing PAA for up to 5 min, which produced a 5-log reduction, followed by NaOCl at 100 ppm and Prosan ® (ACID) in which approximately 4-log were reduced after 2 min of contact time. The least efficient treatment was with 50 ppm of NaOCl for 2 min in which 2.5-log reduction was achieved. Watermelons rind that were only rinsed with water, without mechanical scrubbing, had a log reduction limited to 1-log. For all treatments a 2 min of water rinsing was done to remove disinfectant residuals before bacterial recovery. Although this study was not intended to provide a recommendation for Listeria mitigation, it is important to point out that these results provide a further basis for the practical knowledge that watermelon appears to be amenable to postharvest operations involving a disinfectant agent such as brush-bed sprays or drenching with extended contact. As described below, the experiment was evaluated in both yellow ground spot and green surface and no significant difference was determined ($p>0.05$)

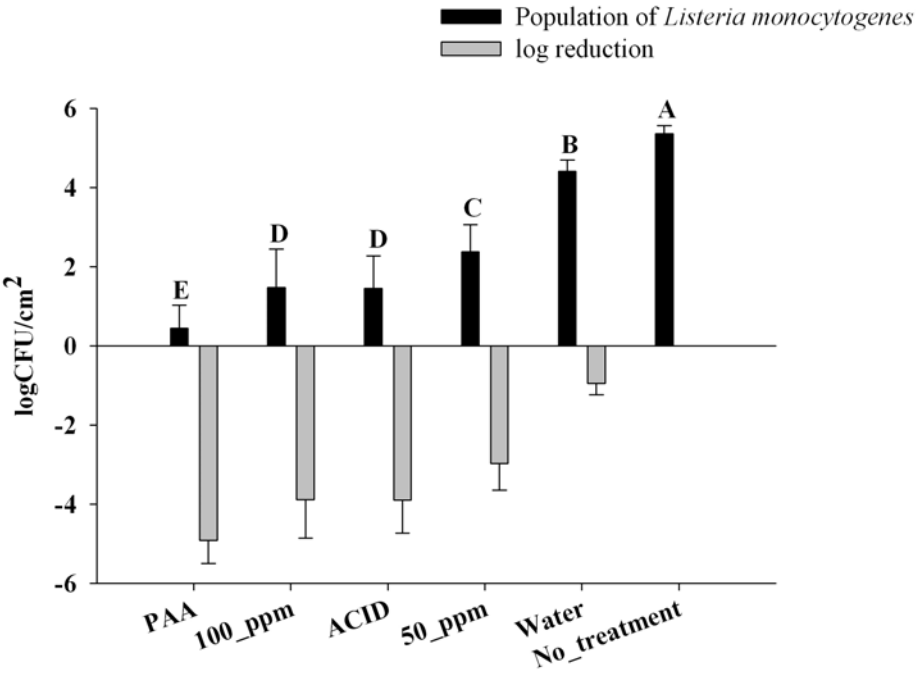

Figure 5. Effect of disinfectants in the log-reduction of *Listeria monocytogenes*. Different letters denote significant difference among treatments after analysis of variance. No difference by t-test was found between yellow and green surfaces therefore results were averaged (n=13).

Table 5. Effect of Peracetic Acid and Prosan ® applied to soaked paper towels in the mitigation of *Listeria spp.* inoculated on watermelon surfaces.

Strain	PTVS333	PTVS334	PTVS335	TVS451	N
	Population of Listeria in log CFU/cm^2 ∞				
Treatment					
Control[§]	5.19 ± 0.73	5.94 ± 0.66	5.94 ± 0.73	5.65 ± 0.13	4
PAA	3.04 ± 0.52^{A,*}	3.79 ± 0.86^{A,*}	3.74 ± 0.27^{A,*}	3.59 ± 0.55^{A,*}	10
Log-reduction	2.15	1.66	2.20	2.04	
Prosan	4.16 ± 0.53^{B}	4.78 ± 0.47^{A,*}	4.57 ± 0.18^{A,B*}	4.57 ± 0.35^{A,B*}	9
Log-reduction	1.03	0.66	1.37	1.08	

(∞) Results represent the mean and standard deviation with N replicates, same letter within the row indicates significant differences among *Listeria* strains $p<0.05$.
(§) Control indicates that no treatment was applied and it was utilized to calculate the log-reduction after application of each disinfectant treatment
(*) Denotes significant differences in the control after Dunnet's test comparison within each strain

The second application method, utilizing soaked paper towels, showed an overall log reduction of approximately 2-2.5 log when peracetic acid was utilized and about 1-log when Prosan (ACID) was applied with this method (Table 5).