

## Final Report



Search and innovation of new food safety improvement alternatives for the mango industry

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## II. Introduction

The ever growing production and marketing of contaminated fruits and vegetables has brought about an unusual increase in the risk of disease propagation. Cross-contamination has become a channel for bacteria, parasites, and viruses to gain access to fruits and vegetables (Beuchat, 1996). In the United States of America (USA), several outbreaks of gastrointestinal diseases have been reported in recent years, that have been associated with the consumption of contaminated fresh produce. Prior studies, as well as more recent ones, that have tracked microbial sources indicate that the contamination of fresh produce occurs in the field and/or during the packing process, especially when Good Agricultural and Manufacturing Practices are not rigorously applied (CDC 2001; CDC 2002; CDC 2003; CDC 2005; CDC 2008). Several cases of infectious *Salmonella* outbreaks associated with fresh mango consumption have been reported. In 1999, mangos from Brazil caused an outbreak of *Salmonella enterica* in Newport, NJ in the US. 78 patients in 13 states were infected with the outbreak strain, 15 of them were hospitalized and 2 died (Sivapalasingan et al. 2003). *Salmonella enterica* of the Saintpaul serotype was deemed to be the culprit of an outbreak that occurred in the US in 2001, and that resulted from the consumption of fresh mangos imported from Peru that were contaminated (Beatty et al. 2004). In 2012, mangos imported from Mexico that were contaminated with *Salmonella* Braenderup lead to an outbreak that involved a total of 127 people infected by the strain in 15 states in the US (CDC, 2012). The purpose of this research is to assess the effectiveness of applying chlorine, chlorine dioxide, and copper ions, during the mango washing, hot water treatment and hydrocooling processes, on the reduction of *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*, as well as on the bacterial transfer rates from the water to the fruit, and vice versa. For this purpose, laboratory trials were conducted under simulated conditions associated with the processes that occur in mango packinghouses. The results show relevant information that could contribute to the development of alternative disinfection measures that could serve to improve the food safety practices, currently being employed by the mango industry.

## III. Objectives

### a. Overview

Develop better food safety practices for the mango industry, related to water use in packinghouses to ensure the safety of the fruit. Ensuring a positive impact on consumer's health and on marketing of the product.

### b. Specific objectives

- i. Determine the effectiveness of each stage of the mango washing process on the reduction of *Salmonella enterica* and *Listeria monocytogenes* from surface the fruit.
- ii. Determine the transfer rates of *Salmonella enterica* and *Listeria monocytogenes* from the water tanks to the mangos, and vice versa.
- iii. Determine the effectiveness of chemical disinfectants as chlorine and copper against *Escherichia coli* when used in the hot water treatment.
- iv. Determine which disinfectants are more effective in controlling and ensuring the microbial quality of the water used for the mango washing process in order

to maximize the control, reduction, or elimination of any cross-contamination risks with *Salmonella enterica* and *Listeria monocytogenes*.

## IV. Materials and Methods

### IV.1 Selection and Disinfection of Fruit

The mangos evaluated in this study were *Tommy Atkins* variety, physiologically mature, with no mechanical damage and evenly weighted. The fruit was provided by a mango grower that operates in the region and was taken to the CIAD National Laboratory for Food Safety Research (*Laboratorio Nacional para la Investigación en Inocuidad Alimentaria de CIAD Unidad Culiacán*) – Culiacan Division to carry out the trials.

The mangos were disinfected using a solution of sodium hypochlorite (NaClO) at a concentration of 200 mg L<sup>-1</sup> of free chlorine; to eliminate chlorine residue, the fruit was washed with sodium thiosulfate at 1% and subsequently rinsed with purified sterile water. After each one of these 3 steps the fruit was rubbed for one minute. Finally, the fruit was allowed to dry out on a sterile grid for 60 minutes in a stainless steel laminar flow hood.

### IV.2 Chemical Disinfectants

The disinfectants to be evaluated in this study were sodium hypochlorite (Cloralex<sup>MR</sup>) and chlorine dioxide (TwinOxide<sup>®</sup>), with concentrations and preparation methods described as follows:

#### Sodium hypochlorite

This disinfectant has a spectrum of activity that includes bacteria, viruses, algae, and fungi. Its composition is made up of sodium hypochlorite at 5.25% of free chlorine, and it stabilizes at a pH range of 6.5-7.5. The concentrations of Cloralex<sup>MR</sup> used in this study were 100 and 200 ppm. In order to reach these concentrations, we proceeded to prepare the desired solutions separately by using 1.90 and 3.80 mL of the disinfectant in concentrated form and depositing them in containers with 1000 mL of purified sterile water. To determine the concentration levels of free chlorine contained in each one of the preparations, we used a HACH model DR 3900 spectrophotometer with a HACH brand DPD agent for total chlorine in the manner described as follows:

1. The spectrophotometer was turned on and set to program 83 (with a reading range from 0.2 to 3 mgL<sup>-1</sup>).
2. In order to corroborate the final concentration, we obtain an aliquot of 1 mL of the solution and diluted (gaged) it to 100 mL with distilled water. 10 mL were taken from this dilution in a measuring cell.
3. The cell was placed in the device to determine the zero reading and subsequently removed from the device.

4. 10 mL of the same dilution was taken and placed in another measuring cell, and a packet of DPD (N,N-Diethyl-p-phenylene-diamine) containing agents for determining total chlorine was added.
5. The measuring cell was covered with parafilm to allow it to dissolve and it was left to rest for 1 min.
6. Immediately after the 1minute rest period, the measuring cell was placed inside the equipment to carry out the sample measurement.
7. The result is expressed in mg of free chlorine per liter of sample ( $\text{mgL}^{-1}$ ) (APHA, 1998).

#### Chlorine dioxide

This disinfectant has a spectrum of activity that includes bacteria, viruses, algae, and fungi. Its composition is made up of chlorine dioxide at 3,000 ppm, and it stabilizes at a pH range of 3-9. The chlorine dioxide (TwinOxide<sup>®</sup>) was prepared in accordance with the manufacturer's instructions. Briefly, we added agent A, which contained sodium chlorite, into an amber color volumetric flask containing 1 L of distilled water. Then we added agent B, a sodium bisulfate compound, into the volumetric flask. This compound solution was allowed to rest for 3 hours which, theoretically, would put it at a final concentration of 3,000 ppm of chlorine dioxide. The concentrations that were used for this disinfectant during the study were 3 and 5 ppm, which were adjusted using the following formula (1)

$$V_1C_1 = V_2C_2 \quad (1)$$

A HACH model DR 3900 spectrophotometer with a HACH brand DPD agent for free chlorine was used in order to determine the chlorine dioxide concentrations in each one of the samples. The procedure used for that purpose is described as follow:

1. The spectrophotometer was turned on and set to program 76 for chlorine dioxide (0 to 5.00  $\text{mg L}^{-1}$ ).
2. 10 mL were removed from the chlorine dioxide solution that was prepared to the desired concentration, then added to a measuring cell of 10 mL.
3. The measuring cell was placed in the device to determine the zero reading, and subsequently removed from the device.
4. 10 mL of the same solution were placed in another cell, to which four drops of glycine were added.
5. The measuring cell was covered with parafilm to facilitate the mixing process, then a packet of DPD (N,N-Diethyl-p-phenylene-diamine) containing agents for determining free chlorine was added.
6. This mixture was shaken and a pink color was observed. It rested for 1 minute.
7. Once the minute of rest passed, the measuring cell was immediately placed inside the device to conduct the sample measurement.

The result was expressed in mg of chlorine dioxide per liter of sample ( $\text{mgL}^{-1}$ ) (APHA, 1998).

### IV.3 Preparation of Turbidity

The turbidity of the water used in the experiments during the hydrocooling treatment process in this study was adjusted to 2 and 50 UNT using a Hach 2100 P Turbidity Meter. Soil from mango farms in Escuinapa, Sinaloa previously subjected to a physiochemical characterization (Attachment 3) and sterilization was used for that purpose.

### IV.4 Preparation of the Inoculum of the Bacterial Strains Used in the Atudy

The *Salmonella enterica* ser. Choleraesuis ATCC 10708, *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 7644 strains used in this study were sourced from the collection stored at the National Laboratory for Food Safety Research at the Nutrition and Development Research Center (*Laboratorio Nacional para la Investigación en Inocuidad Alimentaria del Centro de Investigación en Alimentación y Desarrollo (CIAD)*), Culiacán Campus, and used in different hydro-treatment processes. The *Salmonella* Choleraesuis strain was used in these trials because it is often employed in the assessment of antimicrobial and disinfectant agent trials.

Preparation of the inoculum of the bacteria was carried out by taking a colony of each strain stored under ultra-freezing conditions at  $-80\text{ }^{\circ}\text{C}$ . Each colony was planted separately in xylose-lisine-deoxycholate agar (XLD) (Bioxon, Estado de México, México), CHROMagar ECC and Agar Palcam (Fluka, Suiza) for *Salmonella*, *Escherichia coli* and *Listeria*, respectively. The boxes incubated at  $37\text{ }^{\circ}\text{C}$  for 24 and 48 h  $\pm$  2 h, respectively. After this incubation period, a characteristic colony was selected for each bacterial genus and planted in Trypticasein Soy Broth (TSB) (Difco, México), for *Salmonella* and *E. coli*, and in TSB-YE (Trypticasein Soy Broth and Yeast Extract at 0.6%) (Becton Dickinson Sparks MD, USA), for *Listeria*, with incubation at  $37\text{ }^{\circ}\text{C}$  for  $18 \pm 2$  h. Thereafter, the culture broths from both bacterias were centrifuged at 13,800 *g* for 10 min at  $4\text{ }^{\circ}\text{C}$ . The supernatant was discarded and the cell pellet was washed twice using an attenuation solution, containing phosphates (solution of monobasic potassium phosphate, pH 7.2) to minimize the noncellular constituents associated with the solution (APHA, 2001).

The concentration of the bacterial suspension was determined through the use of the spread plate method. Consequently, bacterial pellets were resuspended in a phosphate buffer and subjected to a serial dilution process using a decimal factor ( $10^{-1}$ ,  $10^{-3}$ ,  $10^{-5}$ ,  $10^{-7}$ ). Afterwards, 0.1 mL of the  $10^{-5}$  and  $10^{-7}$  dilutions were removed and extended over XLD Agar, CHROMagar ECC and PALCAM Agar. The Petri dishes were incubated at  $37\text{ }^{\circ}\text{C}$  for  $24 \pm 2$  h and  $48 \pm 2$  h to allow for the quantification of *Salmonella* Choleraesuis, *Escherichia coli* and *Listeria monocytogenes*, respectively. The concentration of the bacterial inoculum used was  $7.0 \text{ Log}_{10} \text{ UFC mL}^{-1}$  (APHA, 2001).

### IV.5 Inoculation of Mango Fruit

The inoculation of mango fruit was carried out under the method described by Ukuku and Sapers (2001), with some modifications. The mangos were placed in a tub that contained 4 L of the bacterial suspension ( $7 \text{ Log}_{10} \text{ UFC mL}^{-1}$  of *Salmonella* Choleraesuis, *Escherichia coli* or *Listeria monocytogenes*), which was maintained at constant agitation, and were allowed to remain there for a period of 60 minutes of contact, after which they were removed and placed in a laminar flow cabinet for drying.

In order to determine the bacterial inoculum attached to the surface of the mangos, three inoculated mangos were randomly selected and placed independently in hermetically sealed bags containing sterile distilled water at a 1:1 weight/volume ratio; they were manually shaken for one minute and an aliquot of 0.1 µL was taken and planted directly on the agar, as well as 1 mL from which dilutions were made and planted in duplicate fashion using the spread plate method in XLD agar for *Salmonella*, CHROMagar for *E. coli* or Palcam Agar for *Listeria*, with incubation at 37 °C for 24 y 48 h, respectively. Once the time had elapsed the UFC mL<sup>-1</sup> was quantified to determine the inoculum that was attached to the surface of the fruit, which was expressed as UFC Log<sub>10</sub>.

The following describes the experimental procedure used to obtain the measurements of the response variables related to each one of the objectives outlined in this study:

#### **IV.6 Determining the efficiency of disinfectants for each stage of the washing process to remove microorganisms from the surface of mango fruit (Objective 1)**

The mango washing stage after its arrival at the packing house will be considered in three modes: 1) Washing in tanks, 2) Washing with sprayers, and 3) Hydrocooling

##### 1. Wash tanks

The 80 L wash tanks (used at arrival and during the hydrocooling process) were subjected to adjustments in their concentration levels of sodium hypochlorite, 100 and 200 ppm, respectively, turbidity, 2 and 50 UNT, respectively, and a temperature of 25 °C. Once these conditions were standardized, a batch of mangos, previously inoculated with a bacterial solution (*Salmonella enterica* ser. Choleraesuis and *Listeria monocytogenes*) at a concentration of 8 log<sub>10</sub> UFC, was introduced into the wash tank for an exposure/contact period of 1 minute. Once the contact period ended, a representative sample of mangos was removed from the tanks for the purposes of conducting microbiological analysis, placed in a hermetically sealed bag containing sterile distilled water at a 1:1 weight/volume ratio and 0.5 mL of sodium thiosulfate (3 %), and manually shaken for one minute. The bacterial concentration was determined after the application of the disinfectant treatment. The analysis of this data yielded the efficiency of the applied disinfectant. In the case of the chlorine dioxide, the procedure followed was the same as the one conducted previously, however, the concentrations used were 3 and 5 ppm, respectively. The experiment was conducted three times.

##### 2. Sprayers

A batch of mangos, previously inoculated with a bacterial solution (*Salmonella enterica* ser. Choleraesuis and *Listeria monocytogenes*) at a concentration of 8 log<sub>10</sub> UFC, was placed in a mechanical rotator (ATR, RKVSD) at a velocity of 60 rpm, and was sprayed from a distance of 30 cm (Chaidex et al 2007) with each disinfectant solution (sodium hypochlorite, 100 and 200 ppm, chlorine dioxide, 3 and 5 ppm) for 20 seconds at 25 °C. In order to cease the action of the disinfectant and quantify the bacterial concentration on each mango, they were placed in a hermetically sealed bag containing sterile distilled water at a 1:1 weight/volume ratio and 0.5 mL of sodium thiosulfate (3%), and manually shaken for a



period of 1 minute. Afterwards, an aliquot was removed from the bag to carry out the bacterial count as described in the section that refers to the preparation of the inoculum (APHA, 2001). Both disinfectants were prepared with a turbidity of 2 UNT. The experiment was conducted three times.

### 3. Hydrocooling

For analysis purposes, this objective was divided into 2 segments (inactivation kinetics and factor effect). The treatment consists of combining various factor levels: type of disinfectant, turbidity, and contact time, all of which appear in table 1.

| Disinfectant and Dosage <sup>1</sup> | Contact Time (min) |        |       |        |
|--------------------------------------|--------------------|--------|-------|--------|
|                                      | 0                  | 1      | 5     | 10     |
|                                      | 2 UNT <sup>2</sup> | 50 UNT | 2 UNT | 50 UNT |
| NaClO 100                            | xxx                | xxx    | xxx   | xxx    |
| NaClO 200                            | xxx                | xxx    | xxx   | xxx    |
| ClO <sub>2</sub> 3                   | xxx                | xxx    | xxx   | xxx    |
| ClO <sub>2</sub> 5                   | xxx                | xxx    | xxx   | xxx    |

Disinfectant; NaClO: Sodium Hypochlorite; ClO<sub>2</sub>: Chlorine Dioxide and X: replica.

<sup>1</sup>ppm

<sup>2</sup> Nephelometric Turbidity Units

The application of the treatments described above was conducted as follows: mangos that were previously inoculated with a bacterial solution of known concentration of *Salmonella Choleraesuis* or *Listeria monocytogenes* were immersed in a container filled with 1 L of sterile distilled water, a type of disinfectant (chlorine dioxide at 3 or 5 ppm, sodium hypochlorite at 100 or 200 ppm) and sufficient sterile soil to generate a turbidity (2 or 50 UNT), for a determined contact period (0, 1.5 or 10 min). Once the trial ended, each mango was placed in sterile distilled water along with 0.5 mL of neutralizing solution. Thereafter, we proceeded to determine the remaining bacterial concentration on the surface of the fruit, by the procedures outlined in the section referring to *The inoculation of mango fruit*. The

recorded response variable was bacterial survival expressed as  $\text{Log}_{10} \left( \frac{N_t}{N_0} \right)$ .

#### IV.7 Transfer rates for *Salmonella Choleraesuis* and *Listeria monocytogenes* (Objective 2)

In order to determine the bacterial transfer from the fruit to the water, mangos inoculated previously at a concentration of 7 Log<sub>10</sub> of *Salmonella Choleraesuis* or *Listeria monocytogenes* were used, as described in the section referring to the *Inoculation of Mango Fruit*. Three of these mangos were used to quantify the bacteria and determine the initial inoculum that attached to the surface of the mango, as described in the section referring to the *Inoculation of Mango Fruit*. The remaining mangos were placed in a

stainless steel tub with 12 L of sterile distilled water and subjected to constant agitation for a period of 30 minutes. Once the time elapsed, three randomly selected mangos were removed from the tub in order to determine the remaining bacterial concentration on the surface of each fruit. For this purpose, each mango was individually placed in hermetically sealed bags containing sterile distilled water with a 1:1 weight/volume ratio; the bags were manually shaken for a period of 1 minute. Thereafter, an aliquot of 0.1 µL was taken and placed directly on agar, whereas another aliquot of 1 mL was taken to conduct three dilutions until a concentration level of 10<sup>-3</sup> was obtained. Following this, the dilutions at 10<sup>-1</sup> and 10<sup>-3</sup> were planted in duplicate fashion using the spread plate method in XLD agar for *Salmonella* or Palcam Agar for *Listeria*, with incubation at 37 °C for 24 and 48 h, respectively. Once the time had elapsed, the UFC mL<sup>-1</sup> were quantified and an average was calculated using the results of the two boxes that were planted in order to determine, based on the differential, the bacterial concentration that was transferred (CBT) from the fruit to the water. The CBT was calculated by using the following formula (Formula 2):

$$CBT = \frac{\text{Average UFC (In both boxes)}}{(\text{Dilution reading}) (\text{Volume planted})} \quad (2)$$

Finally, the result was converted to logarithmic units, expressed as Log<sub>10</sub>(UFC).

In order to determine the transfer rate (TR), the following mathematical operation was used (Formula 3):

$$TR = \frac{\log_{10} \text{receptor}}{\log_{10} \text{donor}} * 100 \quad (3)$$

Whereby:

“Log<sub>10</sub>(receptor)” is the UFC Log<sub>10</sub> that remains on the mango after having been immersed in water for 30 minutes, and “Log<sub>10</sub>(donor)” is the initial UFC Log<sub>10</sub> of the inoculated mango control group.

In order to determine the transfer rate of bacteria from the water to the fruit, we poured 11 L of sterile distilled water and 1L of *Salmonella Choleraesuis* or *Listeria monocytogenes* inoculum into a stainless steel tub. We then proceeded to determine the bacterial concentration of the solution by conducting a serial dilution using an odd factor (from 10<sup>-1</sup> to 10<sup>-7</sup>), the products of which were planted using the spread plate method on Petri dishes in XLD agar or Palcam agar for *Salmonella* y *Listeria*, respectively. The boxes with XLD were incubated at 37 °C for 24 h, and the boxes with Palcam Agar for 48 h. Once the incubation period ended, the colonies were quantified in order to determine the final concentration of the inoculum in the solution, which was expressed as UFC Log<sub>10</sub>.

Once the bacterial inoculum in the water was determined, the fruit, which was independently disinfected, numbered and weighed, was placed in the tub for a period of 30 min. When the contact

time ended, randomly selected mangos were removed and placed separately in hermetically sealed bags containing sterile distilled water with a 1:1 weight/volume ratio, and were manually shaken for 1 minute. Thereafter, serial dilutions were conducted in the manner described previously, and the samples were planted in XLD agar for *Salmonella* and Palcam agar for *Listeria*. The Petri dishes were incubated at 37 °C for 24 h for *Salmonella* and 48 h for *Listeria*.

When the period elapsed, the colonies were counted and the Log<sub>10</sub> computations were carried out using formula (3) to determine the transfer rate of bacteria from the water to the fruit.

#### **IV.8 Trial to assess the effectiveness of copper as a disinfectant in a hot water treatment simulator, using water with varying degrees of quality (Objective 3)**

In a Thermo Scientific Model 2872 thermo bath, equipped with an agitator and a capacity of 26.5 litres, conditioned with sterile purified water, with turbidity adjusted to 2 UNT or 50 UNT at a temperature of 46.1 °C, the dose of copper disinfectant was added (8.5, 12 or 17 ppm). Extreme conditions of contamination were simulated with the addition of the *E. coli* microorganism at a concentration of 10<sup>6</sup> UFC mL<sup>-1</sup>, after which the mangos were placed in the tub. The disinfectant was then monitored for a period of 90 minutes. Different time intervals were used to obtain the samples (45 and 90 minutes, respectively), from which aliquots of 0.1 mL (direct concentrations) were extracted, subjected to serial dilution, and planted directly in CHROMOagar ECC using the spread plate method. Afterwards, the boxes were incubated at 37°C for 24 ± 2 h, and the resulting bacterial concentration was determined after the application of the disinfectant treatment. The experiment was conducted twice. At the conclusion of the assessment, samples were taken of hot-water-treated mangos, as well as of the water itself, in order to conduct the analysis to determine the amount of disinfectant residue.

##### **IV.8.1 *In vitro* assessment of the mortality constant (K) of the *E. coli* microorganism using copper under adjusted conditions.**

The following minimum effective concentrations were selected for the copper disinfectant in ionic form in water with two qualities: 8.5 ppm at 2 UNT and 12.5 ppm at 50 UNT. The assessment was carried out in the following manner: In a Thermo Scientific Model 2872 thermo bath, equipped with an agitator and a capacity of 26.5 litres, 20 litres of sterile water were added at an adjusted turbidity of 2 UNT or 50 UNT (based on trial specifications), extreme contamination conditions were simulated with the addition of *E. coli* microorganism at a concentration of 10<sup>6</sup> UFC mL<sup>-1</sup>, agitation was applied and the temperature was maintained at 46.1 °C, and the disinfectant under study was added. Once the conditions were adjusted, water samples were taken every 5 minutes during a period of 90 minutes, aliquots of 0.1 mL (direct concentrations) were extracted, subjected to serial dilution, and planted directly in CHROMOagar ECC using the spread plate method (APHA, 2001). Afterwards, the boxes were incubated at 37°C for 24 ± 2 h, and the resulting bacterial concentration was determined after the application of the disinfectant treatment. The survival rate for the *E. coli* microorganism was observed, and graphs showing microbial decay curves as well as the K values were developed.

## IV.9 Statistical Analysis

IV.9.1 Objective 1. Determine the effectiveness of disinfectants on the reduction of *Salmonella enterica* and *Listeria monocytogenes* at each stage of the mango washing process

IV.9.1.1 The effectiveness of disinfectants on the reduction of *Salmonella* and *Listeria* during the mango washing process was analyzed using the Tukey test to obtain the means difference.

IV.9.1.2 The effectiveness of disinfectants on the reduction of *Salmonella* and *Listeria* and during the mango hydrocooling process

In the case of the inactivation kinetics for NaClO and ClO<sub>2</sub> at 2 UNT, these were estimated using the Weibull model for survival curves utilizing non-linear regression, with the MINITAB statistical package.  $\text{Log}_{10}\left(\frac{N_t}{N_0}\right)$  was the dependent variable and t the independent variable.

In the case regarding the effects of the various factors: type of disinfectant, turbidity, and contact time, we proceeded to determine an ANOVA for a random three factor design, with three replicas per treatment. The response variable was bacterial survival expressed as

$$\text{Log}_{10}\left(\frac{N_t}{N_0}\right).$$

The means differences between the treatments that were deemed to be significant ( $p < 0.05$ ) were obtained using the Tukey test ( $P < 0.05$ ).

IV.9.2 Objective 2. Transfer rates

Transfer rates were estimated through the use of descriptive statistical techniques based on three replicas.

IV.9.3 Objective 3. The effectiveness of copper during the hot water treatment.

In order to obtain the (K) values, a linear regression statistical estimate was used where the slope of the survival curve represented the values of the kinetic constant (K). Two repetitions of the experiment were conducted. Once the (K) values were obtained, the Chick kinetic model was applied as follows:

$$\frac{dN(t)}{dt} = -kN(t)$$

Whereby:

N(t) represents the number of cells at time t, and k is the inactivation velocity constant.

## V. Results

This section shows the results of this research broken down by objectives.

### V.1 Objective 1. Results of the trials conducted to determine the efficiency of the disinfectants in reducing *Salmonella enterica* ser. Choleraesuis and *Listeria monocytogenes* during the various stages of the wash process.

#### Tanks

Table two shows the variance analysis for the results related to the bacterial reduction efficiency for sodium hypochlorite and chlorine dioxide in the wash tank under different conditions. The Turbidity, Time, and Disinfectant-Contact Time Interaction factors were shown to be statistically significant with a value of P<0.05.

Table 2. Variance Analysis for the effect of the disinfectant, turbidity, and contact-time factors on the reduction of *Salmonella* Choleraesuis on the surface of the mango fruit in the wash tanks.

| Source                 | GL | SC Ajust. | MC Ajust. | Value F | Value p |
|------------------------|----|-----------|-----------|---------|---------|
| Disinfectant           | 3  | 0.3284    | 0.10946   | 0.64    | 0.594   |
| Turbidity              | 1  | 1.2675    | 1.26750   | 7.42    | 0.010   |
| Time                   | 1  | 8.0688    | 8.06880   | 47.21   | 0.000   |
| Disinfectant*Turbidity | 3  | 1.3281    | 0.44268   | 2.59    | 0.068   |
| Disinfectant*Time      | 3  | 2.5635    | 0.85448   | 5.00    | 0.005   |
| Turbidity*Time         | 1  | 0.0008    | 0.00083   | 0.00    | 0.945   |
| Error                  | 35 | 5.9816    | 0.17090   |         |         |
| No adjustment          | 3  | 0.5990    | 0.19965   | 1.19    | 0.330   |
| Pure error             | 32 | 5.3826    | 0.16821   |         |         |
| Total                  | 47 | 19.5386   |           |         |         |

Starting with the P values obtained through the variance analysis, we proceeded to analyze the interactions between the factors that were observed to be statistically significant, since it was the combination of these that registered the biggest impact on the effectiveness of any of the disinfectants during the entire process. Figure 1 shows us the Disinfectant–Time tendency, which was deemed to be significant, on the reduction of *Salmonella* in the various treatments. On it you can see that the highest treatment was represented by the chlorine dioxide at 5 ppm with a reduction of 1.50 log<sub>10</sub>, whereas the treatment that exhibited a lower reduction was that of the sodium hypochlorite with 0.21 log<sub>10</sub>. The sodium hypochlorite at 200 ppm and the chlorine dioxide at 3 ppm exhibited similar reductions, with 0.83 and 0.75, respectively.

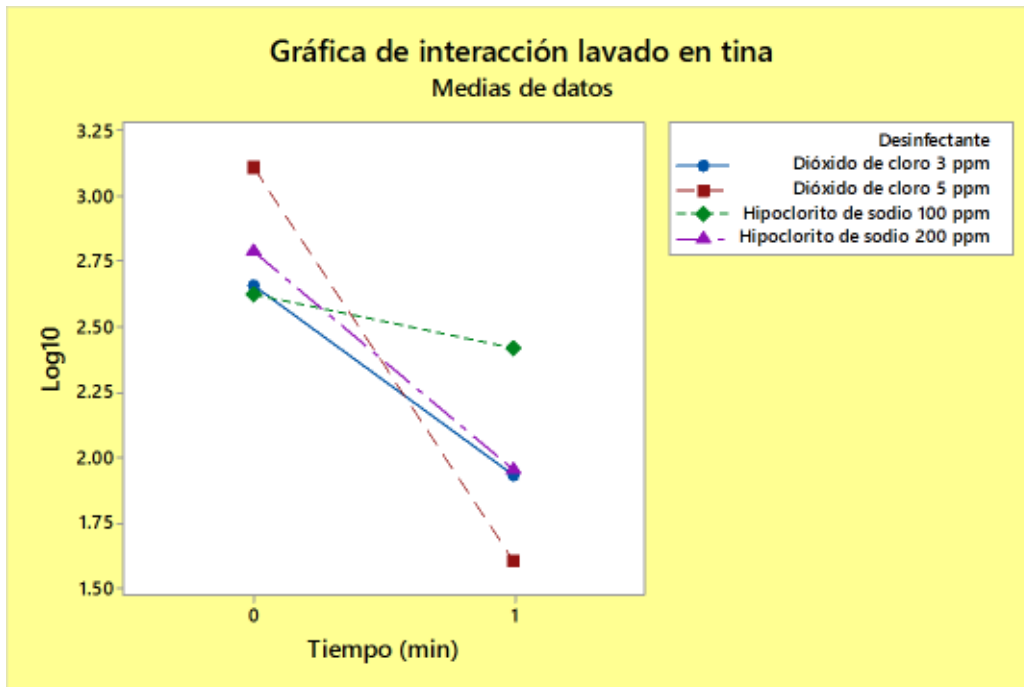


Figure 1. Graph of the effects of the interaction between the Disinfectant–Contact Time factors on the reduction of *Salmonella enterica* ser. Choleraesuis on mangos washed by way of immersion in tanks.

The means comparison for the Disinfectant-Time interaction was carried out using the Tukey test with a 95% confidence interval obtaining the following:

| Disinfectant*Time | Mean    | Group |
|-------------------|---------|-------|
| 5 0               | 3.11167 | A     |
| 200 0             | 2.78500 | A     |
| 3 0               | 2.65667 | A B   |
| 100 0             | 2.62333 | A B   |
| 100 1             | 2.41333 | A B   |
| 200 1             | 1.95167 | B C   |
| 3 1               | 1.92833 | B C   |
| 5 1               | 1.60333 | C     |

The means that do not share a letter are significantly different.

The bacterial reductions in the treatments with turbidity at 50 UNT more lower than those registered at 2 UNT. Given that chlorine reacts with organic material, the organic material can neutralize the disinfectant before it reaches the microbial cells, which would therefore reduce its effectiveness (*Zhang y Farber 1996*). Additionally, any cavities, cracks, or small fissures on the surface of fruit, along with the hydrophobic nature of the waxy cuticle of many fruits and vegetables, can block the chlorine and other sanitizers and keep them from reaching the microorganisms (*Zhang and Farber 1996*).

The results for the treatments with both disinfectants on the reduction of *Listeria monocytogenes* show a percentage of reduction of 100%, which could partially be due to the low initial adhesion of the bacteria to the surface of the fruit and to the fact that *Listeria* showed a greater susceptibility than *Salmonella* to

the disinfectants under study. According to the literature, Gram + bacteria, such as *Listeria*, exhibit less resistance to the disinfectants que Gram - bacteria, such as *Salmonella* (Russell et al. 1997; Maillard, 2002).

### Sprayers

Table 3 shows the variance analysis for the results of the efficiency of sodium hypochlorite and chlorene dioxide on bacterial reduction during the trials conducted with sprayers. The disinfectant and time factors were observed to be statistically significant with a value of  $P < 0.05$ .

Table 3. Variance analysis of the disinfectant and contact time factors and their effect on the reduction of *Salmonella* Choleraeausis on the surface of mangos using sprayers.

| Source            | G.L. | SC Sec. | SC Ajust. | F     | P     |
|-------------------|------|---------|-----------|-------|-------|
| Disinfectant      | 3    | 2.5969  | 0.8656    | 3.49  | 0.040 |
| Time              | 1    | 8.1667  | 8.1667    | 32.92 | 0.000 |
| Disinfectant*Time | 3    | 0.7169  | 0.2390    | 0.96  | 0.434 |
| Error             | 16   | 3.9690  | 0.2481    |       |       |
| Total             | 23   | 15.4495 |           |       |       |

The information, obtained from the treatments applied during the mango wash process using the spraying method, was grouped for the purpose of conducting data analysis using the Tukey method and with 95% confidence showed the following results:

| Disinfectant | Mean    | Group |
|--------------|---------|-------|
| 100          | 3.24667 | A     |
| 200          | 3.24333 | A     |
| 3            | 2.61667 | A     |
| 5            | 2.56000 | A     |

| Time | Mean    | Group |
|------|---------|-------|
| 0    | 3.50000 | A     |
| 20   | 2.33333 | B     |

Valores que comparten la misma letra no presentan diferencias estadísticamente significativas.

The data analysis conducted using the Tukey method shows that there were no statistically significant differences observed between the disinfectants, but the same did not apply to the contact times which were significant. Despite the fact that the bacterial reduction produced by the various disinfectants did not show any significant differences, we will discuss the results of the effects of the principal factors for the purpose of determining practical differences.

The graphs of the principal effects show the trends exerted on the response variable by the treatment factors. In this sense, figure 2 shows that despite the fact that the statistical differences were insufficient to designate both disinfectants as different, they do show that the bacterial reductions registered by the sodium hypochlorite treatments were inferior to those registered by the chlorine dioxide treatments

during the mango wash process using the spraying method. According to the results shown for *Salmonella*, the chlorine dioxide at 5 ppm was determined to be the best treatment producing a final bacterial concentration of 2.56 Log<sub>10</sub>, which represents a reduction of 0.94 log<sub>10</sub>, whereas the sodium hypochlorite at 100 ppm was determined to be the less effective treatment with 3.24 Log<sub>10</sub>, representing a reduction of 0.25 log<sub>10</sub>. With regard to the contact time factor, which was determined to be statistically significant, it showed a bacterial reduction of 1.16 log<sub>10</sub> during the 20 s of treatment with the disinfectant.

In addition, the results for *Listeria monocytogenes* showed a 100% bacterial reduction for both disinfectants. Once again, the low initial adhesion capacity of *Listeria monocytogenes* to the surface of the mango, as well as its sensitivity to the disinfectants that were tested, contributed to the total elimination of this pathogen during this procedure.

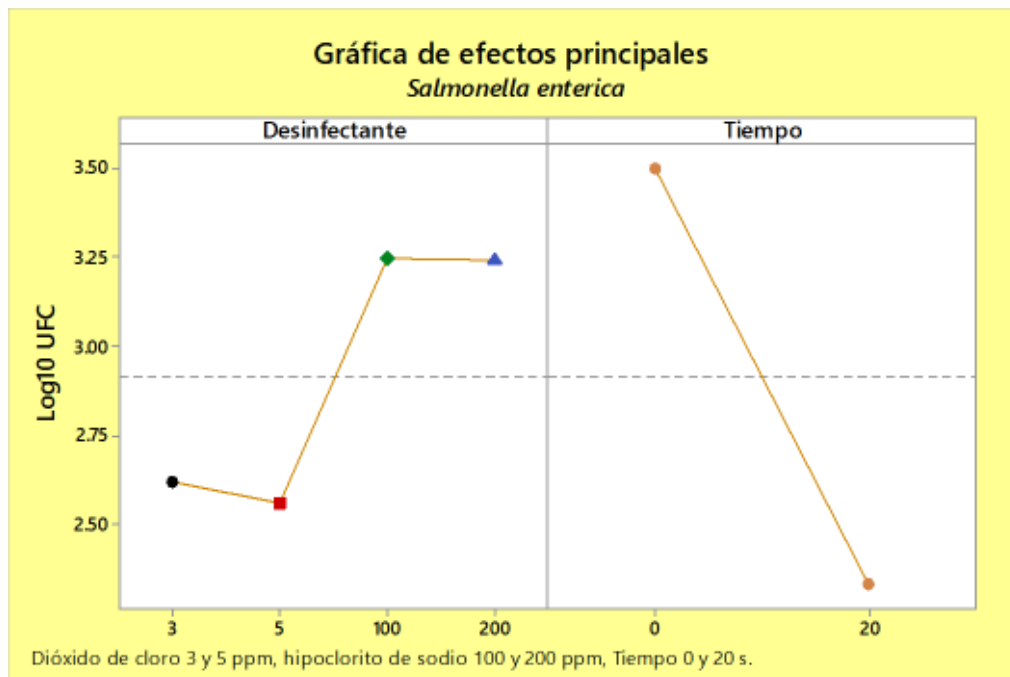


Figure 2. Graph showing the principal effects derived from the disinfectant and contact time factors on the reduction of *Salmonella enterica* ser. Choleraesuis on mangos washed using the spraying method.

The comparison of both wash methods, that is, immersion in tanks (2 UNT) and spraying, in the application of the disinfectant proved to be effective and without any differences for the reduction of *Listeria monocytogenes* on the order of 100%. However, in the case of *Salmonella*, the immersion in the tank registered a slight difference registering greater reductions, possibly due to an extended contact time period, than the spraying method. Nevertheless, these were not statistically significant.

The use of the spraying method can favor reduction in cases, where fruit surfaces have bacteria attached, due to the dragging action exerted by the pressure of the water sprayer with the addition of the disinfectant used in this wash process. Moreover, in the spray wash method, the fact that the water is not reused favors the oxidizing power of the disinfectant, since it doesn't permit the accumulation of organic material.



Additionally, as we mentioned previously, the morphology of the fruit that can exhibit irregularities such as cavities, cracks, and small fissures on the surface of the fruit, along with the hydrophobic nature of the waxy cuticle of many fruits and vegetables, can block the chlorine and other sanitizers and keep them from reaching the microorganisms (*Zhang and Farber 1996*), which is why it's important to point out that, generally speaking, in fresh produce packing houses, the use of the spraying method is accompanied by the use of brushes that are attached to the conveyor belts that move the fruit, and that remove the soil and organic material, so that in this way the disinfectants can exert their maximum action on the microbial cells, and not on the residue. In this sense, *Adama et al (1989)* and *Zhang and Farber (1996)* mentioned that the use of detergents, disinfectants, and other products coupled to some form of physical handling such as brushing, can be used to lower the hydrophobicity and remove part of the wax to increase the exposure of the microorganisms to the sanitizers.

### **Hydrocooling**

For this section related to the hydrocooling treatment, the results were analyzed based on two different perspectives:

- 1) Bacterial inactivation kinetics, where an analysis was conducted of the amount of time required for the effect of the disinfectant to produce a bacterial reduction of 1 Log<sub>10</sub>, that is, of 90% of the bacterial population.
- 2) The effect of the disinfectant, turbidity, and contact time factors on the reduction of bacterial survival.

### **1) Bacterial Inactivation Kinetics**

#### **Inactivation kinetics for *Salmonella Choleraesuis*.**

The application of non-linear regression analysis, based on the Weibull model, to determine the survival of *Salmonella Choleraesuis* using chlorine dioxide yielded the following estimated parameters:

| Parameter                  | Estimate | Estimate Error |
|----------------------------|----------|----------------|
| <b><math>\alpha</math></b> | 0.00015  | 0.00116        |
| <b><math>\beta</math></b>  | 0.15081  | 0.11240        |

The lack of adjustment to the model did not yield any significant results ( $P = 0.844$ ) (Table 4), therefore, we can affirm that the estimated Weibull model is adequate.

Table 4. ANOVA test to adjust the model using chlorine dioxide.

| Source             | Degrees of freedom | Sum of squares | Mean squares | F value | P value |
|--------------------|--------------------|----------------|--------------|---------|---------|
| Error              | 6                  | 2.16071        | 0.36011      |         |         |
| Lack of adjustment | 1                  | 0.01844        | 0.01844      | 0.04    | 0.844   |
| Pure Error         | 5                  | 2.14227        | 0.42845      |         |         |

The estimated prediction model is

$$\text{Log}\left(\frac{Nt}{No}\right) = -\frac{1}{2.303} \times \left[\left(\frac{\text{Tiempo}}{0.00015}\right)\right]^{0.15081} \quad (4)$$

Solving Equation (4) for the variable *Time*, we can estimate the time at which we achieve a reduction of 1 Log<sub>10</sub> in survival for *Salmonella* using chlorine dioxide at 5 ppm, and which occurs at 0.39 min. The graph of the inactivation kinetics is shown in Figure 3.

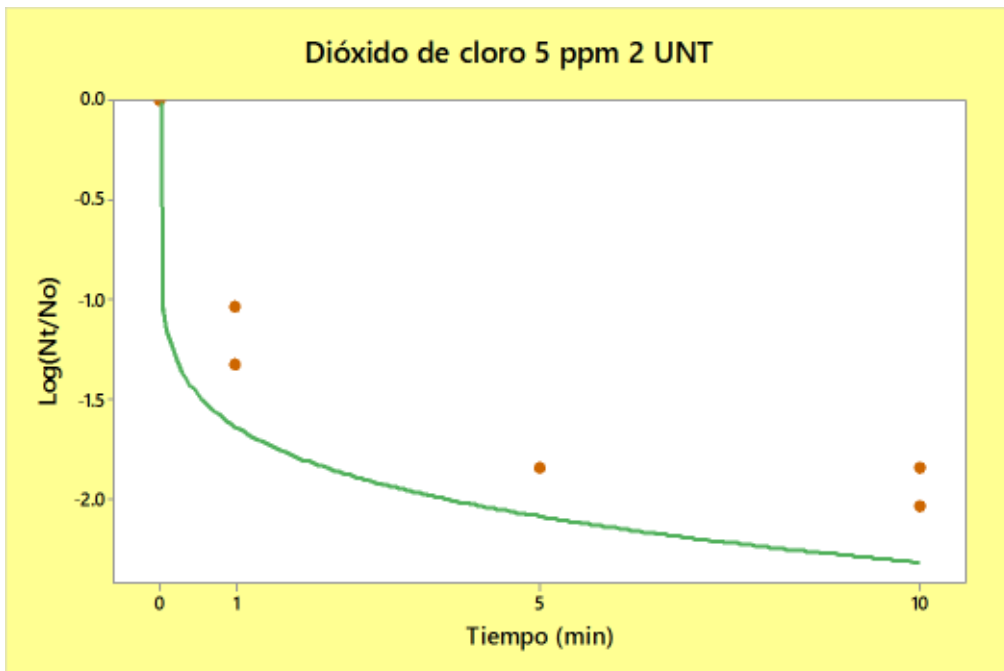


Figure 3. Disinfection kinetics for *Salmonella Choleraesuis* using chlorine dioxide at 5 ppm

Other research using inactivation kinetics, such as *Mahmoud et al., 2007* achieved a reduction of 1 Log<sub>10</sub> UFC in 2.7 min on the surface of strawberries, with chlorine dioxide gas at 5 ppm, and *Mahmoud et al., 2008*, achieved a reduction of 1 Log<sub>10</sub> UFC of *Salmonella* Poona in 1.5 min per 5 cm<sup>2</sup> on cantaloupe melons using chlorine dioxide gas at 5 ppm. This research was able to achieve a reduction of 1 Log<sub>10</sub> of *Salmonella* Choleraesuis in 0.39 min, demonstrating that the cause for this divergence could be attributed to the fact that chlorine dioxide in aqueous form was used for the inactivation of *Salmonella* Choleraesuis adhered to the mangos, and that the microbial adhesion is related to the type of surface for each fruit, since there are differences between the cuticles of strawberries, melons, and mangos, which allow for greater reductions of microorganisms on mangos due to their smoother surface, and the application of the disinfectant in aqueous, instead of gaseous, form results in better contact with the entire surface of the fruit.

Alternatively, the application of non-linear regression analysis, based on the Weibull model, to determine the survival of *Salmonella* Choleraesuis using sodium hypochlorite yielded the following estimated parameters:

| Parameter | Estimate | Estimate Error |
|-----------|----------|----------------|
| <b>A</b>  | 0.02807  | 0.06028        |
| <b>B</b>  | 0.13521  | 0.05598        |

The lack of adjustment to the model did not yield any significant results ( $P = 0.608$ ) (Table 5), therefore, we can affirm that the estimated Weibull model is adequate.

Table 5. ANOVA test to adjust the model using sodium hypochlorite.

| Source             | Degrees of freedom | Sum of squares | Mean squares | F value | P value |
|--------------------|--------------------|----------------|--------------|---------|---------|
| Error              | 4                  | 0.04302        | 0.01076      |         |         |
| Lack of adjustment | 1                  | 0.00421        | 0.00421      | 0.33    | 0.608   |
| Pure error         | 3                  | 2.14227        | 0.42845      |         |         |

The estimated prediction model is

$$\text{Log} \left( \frac{Nt}{No} \right) = -\frac{1}{2.303} \times \left[ \left( \frac{\text{Tiempo}}{0.02807} \right) \right]^{0.13521} \quad (5)$$

Solving Equation (5) for the variable *Time*, we estimated a lower reduction in survival of 1 Log<sub>10</sub> at 13.42 min using sodium hypochlorite at 200 ppm, which demonstrates that this microorganism was adhered to the surface of the mango and the roughness of the cuticle protected it from the disinfectant's action. The graph of the inactivation kinetics is shown in Figure 4.

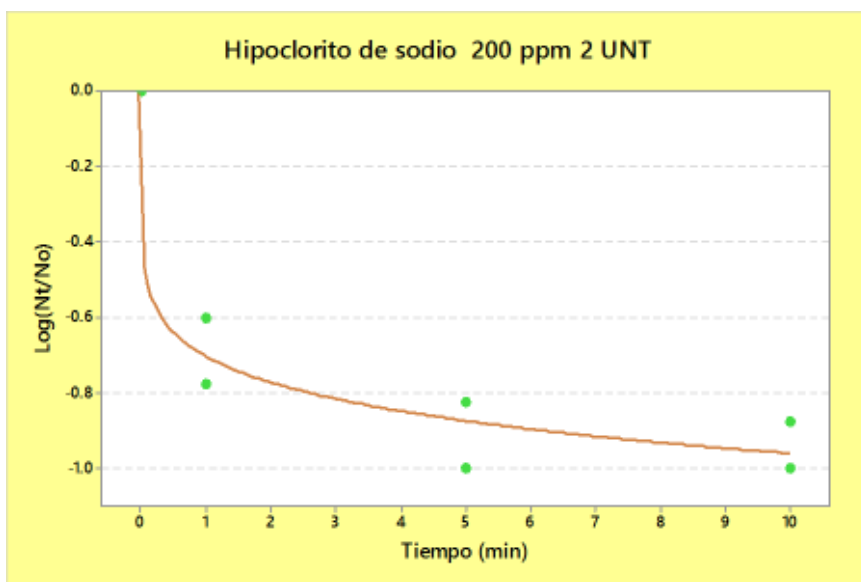


Figure 4. Disinfection kinetics for sodium hypo chlorite at 200 ppm for *Salmonella Choleraesuis*.

The reduction in survival of 1  $\text{Log}_{10}$  of *Salmonella Choleraesuis* at 13.42 min using sodium hypochlorite at 200 ppm demonstrates that this microorganism that is adhered to the surface of the fruit is less affected by this disinfectant, and that the chlorine dioxide at 5 ppm was a faster and better disinfection alternative for *Salmonella Choleraesuis*.

Other work carried out with *Salmonella Typhimurium* adhered to the surface of mangos and mediated with chlorinated products at 200 ppm showed a reduction of 2.15  $\text{Log}_{10}$  UFC  $\text{cm}^{-2}$  in 10 min (Fernandes et al., 2014). Additionally, authors such as (Richards and Beuchat, 2004; Ukuku and Fett, 2006) reported on the reduction of 1-2  $\text{Log}_{10}$  UFC on fresh produce washed in chlorinated water at 200 ppm.

#### **Inactivation kinetics for *Listeria monocytogenes*.**

The inactivation kinetics for *Listeria monocytogenes* could not be estimated, since in the case of both disinfectants, the complete reduction of the microorganism was produced in less than one minute of contact. This could be explained by the weak adherence that *Listeria monocytogenes* exhibited with the surface of the mango fruit.

#### **2) Effect of the disinfectant, turbidity, and contact time factors on the reduction of bacterial survival.**

The following shows the variance analysis, means comparison of the effects that were deemed statistically significant, and a graphical analysis of the factors.

### Analysis of the results for *Salmonella Choleraesuis*

Table 6 shows that the effects related to contact time, type of disinfectant, and turbidity\*disinfectant and time\*disinfectant factors were statistically significant ( $P < 0.05$ ).

Table 6. Variance analysis (ANOVA) of the reduction of *Salmonella Choleraesuis* using chlorine dioxide at 3 and 5 ppm and sodium hypochlorite at 100 and 200 ppm.

| Source                 | G.L. | SC Sec. | SC Ajust. | F     | P     |
|------------------------|------|---------|-----------|-------|-------|
| Turbidity              | 1    | 0.2240  | 0.2240    | 0.11  | 0.746 |
| Time                   | 3    | 30.6136 | 10.2045   | 48.01 | 0.000 |
| Disinfectant           | 3    | 10.7068 | 3.5689    | 16.79 | 0.000 |
| Turbidity*Time         | 3    | 0.1743  | 0.0581    | 0.27  | 0.844 |
| Turbidity*Disinfectant | 3    | 3.5071  | 1.1690    | 5.50  | 0.002 |
| Time*Disinfectant      | 9    | 4.8687  | 0.5410    | 2.54  | 0.013 |
| Lack of adjustment     | 9    | 1.9017  | 0.2113    |       |       |
| Error                  | 64   | 13.6152 | 0.2127    | 0.99  | 0.455 |
| Total                  | 95   | 65.4099 |           |       |       |

$R^2 = 76.28\%$ , GL: degrees of freedom. SC Sec: sum of squares, SC Ajust: sum of adjusted squares, F: statistical test values F and P: level of significance.

### Principle effects for *Salmonella Choleraesuis*

Figure 5 shows the averages of the principle effects for the treatments as well as the results of the means comparison. With regard to the disinfectants, the chlorine dioxide at 5 ppm produced the highest reduction ( $0.96 \text{ Log}_{10}$ ), whereas the sodium hypo chlorite at 100 ppm proved to be the least effective ( $0.62 \text{ Log}_{10}$ ). There were no differences observed between the chlorine dioxide at 3 ppm and the sodium hypochlorite at 100 ppm. With regard to contact time, the reduction in microbial survival yielded different results at the 0, 1 and 5 min marks (Tukey,  $P < 0.05$ ), with no statistically significant differences found between the results at 5 and 10 minutes, showing logarithmic reductions at the 0, 1, 5 and 10 min marks of 0.0, 0.62, 1.15 and 1.49  $\text{Log}_{10}$ , respectively. The turbidity was not significant.

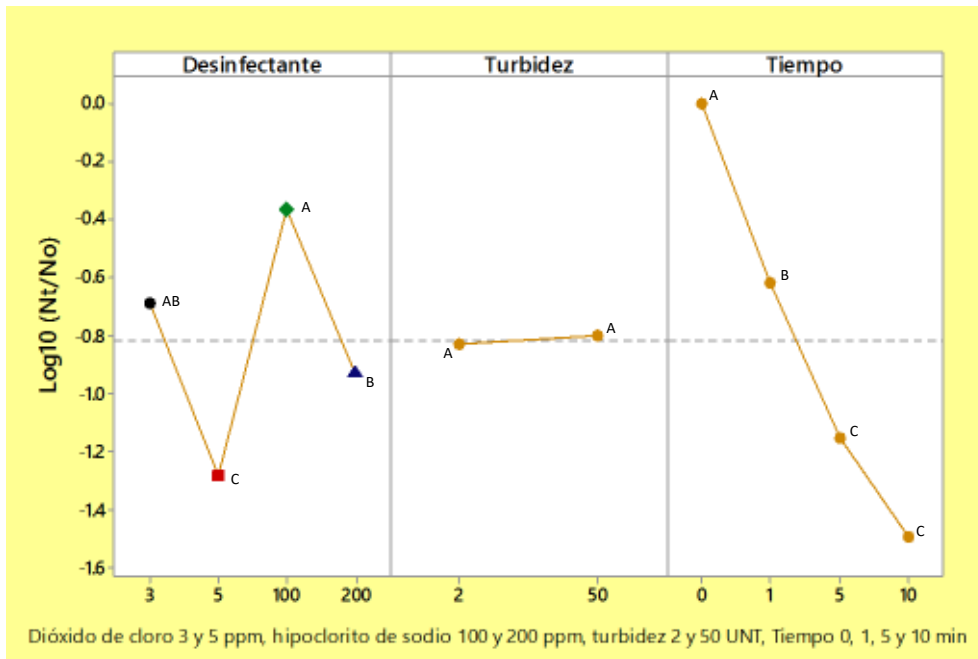


Figure 5. Principal effects of the Time\*Disinfectant\*Concentration factors on *Salmonella Choleraesuis*. Different letters indicate significant differences ( $p < 0.05$ ), based on the Tukey test. Average values for three repetitions  $\pm$  Standard deviation.

### The effects of the Turbidity\*Disinfectant interaction on *Salmonella Choleraesuis*

Figure 6 shows the results of the effects of the interactions between Turbidity and Disinfectant Type. The chlorine dioxide at 3 ppm and 2 UNT yielded significantly lower results ( $P < 0.05$ ) compared with the other combinations of factors at these levels on the reduction of the survival of *Salmonella Choleraesuis*. In the case of sodium hypochlorite, some significant differences were found ( $P < 0.05$ ) between the 100 and 200 ppm concentrations at 50 UNT, though that wasn't the case for the other combinations of factors at these levels. In general, chlorine dioxide at 5 ppm and 2 y 50 UNT and sodium hypochlorite at 200 ppm and 50 UNT yielded the best treatment results for the reduction of the logarithmic survival of *Salmonella Choleraesuis*.

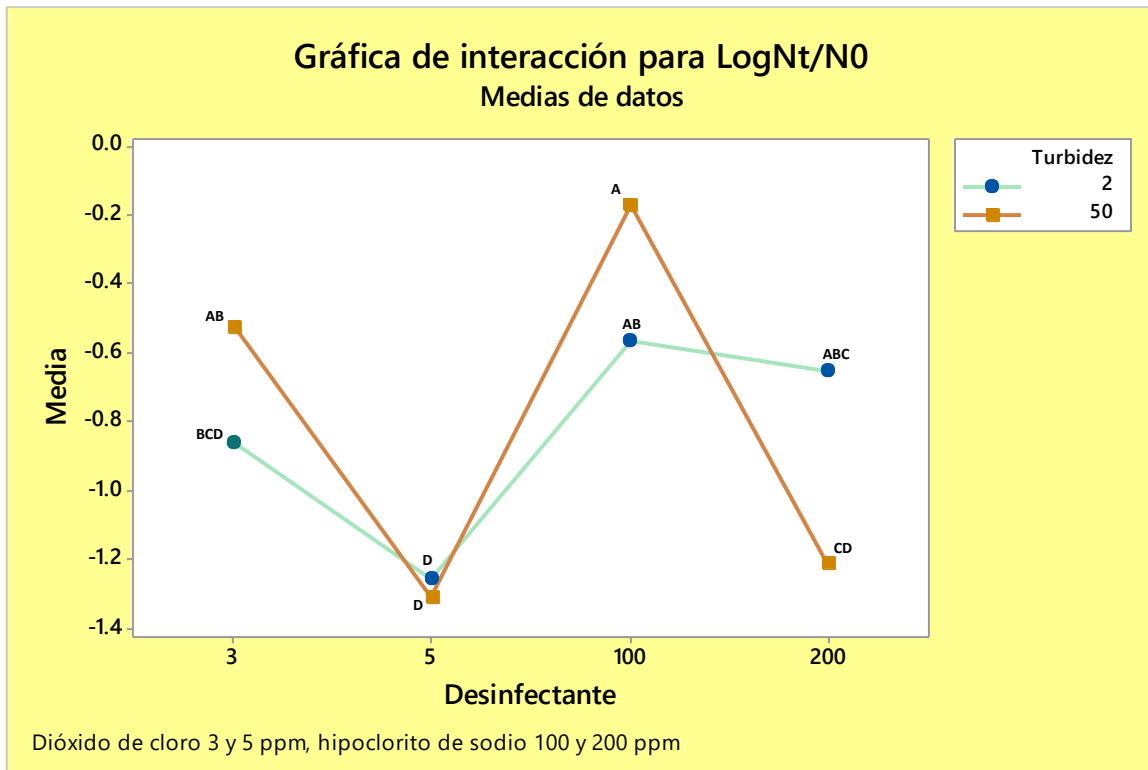


Figure 6. The effects of the Turbidity\*Disinfectant interaction on *Salmonella Choleraesuis*. Different letters indicate significant differences ( $P < 0.05$ ), based on the Tukey test. Average values for 3 repetitions  $\pm$  Standard deviation.

The results shown in Figure 6 can be explained by pointing out that the turbidity at 50 UNT exacts a greater demand for disinfectant than the turbidity at 2 UNT, due to the fact that the presence of organic material, as well as other compounds, in the water react with the oxidizing power of the disinfectant, in addition to the microorganisms. In this sense, the higher concentrations of the disinfectants considered in this study (chlorine dioxide at 5 ppm and sodium hypochlorite at 200 ppm) were able to provide coverage for the required demand by these noncellular compounds, leaving some availability to resolve the demand for sufficient disinfectant to inactivate the bacteria present in the sample. Contrary to this, the lower concentrations of disinfectant (chlorine dioxide at 3 ppm and sodium hypochlorite at 100 ppm) were not sufficient to supply the required oxidant of these two elements present in the water at 50 UNT, affecting the logarithmic reduction of *Salmonella Choleraesuis*.

The turbidity in the water is made up of inorganic (example: mud, clay, iron oxides) and organic material, as well as microbial cells (*Silverman et al. 1983; LeChevallier et al. 1988*). Factors such as the amount of organic material surrounding the white organisms probably influence the adhesion characteristics of the cells and weaken the lethal effect of the disinfectants.

## The effect of the Time\*Disinfectant interaction on *Salmonella Choleraesuis*

Figure 7 shows that chlorine dioxide at 5 ppm was the best disinfectant registering reductions of 1.128, 1.867, 2.12 Log<sub>10</sub> at 1, 5 and 10 minutes of contact time, respectively. The sodium hypochlorite at 100 ppm proved to be the least effective treatment, registering values of 0.104, 0.559, 0.805 Log<sub>10</sub> at 1, 5 and 10 minutes, respectively. Alternatively, an analysis of the disinfectants for each contact time period revealed that at the 1 and 5 minute marks only the chlorine dioxide at 5 ppm exhibited a statistically significant difference, meaning that the concentration of this disinfectant was the one that resulted in the greatest reductions during those contact time periods, whereas at the 10 minute mark only the sodium hypochlorite at 100 ppm registered a statistically significant difference, meaning that it was the treatment that registered the lowest reductions at the 10 minute mark of the contact time period, while the other three did not register any statistically significant results. These differences were calculated using the Tukey test.

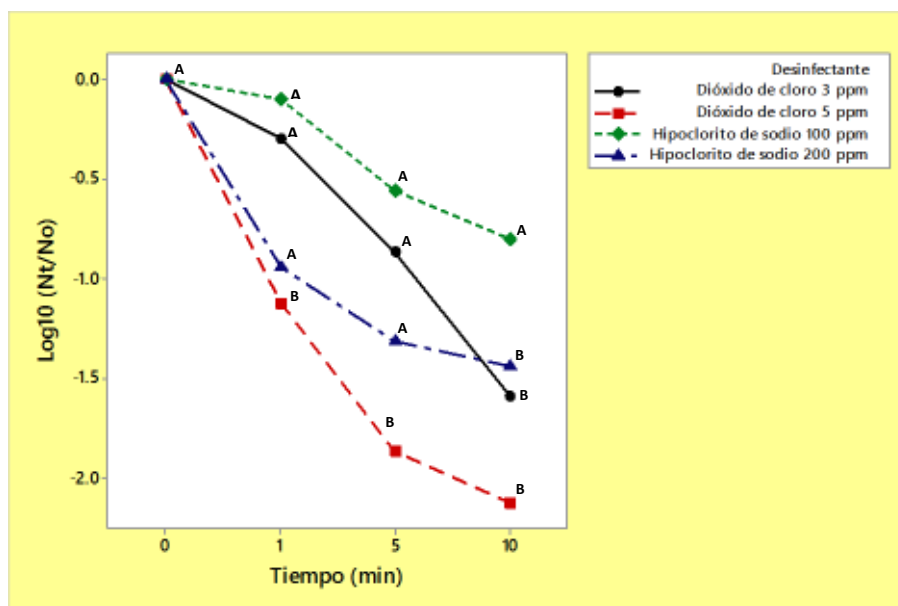


Figure 7. Effects of the Time\*Disinfectant interaction for *Salmonella Choleraesuis*. Different letters indicate significant differences ( $p < 0.05$ ) based on the Tukey test. The means comparison was conducted for each time period (columns). Average values for three repetitions  $\pm$  standard deviation.

The chlorine dioxide exhibited a greater logarithmic reduction of *Salmonella Choleraesuis* due to the fact that chlorine dioxide has 2.5 times more oxidizing power than the hypochlorous acid, which makes it more effective during the disinfection process.

According to the EPA (1997) an antimicrobial agent is one that reduces pathogens by 2 Log UFC g<sup>-1</sup> o mL<sup>-1</sup>, therefore, chlorine dioxide at 5 ppm represents an alternative for the disinfection process, since it registers a reduction of 2.24 Log<sub>10</sub> for *Salmonella Choleraesuis*.

The results show that the disinfectants that are the objects of this study were effective against the microorganism strains that were tested in the trial. This is because their composition is made up of chlorine compounds with an action mechanism that inhibits essential enzymes when the thiol groups



are oxidized (S-H). Additionally, these can also cause changes in the permeability of the cell membrane (Henao S, 2003).

### **Analysis of the results for *Listeria monocytogenes***

The concentration of *Listeria monocytogenes* that adhered to the surface of the mango was low and was eliminated in a few seconds by all of the disinfectants that were tested, precluding the collection of any data, during the prescribed contact times set up to conduct the statistical analysis of the experiment. This can be attributed to the low adhesion capacity and low transfer rate of the pathogen from the water to the fruit (Objective 2). For all these reasons, we can conclude that *Listeria monocytogenes* is not a relevant pathogen for the mango hydrocooling process.

### **V.2 Objective 2. Transfer of *Salmonella* and *Listeria monocytogenes* from the fruit to the water and from the water to the fruit**

During this trial, we proceeded to inoculate the mango fruit with a known concentration of the bacteria under a study to simulate the arrival of contaminated fruit to the packinghouse, and assess the removal and transfer effect of the bacteria to the tank, in an attempt to duplicate the conversion of this input into a constant contamination vector.

Accordingly, we proceeded to inoculate the water contained in the tank with a known concentration of the bacteria under study in order to simulate the arrival of contamination-free fruit to the packing house and expose them to contaminated water, doing so in an effort to assess the transfer effect of the bacteria to the fruit.

As referenced in Table 7, with regard to the control treatments, absent the application of the disinfectants, the transfer rates for *Salmonella* and *Listeria* from the fruit to the water were 37.45 and 0.00%, respectively. Whereas, inversely, the transfer rate was greater, registering values of 49.17 and 11.20% for *Salmonella* and *Listeria*, respectively. These results demonstrate that the bacterial transfer episodes with *Salmonella enterica* ser. Choleraesuis exhibit a greater likelihood in both directions during the washing process than those that occur with *Listeria monocytogenes*, which were reduced to zero from the fruit to the water.

Transfer rates for bacteria are directly impacted by their capacity to adhere to the surface of the fruit. In this sense, *Salmonella* Choleraesuis demonstrated a greater capacity to adhere to the surface of the mango than *Listeria monocytogenes*. This was corroborated after each bacterial transfer trial, where the first step was to inoculate the surface of the fruit with each bacteria under study, allowing for a 1 hour (up to 4 hours for listeria) period for drying in order to assist the adhesion process. Nevertheless, these adhesions were lower for the *Listeria* ( $0.79 \log_{10}$ ), which is indicative of the low capacity that this bacteria has to adhere to the surface of mangos.

In the case of the bacterial transfer rates using sodium hypochlorite as a disinfectant, the results show that the bacterial transfer did not occur in either scenario. *Listeria*, for its part, due to its low capacity to adhere to the surface of the mango in conjunction with the action of the disinfectant, exhibited a zero transfer rate from the fruit to the water. It's worth mentioning that when conducting the treatment to determine the transfer rate of *Salmonella* from the fruit to the water in the presence of chlorine

hypochlorite in the water contained in the tank, the residual bacterial population was quantified after the treatment and determined to be 29.37%, which is equivalent to a reduction of 0.47 Log<sub>10</sub>, and indicates that, although there was no transfer of bacteria to the water, a certain amount of bacteria population did remain on the surface of the mango, demonstrating that bacterial adhesion to surfaces makes them more resistant to various factors, including disinfectants, since the interaction with these can offer protection due to the bacterial positioning in structures or cavities on the fruit that offset the oxidizing action of the disinfectant.

The behavior observed in the null bacteria transfers, from the water to the fruit and from the fruit to the water, can be explained by the inactivation of the bacterial contamination in the water, which contained 2 UNT, after the application of the disinfectant, which also precluded the transfer from happening in both directions. It should be noted that the low turbidity (2 UNT) is favorable to the action of any disinfectant, and does not represent a barrier to their full effect, which is why we recommend caution and vigilance when it comes to this parameter (Turbidity) in the wash tanks under real conditions, since the slightest increase will have a direct and adverse effect on the microbicide action of any disinfectant.

| <b>Table 7. Transfer Rates from the Water to the Fruit and from the Fruit to the Water for <i>Salmonella Choleraesuis</i> y <i>Listeria monocytogenes</i></b> |                                      |                                      |                            |                            |
|---|--------------------------------------|--------------------------------------|----------------------------|----------------------------|
| <b>Bacteria</b>   | <b>From fruit to water (100 ppm)</b> | <b>From water to fruit (100 ppm)</b> | <b>From fruit to water</b> | <b>From water to fruit</b> |
| <b><i>Salmonella Choleraesuis</i></b>   | 0.00                                 | 0.00                                 | 37.45                      | 49.17                      |
| <b><i>Listeria monocytogenes</i></b>  | 0.00                                 | 0.00                                 | 0.00                       | 11.20                      |

\* Transfer rates are expressed in %

*Salmonella*, a Gram negative bacteria, has two lipidic membranes with a fine cell wall of peptidoglycan located in between, in addition to containing lipopolysaccharides, which are complex polymers with fatty acid residue as a lipophilus and characteristic oligosaccharides and polysaccharides chains that make up the external membrane of these bacteria (Maier et al., 2009). The characteristics pertaining to its cellular wall equip it with adhesive properties which are controlled by proteins, liposaccharides, and lipoteichoic acids.

*Listeria*, a Gram positive bacteria, exhibits only a lipidic membrane and a thick peptidoglycan wall, which negatively charged teichoic acids bond to, contributing to the negative charge of the bacterial cell wall (Maier et al., 2009). The deficient microorganisms in liposaccharides show a reduction in surface hydrophobicity and reduce the capacity to adhere to hydrophilic surfaces (Daved y O'Toole, 2000) like the surface of a mango. Given the low transfer rate exhibited by *listeria monocytogenes*, it is conceivable to consider that this pathogen does not represent a serious microbiological contamination risk during the mango hydrocooling process.

In this study, the transfer rate from the water to the fruit was greater than the transfer rate from the fruit to the water, which coincides with that reported by other authors such as Holvoet et al. (2014);

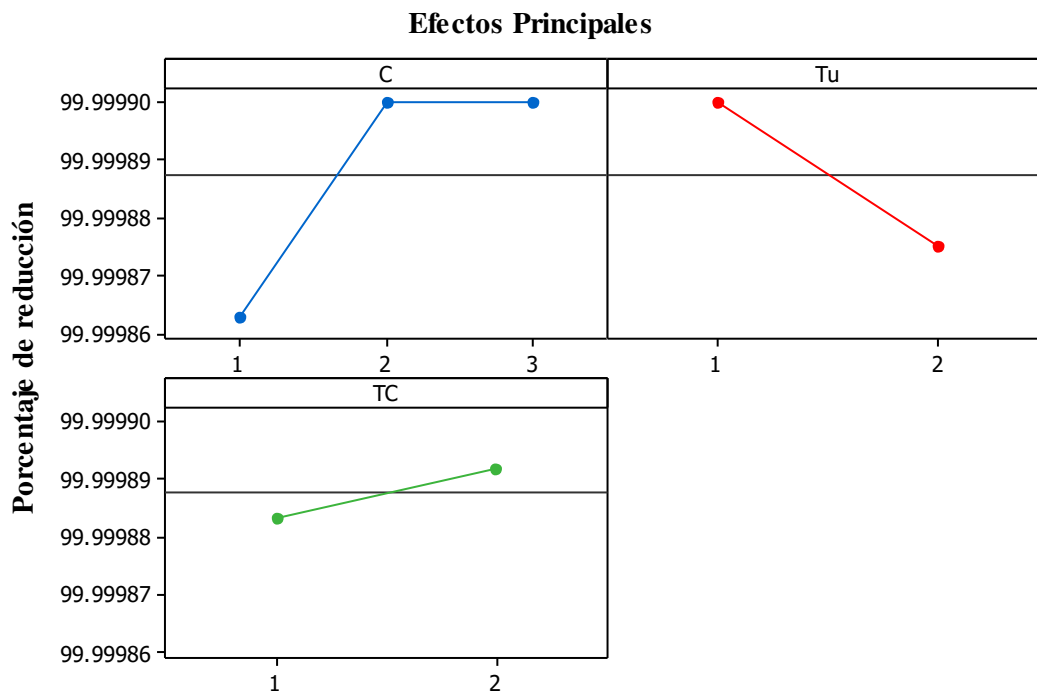
Allende et al. (2008) and Rana et al. (2010) where they describe that the transfer rate of microorganisms from the water to the product occurs in greater proportion, positing that although water is a useful tool for reducing contamination, the use of this resource with inadequate microbiological quality measures has the potential of becoming a direct source of contamination, as well as a vehicle for bacterial transfer.

These results emphasize the inherent vulnerability of the hydrocooling processes to microbial contamination when a disinfectant is not used, or when adequate monitoring is not carried out throughout the work day. The principal effect of a disinfectant is to reduce and control the microbial load contained in the water and prevent the transfer of microorganisms.

**V.3 Objective 3. Trial results for the assessment of copper as a disinfectant in a hot water treatment simulator, using varying degrees of water quality.**

According to the study of the copper-based disinfectant, the ANOVA analysis indicated that there are no significant differences in the turbidity, concentration, and contact time factors. However, the results related to the principal effects are discussed for the purpose of determining practical differences.

Principal effects: Concentration, Turbidity, and Contact Time, on the efficiency of microbiological reduction of *E. coli*, under simulated conditions of hot water treatment (Figure 8).



Tu1 = 2 UNT  
Tu2 = 50 UNT

TC1= Contact time 45 min.  
TC2= Contact time 90 min.

C1 = 8.5 ppm  
C2 = 12.5 ppm  
C3 = 17.0 ppm

Figure 8. Graph showing the principal effects during the hot water treatment.

In addition, the concentrations of the copper disinfectant at 8.5 ppm, 12.5 ppm and 17 ppm were observed to be efficient, and said efficiency was not affected by the turbidity or contact time. However, the two highest concentrations resulted in effects that could not be differentiated. Though the turbidity exhibited a slight difference, it was not deemed to be statistically significant, however, with regard to the contact time, the *E. coli* registered a reduction of 5 log<sub>10</sub> at the 45 minute point. All of which indicates that copper as a disinfectant is efficient when it comes to the microbial reduction of *E. coli*.

Additionally, we proceeded to register the residuality of the copper in the water for the purposes of having a broader understanding of the potential control that copper may have in these scenarios. As seen in Table 8, when the copper is exposed to a higher turbidity its residuality goes down due to the effect that the disinfectant exerts on the organic matter when it reacts with it.

| <b>Table 8. Residuality of copper in water after 90 minutes</b> |                    |               |
|---|--------------------|---------------|
| <b>Concentration<br/>Copper<sup>1</sup></b>                     | <b>Residuality</b> |               |
|   | <b>2 UNT</b>       | <b>50 UNT</b> |
| <b>8.5</b>  | 6.83               | 5.66          |
| <b>12.5</b>   | 9.31               | 6.52          |
| <b>17.0</b>   | 13.77              | 11.35         |

<sup>1</sup>ppm

When evaluating the residuality of the copper in the water at the end of the 90 minute period, provided for in the hot water treatment protocol, we observed that it lost approximately 30% of its initial concentration when the turbidity increased.

#### **Decriptive results for (K) values and removal time for *E. coli*.**

A comparison of the average values of the microbial mortality constant (K) shows that despite the increase in the concentration of the disinfectant which in turn produced a higher turbidity, the average K values were observed to go down, which indicates that the presence of organic material lowers bacterial mortality since the active effect of the copper is diminished.

| <b>Table 9. Time required for the removal of <i>E. coli</i> through the use of copper</b> |             |                            |                         |                         |
|---|-------------|----------------------------|-------------------------|-------------------------|
| Removal of <i>E. coli</i>   |             |                            | Time required (minutes) |                         |
| %   | Units in Ln | Units in Log <sub>10</sub> | k <sup>a</sup> = 0.4334 | k <sup>b</sup> = 0.3994 |
| <b>90</b>   | 2,30258509  | 1                          | 5.31                    | 6.17                    |
| <b>99</b>   | 4,60517019  | 2                          | 11.03                   | 11.53                   |
| <b>99.9</b>   | 6,90775528  | 3                          | 16.34                   | 17.30                   |
| <b>99.99</b>  | 9,21034037  | 4                          | 21.25                   | 23.06                   |
| <b>99.999</b>   | 11,5129255  | 5                          | 26.56                   | 29.23                   |
| <b>99.9999</b>  | 13,5129255  | 6                          | 32.28                   | 34.59                   |
| <b>99.99999</b>   | 16,1180957  | 7                          | 37.19                   | 40.36                   |
| <b>99.999999</b>  | 18,4206807  | 8                          | 42.50                   | 46.12                   |

K<sup>a</sup> : Mortality constant for *E. coli* at 2 UNT and 8.5 ppm of copper

K<sup>b</sup> : Mortality constant for *E. coli* at 50 UNT and 12.5 ppm of copper

Table 9 shows us the amount of time required to remove X number of logarithmic units of *E. coli*, where one unit represents 90% removal, two units represent 99% removal, three units represent 99.9% removal, and so on. Therefore, if the initial concentration of *E. coli* in the water used for the hot water treatment is 7 logarithms and a final concentration of 1 logarithm is desired, 6 logarithms will need to be removed, which represents 99.9999%. This indicates that 32.28 minutes will be required in order for the copper at a concentration of 8.5 ppm and 2 UNT to be able to achieve that removal, and 2.31 minutes more, when the concentration is 12.5 ppm at 50 UNT. Nonetheless, the efficiency of copper as a disinfectant is achieved prior to the 90 minutes established in the hot water treatment protocol.

In this study, we demonstrate that the presence of the copper as a disinfectant at 2 UNT has A significant effect on the survival of *E. coli* registering 90% of reduction in 5.31 minutes at 2 UNT. However, when the contact time with the disinfectant is shortened and the turbidity in the water used for the hot water treatment is increased, it lowers the action for the copper, though not significantly. One of the most relevant advantages of using copper during the hot water treatment process for mangos is its stability when exposed to high temperatures, particularly when in some instances the presence of temperature tolerant bacteria such as *Escherichia coli* could occur.

#### **V.4 Objective 4. Results for the determination of the most effective disinfectant for the control of *Salmonella enterica* ser. Choleraesuis and *Listeria monocytogenes* during the various stages of the mango washing process in the mango packinghouse.**

The disinfectant considered to be the most effective at controlling and ensuring microbial quality during the various steps of the mango washing process inside the packing house, was dependent on the method with which it was applied. If, in fact, there were no statistically significant differences in the percentages of bacterial reduction between the immersion method (2 UNT) and the spraying method, the quantification of the bacterial populations of the treatment showed a slight difference which could be crucial for the elimination of a pathogen. In this sense, chlorine dioxide at 5 ppm registered the maximum bacterial reduction compared to the rest of the treatments using both washing methods, with a percentage of bacterial reduction of 50.08 and 42.46%, both in tank immersion and spraying, respectively.

The inactivation kinetics of *Salmonella enterica* ser. Choleraesuis mediated with chlorine dioxide and sodium hypochlorite were observed to reach a bacterial reduction of 1 Log<sub>10</sub>, that is, 90% of the bacterial population, in 0.39 and 13.42 minutes, respectively.

In summary, chlorine has been used extensively in the food industry for the elimination of pathogenic bacteria. Some examples of this includes the washing of fruits and vegetables as well as the disinfection of contact surfaces. Nevertheless, due to some concerns regarding the environmental and public health risks associated with the formation of dangerous byproducts from the disinfection process (primarily trihalomethanes), the industry has been under intensifying pressure to look for other alternatives to this disinfectant. In fact, the use of chlorine for the disinfection of fresh produce is currently prohibited in some European countries including Germany, Holland, Switzerland, and Belgium, with this being the trend for this disinfectant in the food industry.

## VI. Conclusions

This research project demonstrated that *Salmonella enterica* ser. Choleraesuis possesses a greater capacity to adhere to the surface of mango fruit than *Listeria monocytogenes*. Therefore, if there were pathogens present on the surface of the mango fruit, and this fact was compounded by the use of low quality water, then the transfer could take place. Alternatively, in the case of *Listeria monocytogenes* and its limited capacity to adhere to the surface of the mango fruit, the attachment of the pathogen to the surface of this product is limited, as well as its ability to transfer during the wash processes employed during the mango packing cycle.

This study demonstrates that the use of copper in the mango hot water treatment process has a significant effect on the inactivation kinetics of *E. coli* bacteria. One of the most relevant advantages of using copper during the hot water treatment process is its stability when exposed to high temperatures, particularly when the presence of thermotolerant bacteria such as *Escherichia coli* could exist in these scenarios.

This study is the first report on the use of aqueous chlorine dioxide as a method for disinfecting *Salmonella enterica* ser. Choleraesuis and *Listeria monocytogenes* during different stages of the mango washing process under a variety of conditions. The study of the bacterial inactivation kinetics demonstrated that chlorine dioxide is more effective than sodium hypochlorite during shorter contact periods, making it a viable alternative for the wash process employed during the mango packing cycle.

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## Attachment I. Mango farm and packing house visits

A number of the activities scheduled within the framework of the project included site visits to mango farms from which the fruit would be sourced in order to conduct the laboratory trials. The farms that were visited are located in the municipality of Escuinapa, Sinaloa. During our visit, farm managers expressed some concern at the fact that this season had been an atypical cycle in which production was affected by a scarce amount of fruit, a fact that obliged them to end their season two months earlier than usual.

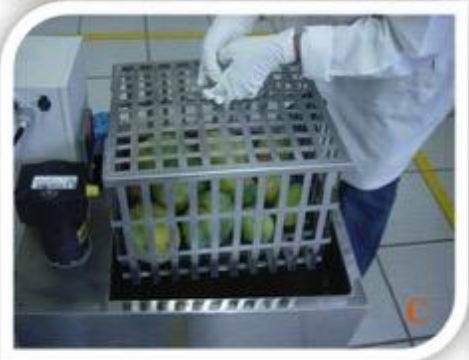


One of the activities carried out prior to conducting the laboratory trials was a visit to a mango packinghouse to update the information related to the mango packing process. During this visit, we were able to observe the entire mango packing process, including the respective hot water treatments that will be assessed as part of the framework for this research project. To date, the preferred disinfecting method for these packing houses relies on the use of chlorine.



## Attachment II. Laboratory activities

The laboratory activities included all of the preparations needed to carry out the trials corresponding to each one of the objectives outlined in the research project. For this purpose, the preparation of broths and agar cultures, bacterial reactivation, propagation, and purification, among others, more steps deemed to be crucial prior to conducting the trial. Once all the necessary elements were in place, we proceeded to apply the treatments taking into account each one of the factors and levels.



### Attachment III. Physiochemical characterization of mango farm soil

Physiochemical analyses conducted on mango farm soil from Escuinapa, Sinaloa, using the simulation with turbidity in the water of the hydrocooling treatment.

| Parameter                                     | Result     | Reference level |
|---|------------|-----------------|
| pH at 25 °C                                   | 6.11       | 6.6-7.3         |
| Electrical conductivity (ds m <sup>-1</sup> ) | 0.13       | 1-2             |
| Organic material                              | 1.09       | 1.5             |
| Phosphorous P-PO <sub>4</sub> (ppm) Bray      | 18.8       | 10-20           |
| Sodium (ppm)                                  | 119.41     | 250             |
| Potassium (ppm)                               | 442.5      | 180             |
| Calcium (ppm)                                 | 1092.3     | 1000-2000       |
| Magnesium (ppm)                               | 278.97     | 60-180          |
| Iron (ppm)                                    | 22.23      | 9-12            |
| Manganese (ppm)                               | 17.7       | 2-12            |
| Zinc (ppm)                                    | 1.55       | 1.3-2.5         |
| Copper (ppm)                                  | 0.645      | 0.9-1.2         |
| CIC (meq 100 g <sup>-1</sup> )                | 9.389      | 25              |
| Texture:                                      | Sandy Loam | -               |
| Clay %  | 13.04      | -               |
| Mud %   | 23.26      | -               |
| Sand %  | 63.68      | -               |

Source: Castellanos, 2000 y Molina y Meléndez, 2002