Evaluation of the Effectiveness of Aeration to Reduce Microbial Risk During Washing and Sanitizing of Fresh Produce

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November, 2020

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Special graduation project presented as partial requirement to obtain a Bachelor of Science degree in Food Science and Technology.

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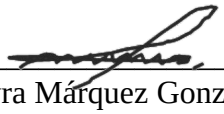

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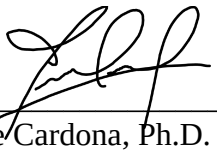

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Evaluation of the effectiveness of aeration to reduce microbial risk during washing and sanitizing produce

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Abstract. The current washing and disinfection methods of the fresh produce industry are guided to use water and chemical agents, the most used is chlorine. Novel techniques are being sought to ensure clean and safe produce to the consumer. A good option is the use of aeration as a source of washing and decontamination. The purpose of this study was to evaluate the effect of the application of sanitizer (chlorine) and an aeration system in *E. coli* O157:H7 to fresh bell peppers. Bell peppers, inoculated with a cocktail of five strains of nalidixic acid resistant mutant of *E. coli* O157:H7 with an initial concentration of 5.41 ± 0.15 Log CFU/g, was immersed during three minutes in a solution with chorine (CB) and combination with bubbles (CBB), water (WB) and water with bubbles (WBB). The antimicrobial effect on the bacterial count in bell peppers and the solution were determined in all treatments. Results indicated that the use of water with bubbles (WBB) in final count after treatment were statistically similar to bell peppers treated with chlorine (CB) and chlorine with bubble (CBB), reporting final counts of (Log CFU/g) 3.35 ± 0.02, 2.62 ± 0.63 and 3.18 ± 0.42, respectively . The aeration helps to reduce the bacterial load being an alternative to use with other chemical substances or to reduce the concentration applied for the washing and sanitization of bell peppers.

Key words: Bubbles, chlorine, *Escherichia coli* O157:H7, food safety.

Resumen. Los métodos actuales de lavado y desinfección en la industria de los productos frescos están orientados al uso del agua y agentes químicos, entre los cuales el más usado es el cloro. Se están buscando nuevas técnicas para garantizar productos limpios e inocuos para el consumidor. Una buena opción es el uso de aeración para el lavado y desinfección. El propósito de este estudio fue evaluar el efecto de la aplicación de desinfectante (cloro) y un sistema de aeración en la remoción de *E. coli* O157:H7 en pimientos frescos. Pimientos, inoculados con un cocktail de cinco cepas resistentes al ácido nalidíxico de *E. coli* O157:H7 con una concentración inicial de 5.41 ± 0.15 Log CFU/g, fueron sumergidos por tres minutos en una solución con cloro (CB) y combinación con burbujas (CBB), agua (WB) y combinación con burbujas (WBB). El efecto antimicrobiano sobre el conteo bacteriano en los pimientos y la solución fue determinado en todos los tratamientos. Los resultados obtenidos indican que el uso de agua con burbujas (WBB) en el conteo final fue estadísticamente igual al conteo obtenido en los pimientos tratados con cloro (CB) y cloro con burbujas (CBB), se reportaron conteos finales de 3.35 ± 0.02, 2.62 ± 0.63 y 3. 18 ± 0.42 (Log CFU/g) respectivamente. La aeración ayuda a reducir la carga bacteriana, siendo una alternativa para el uso junto a otras sustancias químicas o para reducir su concentración aplicada para el lavado y desinfección de pimientos frescos.

Palabras clave: Burbujas, cloro, *Escherichia coli* O157:H7, inocuidad de alimentos.

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1. INTRODUCTION

Fruits and vegetables are part of an essential food group for humans. Their role is essential in the diet by serving as a source of nutrients vital for proper nutrition and health; these, are commonly consumed fresh and raw. Recently, there has been an increasing consumption of fresh produce worldwide due to the consumers' concerns on their health benefits (Cook 2003). However, increasing consumption of fresh produce coincided with the increasing cases of foodborne illnesses and outbreaks (Afari *et al*. 2015). Outbreaks are not only a burden on public health but also cause heavy economic loss to the food industry (Hussain and Dawson 2013). According to the CDC in 2019 sometimes raw fruits and vegetables contain harmful germs that can affect the consumer health, such as *Salmonella* spp, *E. coli*, and *Listeria monocytogenes*.

Food-borne illness is a big problem; the main group of food that is associated with this are fresh produce that includes processed fruit and vegetables, because they are implicated with pathogens like *E.coli* O157:H7, *Salmonella* spp, *Listeria monocytogenes*, and human parasite (Callejón *et al*. 2015; Bhilwadikar *et al*. 20019). Although good strategies have been developed to reduce crop risks, we must continue to look for appropriate strategies to reduce contamination risk and ensure product safety. The need of effective measures for disinfecting the produce is evident to reduce the initial microbial counts, to prevent pathogens from reaching unacceptable levels in fresh produce (Kang and Kang 2019). According to Murray *et* al. 2017 the current philosophy related to ensuring the food safety of fresh produce is to prevent contamination in the field and to minimize cross-contamination during post-harvest handling.

Sanitizers and water have always been the source of decontamination of fresh produce. Washing with potable water removes microorganisms to some degree, a process which can be enhanced by using sanitizers for disinfection of produce, i.e., decontamination (Van Haute *et al*. 2013). Washing can transfer microorganism to wash water and to other materials as the equipment (Gil *et al*. 2009). According to the FSA in 2019 cross-contamination is one of the most common causes of food poisoning. It happens when harmful bacteria are spread onto food from either other food sources. Over the years it has been seen that post-harvest treatments have limited decontamination and can lead to cross contamination (Barrera *et al*. 2012; Gombas *et al*. 2017).

Chlorine is still the most used sanitizer due to its efficacy, cost-effectiveness ratio and simple use (Petri *et al*. 2015). There are several factors that influence the efficiency of chlorine as turbidity and the acidity (pH) of the water, the turbidity should be < 5NTU and the pH level between 7.2 and 6.8. for the best result (World Health Organization 2011). Chlorine has oxidizing properties that destroy nucleic acid and cell membranes providing an attractive option for disinfection, however recovery of chlorine tolerant microbial pathogens at a low chlorine dosage has been reported (Owoseni *et al*. 2017).

Concentrated liquid sodium hypochlorite is the most used chlorine presentation, granulated calcium hypochlorite or chlorine gas are also used (Linares *et al*. 2018*)*. At high pH chlorine can produce reactions with organic nitrogen-based materials to produce chloramines, in addition may cause incomplete oxidizing of organic materials, resulting in potential carcinogenic byproducts as chloroform (CHCl3), trihalomethanes (THMs), Haloactic acids (HAAs), Haloacetylnitrile (HAN)

and nitrosodimethylamine (NDMA), products known for their carcinogenic potential. The formation of these byproducts associated with chlorine have intensified the search for alternatives for disinfection (Suslow 2000; Owoseni *et al*. 2017).

The use of chlorine in the treatment of water for human consumption is generally used in a concentration of 1 to 2 µg/ml, however, for post-harvest handling of fruits and vegetable levels of 10 to 25 times more is generally needed (Suslow 2000). Eliminating chlorine from washing and sanitizing process is a new trend because concerns about its efficacy on the produce, environmental and health risk because of the formation of carcinogenic halogenated disinfection byproducts (Ölmez and Kretzschmar 2009). For this reason, looking for novel techniques to enhance the use of sanitizers and more efficient decontamination for food surface is important.

The use of novel techniques to ensure clean and safe produce to the consumer is necessary; a good option is the use of aeration as a source of decontamination. Since years ago, bubbles have been used in Japan; applied in water used to wash surface or produce destroys 90% more microorganism (Burfoot *et al*. 2017). The use of aeration has been applied more for the treatment of water for human consumption. Bubbles could have an action on the cleaning because of the generation of free radicals or the release of energy associated with the collapse of bubbles (Takahashi *et al.* 2007; Agarwal *et al.* 2011).

According to Fox in 2004, aeration process consists of a large airflow that passes through the water, the contaminant is transferred to the air to be removed from the water. Water treatment by the aeration can be classified into four systems (i) Diffused Aeration, (ii) Mechanical and Submerged Agitators Aeration, (iii) Surface Aeration and (iv) Pure Oxygen Aeration (Mohammed 2013).

As previously described, the use of aeration may be a good option for the food industry. Thereby, it is important to know the effect of this technique against pathogens like *Escherichia coli* O157:H7 on bell peppers, and to validate aeration system's action to enhance the sanitizer effect in addition to rate of contamination of the washing water.

The objectives of this study were:

- Evaluate the use of aeration during washing and chlorine treatment to decontaminate nalidixic acid resistant mutant of *E. coli* O157:H7 from bell peppers.

- To examine the degree of cross-contamination of *E. coli* O157:H7 during treatment.

2. MATERIALS AND METHODS

Experiment location

The evaluation of the effectiveness of aeration to reduce microbial risk during washing and sanitizing fresh produce was carried out in the microbiology laboratory of the School of Nutrition and Food Sciences, Louisiana State University, Baton Rouge, Louisiana, United States of America.

Develop nalidixic acid resistant mutant of *Escherichia coli* O157:H7

Five *E. coli* O157:H7 strains isolated from different sources (Table 1) were used: Odwalla strain 223, F4546, EC 4042, H1730 and ATCC 43895. Nalidixic acid stock solution was prepared in a concentration of 30 mg/mL to be added to broths and media. Microorganisms stored in refrigeration were activated in a two-day process using Tryptic Soy Broth (TSB). Nalidixic resistant mutants were growth in MacConkey agar with sorbitol (SMAC), bottles of the medium were prepared at various nalidixic acid increasing concentrations of 0, 10, 20, 30, 40 and 50 µg/ml. A volume of 100 µL of the activated culture was spread on SMAC plate in increasing concentrations. The plates were incubated at 37 °C for 24 hours. The next day a loopful of the microorganism was taken from the plate with the highest concentration and was added to TSB containing the same highest concentration of nalidixic acid in which the organism survived. It is necessary to continue the process until the microorganism grows 50 µg/mL, however all the strains grew immediately at 50 µg/mL. The tubes were incubated at 37 °C for 24 hours. The nalidixic acid resistant organism was streaked in SMAC containing 50 µg/mL nalidixic acid, incubated and look for culture characteristic to confirm. The nalidixic resistant organism was stored in vials.

Table 1. Origin of strains of *E. coli* O157:H7.

Organism	Strain	Source	Pathogen
E. coli O157:H7	Odwalla strain 223	Apple juice	Yes
	F4546	Sprouts	Yes
	EC 4042	Spinach	Yes
	H1730	Lettuce	Yes
	ATCC 43895	Raw hamburger meat	Yes

Activation of the organism

The activation was carried out in a two-day process. The first day 16.66 µL of nalidixic acid was added with a sterile pipette to medium tubes containing 10mL of TSB to obtain a concentration of 50 µg/mL. The tubes were vortex and 0.1 mL of the microorganism was added afterwards. Tubes were vortexed again. The plates were incubated at 37 °C for 24 hours. The next day the same amount of nalidixic acid (16.66 µL) was added to the medium tubes. With a sterile pipette tip 0.1 mL of the Day 1 organism was added to 10 mL of TSB medium and vortex. The plates were incubated at 37 °C for 24 hours. The process was repeated for each of the strains at the same time.

Preparation for the inoculum

A cocktail was prepared by adding three strains in a falcon tube and vortex. The weight of the falcon tube was taken and the same was added with water in the other tubes to balance the centrifuge. The falcon tubes were centrifugated at 7,924 × g for 10 minutes, the supernatant was discarded preserving the pellet. The process was repeated with the remaining two strains. The two resulting pellets were mixed, 10 mL of PBS was added and vortex. The absorbance of the inoculum was measured with a spectrometer at 600 nm.

Inoculation

The biosafety hood was sanitized with ethanol (70%) and aluminum foil was placed on the surface. The bell peppers (20) were placed on the aluminum foil. Aliquots of 200 µl were inoculated for each pepper, around 40 droplets to fully inoculate the pepper area and two droplets around the stem. Peppers were left to dry for one hour at room temperature.

Application of aeration during washing and chlorine treatment

Twenty-two (22) green bell peppers (*Capsicum annuum*) were purchased a day before the experiment from a local market and kept refrigerated. Green bell peppers are the immature fruit of the pepper plant, that is harvested one month after flowering or 70 days after planting when the pepper has reached the full size, but the seeds are still immature (Pickersgill 2003). According to the USDA in 2005 a ripe green pepper that present similar varietal characteristics belongs to U.S. Fancy grade.

The same day the necessary tools for the experiment were autoclaved. Adequate amounts of medium were calculated, the following media and buffer were prepared: Tryptic Soy Broth (TSB), MacConkey Agar with Sorbitol (SMAC) and Phosphate Buffered Saline (PBS). The stomacher bags, sample bags, dilutions tubes and petri dishes were labeled the day of the experiment.

Chlorine solution

Two 12 L buckets were used for the treatments. To calculate the amount of chlorine at 6% of purity needed to obtain 100 ppm, the appropriate formula was used [1]. The action of the chlorine depends on the pH, for this reason, citric acid was necessary to balance the solution to neutral. Citric acid

at 0.1N was used, the amount was calculated using a pH meter calibrated with buffers at 4 and 7. The results was 20.4 mL of chlorine (6%) and 109 mL of citric acid (0.1 N).

$$\text{Volume of bleach} = \frac{(\text{desired ppm} \times \text{volume of wash water})}{(\%\ \text{of chlorine purity} \times 10{,}000)} \qquad [1]$$

Aeration system description

The construction of the system was carried out in the LSU engineering building. The system was made with 3.16 cm × 30.48 cm PVC pipes and a blower with an air volume of 161.4 m³/h and a speed of 55.88 m/s as is shown in Figure 1. The principle of the system is that the blower fulfills the function of providing a large airflow through the tubes. The tubes are submerged in water, each tube has perforations at one inch which allows the generation of bubbles.

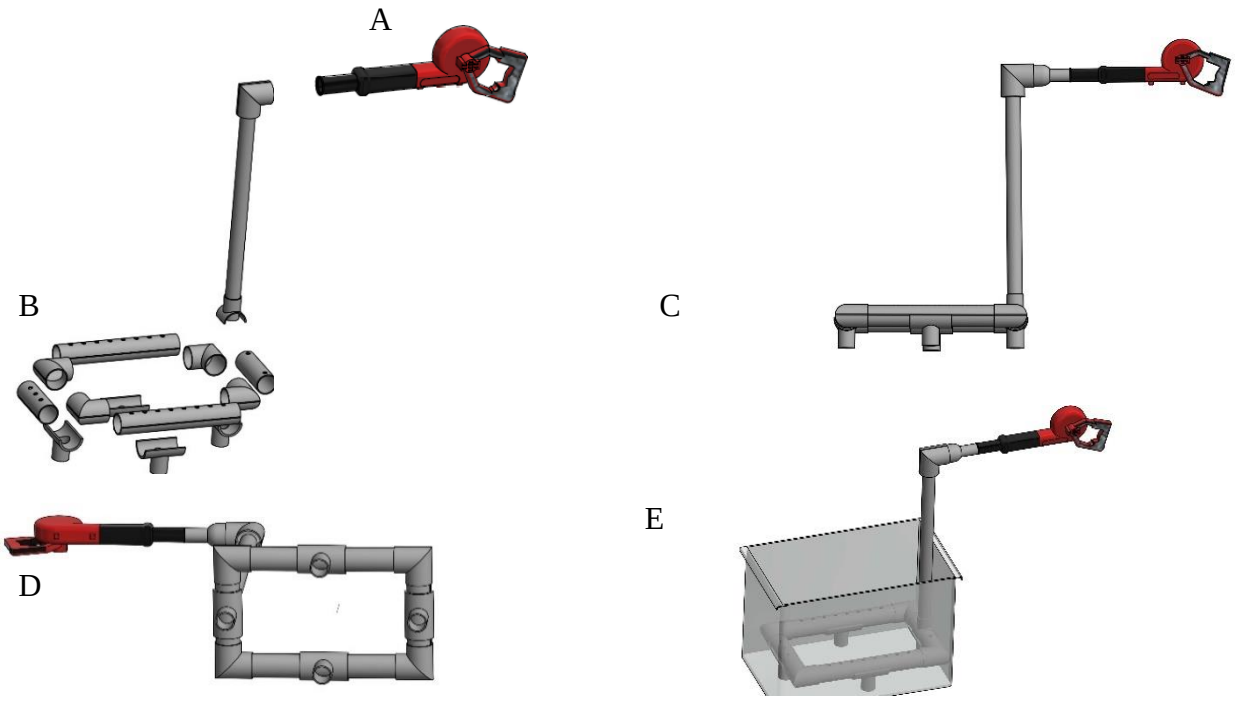

Figure 1. Aeration system (A) Blower, (B) Parts, (C) Side view, (D) Bottom view, (E) General view.

Application of treatments

Two bell peppers were used for the uninoculated (UB) and inoculated control (IB). Four bell peppers were used for the application of each treatment (Table 2) for three minutes. After the application of the treatment, the four peppers were taken at the same time and immersed in a sterile tared bag with 100 mL of PBS.

Table 2. Description of treatments.

Treatment	Code
Bell peppers treated with water	WB
Bell peppers treated with water and bubbles	WBB
Bell peppers treated with chlorine	CB
Bell peppers treated with chlorine and bubbles	CBB

Microbiological analysis

The weight of the bag with the pepper and PBS was recorded. Each bag was homogenized in the stomacher for four minutes. The bags were place under the biosafety hood and dilutions were carry out. A volume of 100 µL of the original sample and dilutions were place on each petri dish with SMAC and nalidixic acid, using the spread plate technique. The plates were incubated at 37 °C for 24 hours. The next day the colonies were identified and counted, the data was presented in CFU/g and converted to Log CFU/g. Additionally, a 10 mL sample of the solution was taken. Volumes of 300 µl, 300 µl and 400 µl were placed in three different petri dishes. The plates were incubated at 37 °C for 24 hours. The next day the colonies were identified and counted.

Experimental design and statistical analysis

For this experiment a Completely Randomized Design (CRD) was used, four treatments with four replicates were established. Two independent replicates were performed on different days. The data was analyzed through an ANOVA and a Tukey test to determinate mean differences ($P < 0.05$), using the Statistical Analysis System (SAS 9.6®). The model proposed for the design is defined by Equation 2.

$$y_{ij} = \mu + T_i + \varepsilon_{ij} \qquad [2]$$

3. RESULTS AND DISCUSSION

The initial level of the inoculum was 5.41 ± 0.15 Log CFU/g (Table 3). There was a significant variation ($P < 0.05$) in the reduction of *Escherichia coli* O157:H7 counts Log CFU/g as a result of the treatments with chlorine (CB), chlorine with bubbles (CBB) and water with bubbles (WBB) (Table 2). No significant reduction of *E. coli* O157:H7 on bell pepper was observed with water only treatment, however, levels of *E. coli* O157:H7 were significantly lower on bell pepper treated with chlorine (CB), chlorine with bubbles (CBB) and water with bubbles (WBB). No significant difference was observed on the reduction of *E. coli* O157:H7 between the chlorine (CB), chlorine with bubbles (CBB) and water with bubbles (WBB) treatments.

Table 3. Mean values for *Escherichia coli* O157:H7 count (Log CFU/g) from bell pepper after washing and chlorine (100 ppm) treatments.

Treatment	**Average Log CFU/g**
Inoculated Control (IB)	5.41 ± 0.15^{a}
Bell peppers treated with water (WB)	4.26 ± 0.08^{ab}
Bell peppers treated with water and bubbles (WBB)	3.35 ± 0.02^{bc}
Bell peppers treated with chlorine and bubbles (CBB)	3.18 ± 0.42^{bc}
Bell peppers treated with chlorine (CB)	2.62 ± 0.63^{c}

*The means followed by different lowercase letters (a - c) on the column indicate significant differences with the Tukey test ($P < 0.05$).
CFU: Colony forming unit.

In the case of chlorine treatments, it was observed that the bubbles did not influence the reduction of microorganisms on the bell peppers' surface. Chlorine is still the most used sanitizer due to its efficacy, cost-effectiveness ratio and simple use (Petri *et al*. 2015). Chlorine-based-sanitizers exhibited the microbial reduction of < 1.12 Log CFU/g on fruits and vegetables (Yoon and Lee 2017).

Washing can transfer microorganism to wash water and to other materials as the equipment (Gil *et al*. 2009). There are several factors that influence the efficiency of chlorine such as turbidity and the acidity (pH) of the water, the turbidity should be < 5NTU and the pH level between 7.2 and 6.8. for the best result. (World Health Organization 2011). Attachment to particulate matter, aggregation, encapsulation of the pathogen, ingestion by protozoa, and water turbidity may also affect chlorine efficacy (CDC 2012).

Several factors influence the action of chlorine on pathogens; therefore, efficiency is affected by the presence of bubbles. Aeration refers to any process where water and air come in contact to remove volatile substances in and out of the water (Salamanca 2016). That is why the disinfectant's effectiveness decreases since it is absorbed by the air due to the turbulence caused by the system and does not allow it to act on the pathogens present on the surface of the bell pepper.

In the water treatment, bubbles increase the reduction of microorganisms on the surface of the product. The aeration process consists of large volumes of air passing through the water to transfer the contaminant from the water to the air and therefore remove the contaminant from the water (Fox 2004). Bubbles could have an action to the cleaning due to a scrubbing action, a release of energy as the bubbles collapse, or the generation of free radicals (Takahashi *et al*. 2007; Agarwal *et al*. 2011). The addition of water aeration for the washing of vegetables increases the ability to remove pathogens from the surface as observed in the reduction of Log CFU/g of the study (Table 2).

Reductions (Log CFU/g) observed according each treatment are presented in Table 4. Results obtained in this study are comparable to data obtained in the study carried out in 2017 by Jiang *et al.* with cold plasma-activated hydrogen peroxide aerosol to inactivate different food borne pathogens in grape tomato, spinach, and cantaloupe. The effect of aerosolized H2O2 on *E. coli* O157:H7 on tomatoes, spinach leaves, and cantaloupe rind were different on each product and part, on the smooth surface of tomatoes were reduced to a level below detection limit (< 0.6 Log CFU/ piece) while the bacterium was only reduced by 1.0 Log CFU/piece on the stem scar area of tomatoes. On the surface of spinach leaves and cantaloupe rind, *E. coli* populations were reduced by 1.5 and 4.9 Log CFU/ piece, respectively. Which is quite similar to the reductions found in this study.

Table 4. Mean values for the reduction in *Escherichia coli* O157:H7 counts (Log CFU/g) from bell pepper after washing and chlorine (100 ppm) treatments[&].

Treatment	**Reduction Log CFU/g[NS]**
Bell peppers treated with chlorine (CB)	2.79 ± 0.77^{b}
Bell peppers treated with chlorine and bubbles (CBB)	2.23 ± 0.27^{bc}
Bell peppers treated with water and bubbles (WBB)	2.06 ± 0.16^{bc}
Bell peppers treated with water (WB)	1.15 ± 0.07^{c}

[&] Initial level of the inoculum 5.41 ± 0.15 (Log CFU/g).
[NS] No significant difference was observed among treatments ($\alpha = 0.05$).
CFU: Colony forming unit.

Similar results were obtained in the study carried out in 2018 by De Oliveira *et al.* with a combination of aerosolized curcumin and UV-A light on fresh produces (spinach, lettuce, and tomato) which shows reductions of approximately 3 Log CFU × cm^{-2} of *E. coli* O157:H7. The study carried out in the same year by Singh *et al.* with peracetic acid in fresh produce (romaine lettuce, lemons, tomatoes, and blueberries) surfaces, achieved moderate level of reductions (1.4 to 2.3 Log CFU/g). Out of all wash treatments, near-neutral electrolyzed water (NNEO) with 2.3 Log CFU/g reductions and peracetic acid (PAA) at 100 mg/L with 2.2 Log CFU/g reductions.
As part of the experiment, samples were taken from the solutions used for the application of each treatment. The number of colonies (Log CFU) were observed after applying each treatment for three minutes, only colony count was obtained in the water solution after each treatment (Table 4).

The treatment with water and bubbles (WBB) is equally effective to chlorine treatment (CB), the first can be used in the washing and sanitizing of bell peppers reducing the concentration of sanitizer or antimicrobial to keep water clean for a longer time. In the same way it was observed that the treatment with water and bubbles (WB) does not present microorganism in the solution after three minutes (Table 5). It is important to remember that the treatments can produce different results with other types of food and sanitizer product.

Table 5. Microbial load in 1mL of the solution after three minutes of immersion or applying the bubble and chlorine (100 ppm) treatment.

Solution	**Log CFU/mL**
Chlorine solution after dumping bell peppers (CS)	ND
Chlorine solution after bubble treatment (CSB)	ND
Water solution after dumping bell peppers (WS)	1.90
Water solution after bubble treatment (WSB)	ND

ND: Not detected. Detection limit of 1 CFU/mL.
CFU: Colony forming unit.

The use of chlorine during washing helps to reduce the levels of cells in the solution, ensuring the quality of the water both at the beginning and at the end of the process, minimizing the risk of cross contamination due to the residuality of pathogens in the solution. According to the FDA in 1998, water itself is a useful means to reduce the possibility of contamination, however it can cause contamination directly or indirectly, microbiological accumulation can occur in addition to the microorganisms that come from the product. The high cost of water has resulted in the industry-wide practice of reuse or recirculation of wash water. The water can contain organic loads, which can detriment the quality of the clean produce and contaminate with pathogens (Allende *et al*. 2008).

In the case of the aeration system, the bubbles act on the microorganism. The phase separation boundary is also the bubble surface, and if bubbles capture the active molecules, they remain on the bubble surface. In other words, bubbles transfer active molecules to the water surface and realize in this manner the purification of water (Smirnov *et al*. 2018). That is why both bubbles, microbubbles and nano bubbles are used in water treatment. The potential of bubbles to the cleaning is associated with the turbulence as microbubbles collapse and the generation of highly reactive free radicals (Agarwal *et al*. 2011).

4. CONCLUSIONS

- The use of aeration reduces the bacterial load being an alternative to use with other chemical substances or to reduce the concentration applied for the washing and sanitization of bell peppers.

- The aeration system helps avoid cross-contamination during the treatment, maintaining the washing solution free from *E. coli* O157:H7.

5. RECOMMENDATIONS

- Carry out an experiment to test the effectiveness of reducing the chlorine concentration and compare it with the air treatment.
- Carry out evaluations at different concentrations of chlorine to determine the reduction and the appropriate amount to keep the water clean for a longer time.
- Evaluate the washing and sanitizing treatments on other vegetables and sanitizer products.
- Carry out evaluations of the washing and sanitizing treatments at different times to know its effectiveness during different periods of time.
- Carry out evaluations of the washing and sanitizing treatments at different pressure to know its effectiveness.
- Evaluate the system with other pathogens such as: *Salmonella spp* and *Listeria monocytogenes*.
- Conduct a red, strikethrough specific evaluation of the solution used for each of the treatments, to know in more detail the amount of microorganism remaining in the solution after each treatment.
- Evaluate the effectiveness of micro and nano bubbles in the fresh product washing and decontamination process.

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7. APPENDICES

Appendix 1. SAS code

```
Data WashTreatments;
Input treatment $ counts;
Datalines;
IB    5.51
IB    5.30
CB    2.18
CB    3.06
CBB   3.47
CBB   2.88
WB    4.31
WB    4.20
WBB   3.34
WBB   3.36
;
RUN;

PROC ANOVA Data=WashTreatments;
class treatment;
model counts=treatment;
means treatment/ tukey alpha=0.05;
RUN;
```

Appendix 2. SAS code Output.

THE SAS SYSTEM

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
treatment	5	CB CBB IB WB WBB

Number of Observations Read	10
Number of Observations Used	10

Continuation appendix 2.

THE SAS SYSTEM

The ANOVA

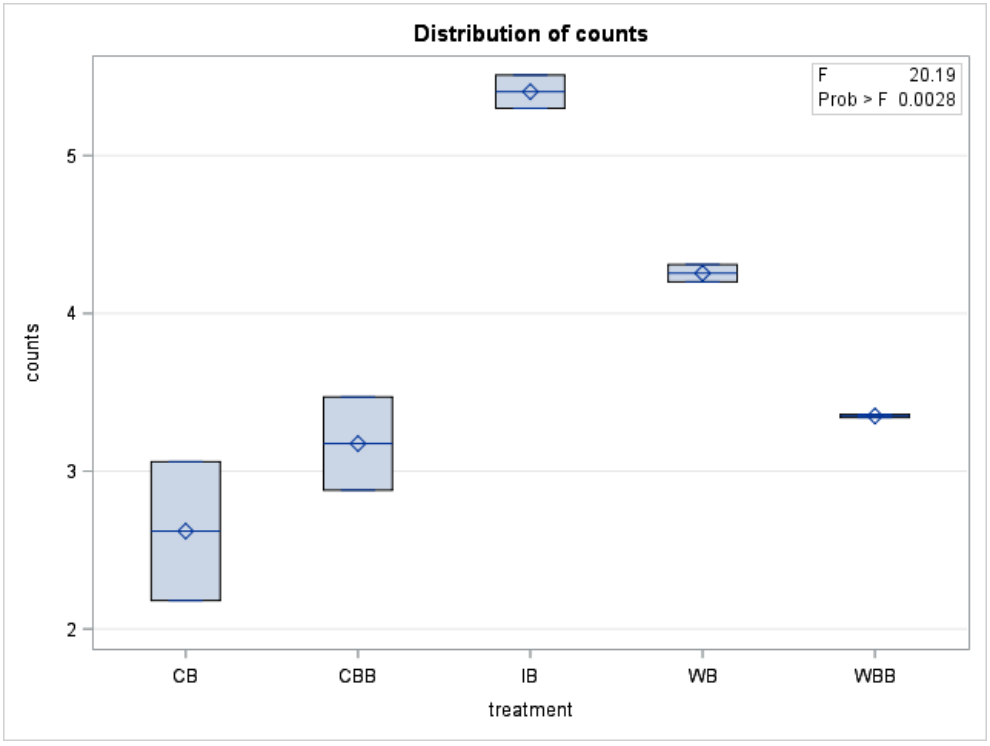
Procedure Dependent

Variable: counts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	9.52194000	2.38048500	20.19	0.0028
Error	5	0.58955000	0.11791000		
Corrected Total	9	10.11149000			

R-Square	Coeff Var	Root MSE	counts Mean
0.941695	9.130025	0.343380	3.761000

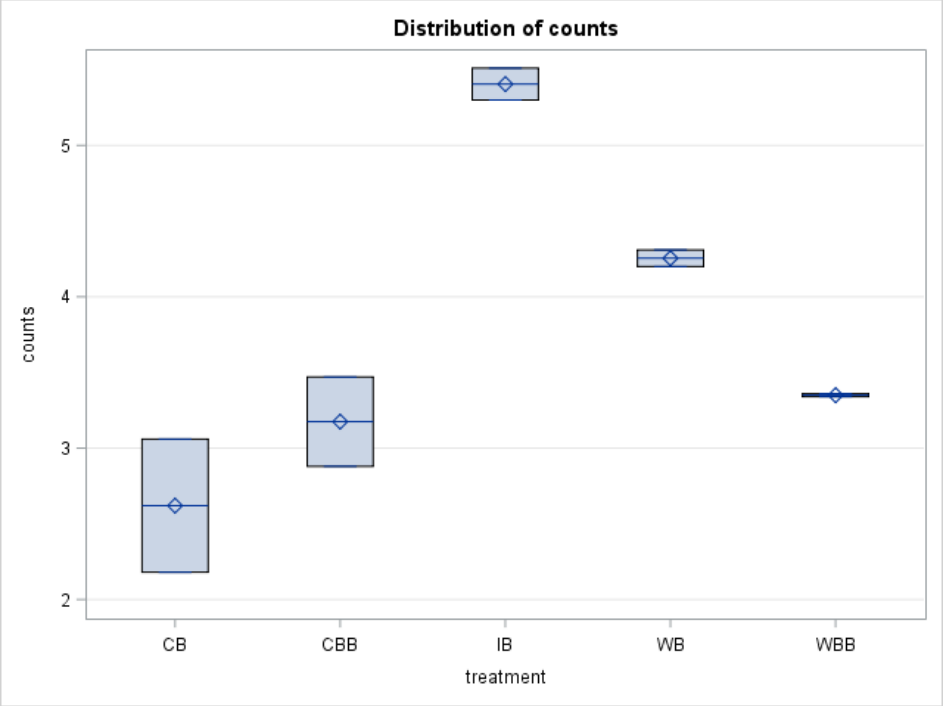
Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	4	9.52194000	2.38048500	20.19	0.0028



Continuation appendix 2.

THE SAS SYSTEM

The ANOVA Procedure



THE SAS SYSTEM

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for counts

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	5
Error Mean Square	0.11791
Critical Value of Studentized Range	5.67302
Minimum Significant Difference	1.3774

Continuation appendix 2.

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	treatment
	A	5.4050	2	IB
	A			
B	A	4.2550	2	WB
B				
B	C	3.3500	2	WBB
B	C			
B	C	3.1750	2	CBB
	C			
	C	2.6200	2	CB