

Application of Lactic Acid Bacteria for Pre- and Post-Harvest Treatment to Control *Listeria Monocytogenes* on the Surface of Produce

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Abstract

Two lactic acid bacterial isolates, C-1-92 (*Lactococcus lactis* subsp. *Lactis*) and #152 (*Enterococcus durans*) with excellent inhibitory activity against *L. monocytogenes* at 4-37°C in various conditions were evaluated. A cocktail of three *L. monocytogenes* strains were used to inoculate green peppers and apples at 21±1°C at a concentration of 3 log CFU/cm². After 48h post-inoculation, green peppers and apples were individually treated with 0.1% peptone, 5 log CFU/ml C-1-92 or #152. These two lactic acid bacteria were further evaluated in hot and green pepper plants by similar methods. After 14 days the population of *L. monocytogenes* on green pepper treated with control, #152, and C-1-92 studies was 3.4, 2.3, and 1.3 log CFU/cm², respectively. The application of C-1-92 treatment reduced the *L. monocytogenes* population by 2.1 log CFU/cm² (P<0.05) when compared to the control. For apple studies, *L. monocytogenes* population treated with C-1-92 or #152 were below detection level (0.69 CFU/cm², P<0.05) and 50% samples were detected by selective enrichment only at day 14; whereas, *L. monocytogenes* control group was 2.6 CFU/cm². Pepper plants studies indicated that the treatment significantly reduced *listeria* count 5 days following treatment. The average count of *listeria* in C-1-92 or #152 treated hot peppers was 0.7 and 0.96 log CFU/cm², respectively, whereas the average count in hot peppers of control group was 2.5 log CFU/cm². *Listeria* reduction from green pepper plants were similar with results obtained from hot pepper plants study. The intervention by *Lactococcus lactis* subsp. *Lactis* (C-1-92) can substantially reduce the existence of *L. monocytogenes* on the surface of fresh produce.

Keywords: Lactic acid bacteria; *Listeria monocytogenes*; Produce.

Introduction

Outbreaks of illness due to foodborne pathogens associated with fresh produce have increased in the past decade [1-6]. Fruits and vegetables contain rich carbohydrate, fibre, and various vitamins. Consumers prefer ready to eat (RTE) food, which fruit and vegetable can be consumed right out of the package without washing, to offer consumers high nutrition, convenience, and value. The contamination of *L. monocytogenes* is common in RTE food, especially fruits and vegetables. Recent recall issued by Food and Drug Administration involved nectarines, peaches, and plums involved a dozen states and many stores for potential *L. monocytogenes* contamination. These recalls gave farmers, processing companies, and retailers tremendous loss and bad reputation, and hurt consumer's confidence for RTE food and bad influence for food industry. A recent outbreak of *Listeria monocytogenes* infections was identified to link to whole cantaloupe contamination, involved 28 States, illnesses to 147 persons with 143 hospitalization and 33 deaths. Its survival mechanisms were not characterized, an effective, practical, cost-effective, and environmental-friendly method for control of *L. monocytogenes* in fruits and vegetables is urgently needed [6-12].

L. monocytogenes is hard to eliminate in the produce processing environment. It can survive many years in the fresh produce processing environment, especially floor drains as a long-term resident. Some specific strains can keep in a dormant status even under strict sanitation condition. These dormant cells can recover and start to grow, even can form the biofilm at space in the processing facilities and their locations may be very hard to reach for regular sanitization processing. These tiny biofilms can accumulate millions of *L. monocytogenes* cells and can become the source of contamination, cross-contamination, and re-contamination in various produce products as long as they get the chance to detach and

initiate attachment to food processing surface. The presence of *L. monocytogenes* in mixed biofilms in produce processing chains has great concern because of their risk for cross-contamination of other surfaces [1,3,11].

The survival ability of *L. monocytogenes* is related with strain specific, contamination level, moisture, temperature, and environmental condition. Accumulation of a large amount of *L. monocytogenes* in a particular contact surface is needed for food contamination in a modern food facility because food containing various gradients, lower water activity, and pH will inhibit the growth of *L. monocytogenes* even contaminated at lower level. To accumulate large amount of *L. monocytogenes* will require biofilm formation. Only in this condition, large amount of *L. monocytogenes* embedded in the form of mixed biofilm can be accumulated. Many of the microbial intervention strategies for produce involve the use of antimicrobial chemicals in rinses or washes; however, the efficacy of most chemical intervention treatments is reduced by the presence of organic matter. More effective antimicrobial treatments are desired that are practical, cost-effective and safe to use [4]. Currently, there is no available intervention procedure for reduction of foodborne pathogens in pre-harvest produce.

Our previous studies have identified a group of bacteria with strong inhibitory zone against the growth of *L. monocytogenes* [13-19]. These isolates were individually evaluated at 4, 8, 15, and 37 °C for their log reduction against the growth of *L. monocytogenes* by tube and biofilm assays and nine isolates were identified. Biochemical and genetic studies reveal that they belong to three bacterial species, including *Enterococcus durans*, *Lactococcus lactis*, subsp. *lactis* and *Lactobacillus plantarum* [16]. Two isolates, including one isolate of *E. durans* and one isolate of *L. lactis*, were validated by applying them in floor drains in a poultry processing plant and a Ready-To-Eat poultry processing plant. Results revealed that biological treatment can greatly ($>4 \log \text{CFU}/100\text{cm}^2$) reduce the number of listeria sp. cells in floor drains at 3 to 26 °C in which fresh poultry is processed [17]. This purpose of this study is to validate this bio-control method for substantial reduction of *L. monocytogenes* contamination on fresh produce at pre-and post-harvest stages and to observe various factors to interfere its efficiency.

Materials and Methods

Green Peppers and apples

Fresh green peppers (150±25g) and apples (unwaxed Gala, 260±20g) purchased from a local retailer. Peppers and apples selected randomly used for tests of food-borne pathogens (*Listeria*, *E. coli*, and *Salmonella*) and aerobic bacteria counts by the methods we reported previously [15].

Bacteria strains

A mixture of three *Listeria monocytogenes* strains (LM 101, serotype 4, salami isolate; H9666, serotype ½C, human isolate; and ATCC 5779 ½C, cheese isolate) were selected for inoculation study. Two isolates of lactic acid bacteria, including C-1-92

(*Lactococcus lactis* subsp. *Lactis*) and #152 (*Enterococcus durans*); one strain of *L. innocua* (produce isolate, lab collection) were used for the study. Each isolate was individually grown in 10ml Brain Heart Infusion medium (BHI, Becton Dickinson Microbiology Systems, Sparks, MD) for 16-18h at 37 °C. Cultures of the three well-grown *L. monocytogenes* strains containing approximately the same population were combined or individually Lactic acid bacteria, washed three times in a 50ml sterilized centrifuge tube in a centrifuge (Thermo, Milford, MA) at 4,000g for 20min, and suspended in 0.1M phosphate buffer, pH 7.2. The final suspension was adjusted to an optical density of 0.5 at 630nm (approximately $10^8 \text{CFU}/\text{ml}$) as determined with a spectrophotometer (Spectronic instruments, Rochester, NY). Cell numbers were confirmed by plating 0.1ml of the suspension serially diluted (1:10) in 0.1% peptone water on tryptic soy agar (TSA, BD) and MOX (BD for listeria) MRS (BD for lactic acid bacteria). All plates were incubated at 37 °C for 24h.

Contamination of peppers and apples

One ml of 3-strain *L. monocytogenes* mixture was washed for 3 times by 0.1% peptone by centrifugation at 4000g and pellet was suspended in 0.1% peptone water solution. The bacterial solution added to 1-L 0.1% peptone water to final concentration of $10^3 \text{CFU}/\text{ml}$ in a 2-L glass beak with a magnetic bar at 150rpm. After mixed for 2minutes a volume of 300ml was transferred to a 500ml hand spray bottle. About $1.2 \pm 0.2 \text{ml}$ was sprayed onto each green pepper or apple in a laminar hood and dried for 40min.

Preparation of treatment bacteria

Each treatment strain, *Lactococcus lactis* subsp. *lactis* (#C-1-92) and *Enterococcus durans* (#152), was grown individually in a 500ml Erlenmeyer flask containing 250ml Lactobacillus MRS broth (MRS, Becton Dickinson) at 32 °C for 24h. Cells were sedimented by centrifugation at 10,000xg for 20min at 4 °C. The bacteria were resuspended in 25ml of MRS broth at ca. $10^9 \text{CFU}/\text{ml}$, serially (1:10) diluted in 0.1% peptone, and plated on MRS agar and tryptic soy agar in duplicate for bacterial counts.

Treatment of peppers by lactic acid bacteria

The solution of C-1-92 (*Lactococcus lactis* subsp. *Lactis*) and #152 (*Enterococcus durans*) was adjusted to $10^5 \text{CFU}/\text{ml}$ and sprayed on the surface of green pepper or apple ($1.2 \pm 0.2 \text{ml}$) and dried for 40min at a laminar hood. The peppers and apples were incubated at 21 °C and samples taken out for bacterial enumeration daily.

Enumeration of *L. monocytogenes*

Each bag with green pepper or apple with same amount of 0.1% peptone water (250ml) was vigorously rubbed by hands for 1min and shake in a horizontal shaker (Thermo Scientific) at 200 rpm for 2min. A volume of 1ml suspension from each bag was serially (1:10) diluted in 9ml 0.1% peptone up to $10^{-6} \text{CFU}/\text{ml}$. A volume of 0.1ml from each diluted tube was surface plated on modified Oxoid agar (MOX, Oxoid) in duplicate for isolation of *Listeria* bacteria according to the methods described above and previously [18, 19].

Selective enrichment

When *L. monocytogenes* was not detected by direct plating, a *Listeria* selective broth was used, and a volume of 1ml was added to 9ml broth. The broth tubes were incubated for 24h at 37 °C. Following incubation, a 10- μ l loopful from the broth tube was plated in duplicate onto MOX plates for *Listeria*, and plates were incubated for 24h at 37 °C. Colonies with typical *Listeria* morphology (black) were selected and transferred once more on MOX plates and incubated for 24h at 37 °C for further confirmation by genetic and immunological methods by the procedures described previously [16].

Determination of aerobic plate counts (APC), fecal coliform, *E. coli*, and *Salmonella*

Serial dilutions of samples described above were surface plated on plate count agar plates (PCA, Becton Dickinson Microbiology Systems) and incubated at 30 °C for 72h for enumeration. For fecal coliform, *E. coli*, and *salmonella* determination similar methods were selected according to the report we described previously [15].

Confirmation of *L. monocytogenes*

Colonies counted as *Listeria* sp. were randomly selected and transferred to MOX plates and confirmed as *Listeria* sp. by biochemical tests (API 20I miniaturized diagnostic test) and lateral flow latex agglutination assay (Oxoid, *Listeria* Rapid Test), and as *L. monocytogenes* by an enzyme-linked fluorescent immunoassay and PCR assay [18].

Selection of pepper plants

Two kinds of pepper plants (hot pepper and green pepper) were selected for pre-harvest treatment studies. Pepper plants (4-6 weeks old) were purchased from a local retailer. These plants were grown for another 3 to 4 weeks at temperature ranged from 62 to 96 ° F under direct sun. Then, they were individually transferred into a 3-gallon pot with all-purpose fertilizer to grow for another 3-5 weeks (height at 4 to 4.7 feet for hot pepper plants and 3.5 to 4

feet for green pepper plants) before experiment.

Contamination of *Listeria*

L. innocua (non-virulent *Listeria* isolate) was used for the contamination of pepper plants. The bacteria was grown in individual test tube containing 10ml of BHI media at 37 °C for 18h. Bacteria from 5 individual tube were combined in a 50ml centrifuge tube and washed by centrifuge at 4,000rpm for three times. The pellet was suspended in 50ml 0.1% peptone water. Then, all of them were poured into a tank containing 600ml 0.1% peptone water. Under pressure the bacterial were surface-contaminated the pepper plants.

Treatment of pepper plants

Twenty-four hours after contamination of *listeria*, two lactic acid bacteria, including C-1-92 and #152 at similar concentration with the similar growth condition was individually sprayed on all pepper plants.

Collection of pepper

Peppers were individual collected by hand with sterile glove and placed in individual whirl-Pak bag (55oz, 1,627ml, 19 x 30cm, Nasco). A solution of 25ml of 0.1% peptone water was added into each bag and mixed with a shaker at 150rpm for 2min. The solution in each bag was serially (1:10) diluted to 10⁻⁶CFU/ml. The solution from each dilution tube was plated on the surface of MOX and PCA plates in duplicate. The plates were incubated at 37 °C for MOX or 30 °C for PCA at 48 to enumerate the *listeria* and aerobic bacteria according to the method described previously [15].

Statistical analysis

Statistical analysis was performed with SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). The data were presented as means \pm standard error. Data were analyzed for analysis of variance (ANOVA) to determine significant difference ($P < 0.05$) and for correlation coefficients (CORRELL) to determine the correlation between two group data.

Results

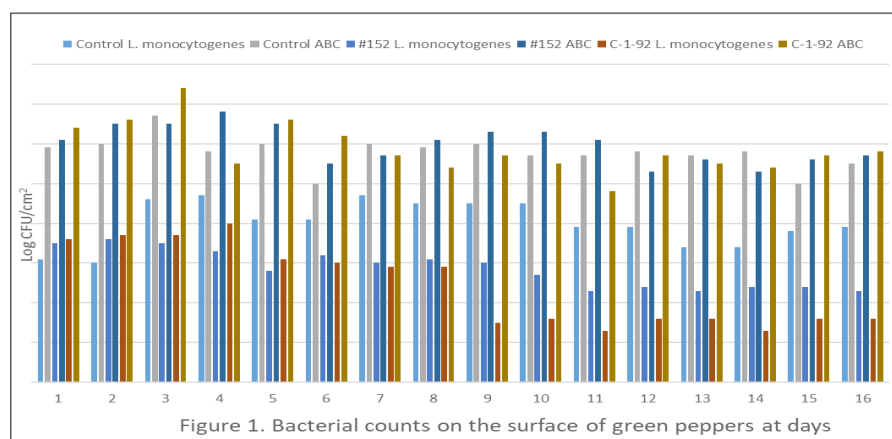


Figure 1: ABC, aerobic bacteria count.

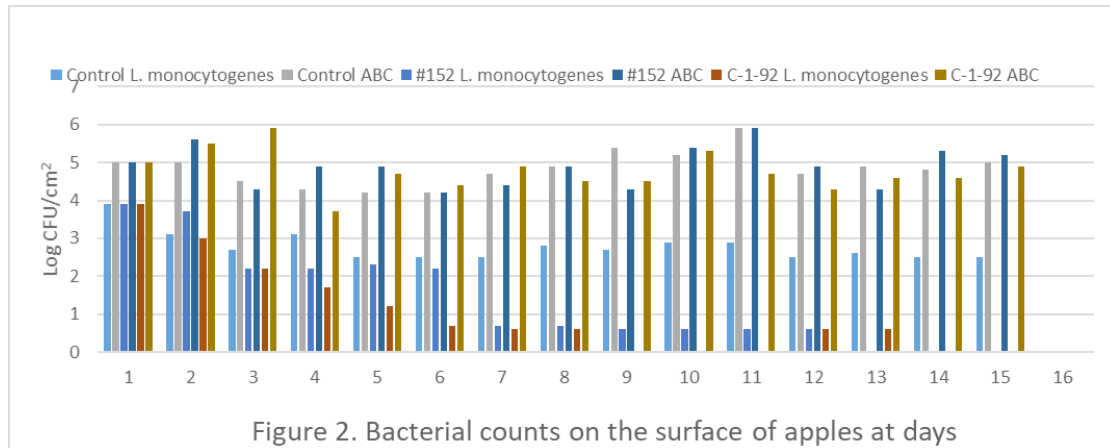


Figure 2. Bacterial counts on the surface of apples at days

Figure 2: ABC, aerobic bacteria count.

All peppers and apples were negative for isolation of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* before experiment. The total aerobic bacteria counts were 4.7 ± 0.4 for green peppers and 4.2 ± 0.2 for apples. After 14 days, the population of *L. monocytogenes* on green pepper treated with 0.1% peptone water, *Enterococcus durans*, and *Lactococcus lactis subsp. Lactis* studies was 3.4, 2.3, and 1.3log CFU/cm², respectively. The application of *Lactococcus lactis subsp. Lactis* treatment reduced the *L. monocytogenes* population by 2.1log CFU/cm² ($P < 0.05$) when compared to the peptone water only (Figure 1). For apple studies, *L. monocytogenes* population treated with *Lactococcus lactis subsp. Lactis* C-1-92 or *Enterococcus durans* were below detection level (0.69CFU/cm², $P < 0.05$) and 50% samples were detected by selective enrichment only at day 14; whereas, *L. monocytogenes* in control group was 2.6CFU/cm² (Figure 2). The aerobic bacterial counts on peppers and apples revealed there were no difference among control and lactic acid-treated groups. The results demonstrated that the treatment by *Lactococcus lactis subsp. Lactis* (C-1-92) can substantially reduce the existence of *L. monocytogenes* on the surface of fresh produce.

The baseline determination of aerobic bacteria counts for hotter and green pepper before studies were 4.8 ± 0.7 and 4.9 ± 0.5 , respectively. *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 were not isolated from these pepper plants. Results obtained from pre-harvest treatment by *Enterococcus durans* or *Lactococcus lactis*

subsp. Lactis on hot pepper plants demonstrated that application of both lactic acid significantly reduced the population of *Listeria* on the surface of hot pepper plants. Starting from day 5, significant difference ($P < 0.05$) of *listeria* count between control and treated hot pepper groups were demonstrated. The effect of treatment in either *Lactococcus lactis subsp. Lactis* or *Enterococcus durans* became more significant after 12 days. All samples collected from either treatment group turned to be negative for isolation of *listeria*. However, the positive isolation of *listeria* lasted to the end of the study (Table 1).

The aerobic bacteria count indicated that their population was increased at the first 5 days after the treatment was completed. However, this increase did not continuously increase, their number returned to their pre-treatment condition (10^4 - 10^5 CFU/cm²) and kept this trend to the end of the study. Similar results were obtained with the green pepper plant study. Starting from day 6, the *listeria* counts from samples collected from either treated groups were reduced significantly ($P < 0.05$, Table 2) when compared with samples collected from control group. This tendency was continuously lasted to the end of the study. As same as hot pepper results demonstrated. The counts of total aerobic bacteria were backed from the pre-treated condition (10^5 - 10^6 CFU/cm²) after initial increase (Table 1).

Table 1: Bacterial counts on the surface of hot pepper plants.

^a (+) positive selective enrichment.

^b (-) negative selective enrichment.

Days Following Treatment	Bacterial Counts (Log10 CFU/Cm ²) in Different Treatment Groups					
	Control (Treated By 0.1% Peptone Water)		Treated With <i>Lactococcus Lactis Subsp. Lactis</i>		Treated With <i>Enterococcus Durans</i>	
	Listeria	ABC	Listeria	ABC	Listeria	ABC
2	2.2, 2.2, 2.8, 2.3, 2.4, 2.7	7.0, 6.7, 6.9, 7.1, 5.9, 7.3	1.4, 1.9, 1.3, 1.2, 1.7, 1.8	6.7, 6.3, 6.7, 6.5, 6.7, 6.0	1.7, 2.1, 2.2, 0.7, 0.7, 1.2	5.8, 6.7, 6.8, 7.4, 6.5, 5.9
5	3.8, 1.6, 4.0, 2.1, 1.8, 1.7	5.5, 5.3, 6.2, 5.9, 4.7, 5.9	+a, +, -b, 0.7, 0.7, 1.5	4.5, 5.3, 5.1, 4.4, 4.4, 4.8	+, +, -, 2.0, 1.7, 0.7	5.6, 5.6, 5.8, 5.7, 5.8, 5.9
8	0.7, 1.0, 1.0, 3.5, 2.5, 0.7	4.5, 6.1, 4.5, 6.0, 5.1, 4.7	-, -, 0.7, 1.0, +, 1.2	3.9, 5.3, 4.9, 5.1, 5.1, 4.7	-, -, -, 0.7, +, -	4.5, 6.8, 4.8, 5.2, 5.7, 4.7

12	2.1, +, +, 2.9, +, 0.7	6.0, 5.5, 5.3, 4.3, 4.0, 4.9	-, -, -, +, -, -	4.0, 4.7, 4.8, 4.0, 4.8, 1.2	-, -, -, -, -, -	4.5, 4.4, 5.3, 4.4, 5.1, 5.0
15	+, 0.7, +, +, 1.0, +	4.7, 4.1, 4.4, 3.7, 4.3, 3.7	-, -, -, -, -, -	4.8, 3.3, 4.7, 4.5, 5.1, 5.0	-, -, -, -, -, -	4.8, 3.4, 4.5, 4.8, 5.0, 4.8
19	+, -, 1.5, 0.7, 0.7, +	5.0, 3.7, 3.0, 4.1, 4.1, 3.9	-, -, -, -, -, -	3.0, 4.3, 3.2, 4.7, 4.2, 4.4	-, -, -, -, -, -	5.0, 4.2, 5.1, 4.9, 4.3, 5.0
26	-, 1.0, 0.7, +, 0.7, -	4.7, 4.3, 4.0, 4.6, 4.0, 4.8	-, -, -, -, -, -	3.7, 4.8, 3.7, 4.6, 3.7, 4.0	-, -, -, -, -, -	3.7, 3.7, 4.7, 4.7, 4.9, 4.3
40	0.7, +, -, -, 0.7, -	5.0, 4.6, 5.0, 5.4, 4.4, 4.8	-, -, -, -, -, -	5.2, 4.7, 5.7, 4.9, 5.3, 4.7	-, -, -, -, -, -	5.3, 4.5, 4.7, 5.0, 5.8, 5.3

Table 2: Bacterial counts on the surface of green pepper plants.

a (+) positive selective enrichment.

b (-) negative selective enrichment.

Days Following Treatment	Bacterial Counts (Log10 CFU/Cm ²) in Different Treatment Groups					
	Control (Treated By 0.1% Peptone Water)		Treated With <i>Lactococcus Lactis</i> Subsp. <i>Lactis</i>		Treated With <i>Enterococcus Durans</i>	
	Listeria	ABC	Listeria	ABC	Listeria	ABC
2	1.7, 1.5, 0.7, 4.4, 2.7, 4.6	5.4, 5.4, 5.3, 6.3, 6.0, 5.7	0.7, 2.2, 0.7, 1.2, 2.3, 2.5	6.2, 6.1, 6.1, 5.8, 6.3, 6.1	0.7, 0.7, +a, 1.7, 1.7, 0.7	3.5, 5.0, 5.7, 5.9, 6.2, 5.9
6	2.9, 0.7, 2.0, +, 0.7, 1.7	6.2, 4.0, 6.1, 4.8, 5.4, 5.3	1.7, -b, 1.2, 1.0, 0.7, +	5.5, 4.9, 5.7, 5.4, 5.7, 5.7	1.9, 1.0, -, -, 0.7, +	5.5, 5.7, 5.5, 4.2, 4.7, 4.7
9	1.8, 3.2, +, 0.7, 1.8, 1.0	5.4, 6.4, 5.9, 5.2, 5.6, 5.8	+, 0.7, -, 1.5, +, -	5.2, 4.9, 5.0, 5.9, 5.5, 5.7	-, -, 0.7, -, 1.3, +	6.2, 5.4, 5.5, 5.6, 4.8, 5.6
16	2.0, -, 1.2, 0.7, +, -	5.8, 5.6, 5.7, 5.8, 4.7, 5.2	-, -, -, +, -, -	6.8, 6.0, 5.2, 5.0, 5.8, 5.1	-, -, -, -, -, -	6.0, 4.2, 4.3, 5.3, 5.6, 5.0
23	-, 1.4, +, 2.1, -, 0.7	5.3, 5.2, 5.5, 5.4, 5.9, 4.0	-, -, -, -, -, -	5.0, 4.5, 5.1, 4.3, 3.7, 5.6	-, -, -, -, -, +	5.9, 5.3, 5.6, 5.5, 5.6, 4.8

Discussion

Our previous studies identified two lactic acid bacteria; they demonstrated strong ability to inhibit the growth of *L. monocytogenes* at various conditions with different temperatures. In this study, we individually evaluated its ability as a treatment for pre- and post-harvest fresh produce to reduce the *listeria* contamination. The comparison of *listeria* number and growth characteristics between pre-harvest and post-harvest stages indicated that the *listeria* was easier to survive at the surface of peppers and apples at post-harvest stage. Its survival capability might be related with surface temperature, humidity, and nutrition [5]. At pre-harvest condition, surface temperature was high, especially under direct sunshine in the hot summer (>90 ° F). The population of *listeria* reduced quickly, but lower number still could survive for a long time on the surface of hot or green pepper plants as shown in control groups (Table 1 & 2). This survived *listeria* may become the original source for cross-contamination during harvest management [1].

The advantages of traditional chemical method treatment include rapid reduction, cost-effective, and the best of all is chemical concentration can be continuously increased until effective elimination [13,14]. The disadvantage is contaminated *listeria* may form the biofilm at some parts of the produce and a quick wash may be hard to remove all of them. The *L. monocytogenes* can

survive, reappear, and the biofilm becomes more solid than formed previously. This situation has been revealed that the same strain of *L. monocytogenes* is responsible for current outbreak and outbreak happened many years ago. *L. monocytogenes* can survive at the existing biofilms in produce processing environment, especially floor drains, and will be responsible for *L. monocytogenes* outbreaks. The application of biocontrol will ensure fresh produce from the *listeria* contamination before harvest and ensure processing facilities from the contamination of *L. monocytogenes*.

The purpose that we individually evaluate these two candidates is that produce include various products. Their chance to get the contamination is significantly various. Some of them grow on the surface of soil, such as, watermelon. Their chance to get the contamination of *L. monocytogenes* is much high. Thus, *Enterococcus durans* may be a better choice because their survivability is much strong in the bad environment [18]. However, some produce, such as, peppers, they grow on the plants and their chance to get the contamination of *L. monocytogenes* is low. Thus, the application of *Lactococcus lactis* subsp. *Lactis* should be a good selection because of its strong bacteriocin production [7].

The results of pre-harvest treatment indicated that its bactericidal effect could last more than four weeks from contamination by *listeria* suggested that time for application should

start early, 4 to 6 weeks before harvest may be a good choice. From this study, bio-control application for once should be effective. All inoculated *Listeria* was removed starting from week 2 and lasted for at least more than four weeks.

Conclusion

The application of this bio-control procedure demonstrates that they can substantially reduce the existence of *L. monocytogenes* on the surface of fresh produce at pre- and post-harvest stages.

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