Microbial Food Safety and Postharvest Fruit Disinfection

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PROJECT PERIOD OBJECTIVES

- Assessment of sanitizer efficiency in postharvest fruit disinfection.
- Assessment of chlorine dioxide gas as a sanitizer for stone fruit (peaches, plums, nectarines; PPN).
- Assessment of dose requirements of chlorine dioxide gas for effective disinfection of damaged and abrasion-undamaged fruit.
- Evaluate the efficacy of the essential oils cinnamaldehyde and thymol as a wash aide to reduce surface contamination of fruit by *E. coli* and *Salmonella*.

EXECUTIVE SUMMARY OF RESULTS

- The addition of 0.5% lactic acid does not seem to significantly improve the action of chlorine in the reduction of human pathogenic bacteria under the experimental treatment conditions. In some cases, the hypochlorite + 0.5% lactic acid treatment (unadjusted pH at 2.5) worked significantly better than the hypochlorite adjusted to pH 2.5 indicating that the improved effectiveness with lactic acid may not due to the lower pH or an associated elevation of oxidation reduction potential (ORP), alone.

- Chlorine dioxide was determined to be an effective bactericide on stone fruit under treatment conditions that simulated short-term storage and distribution of PPN. Disinfection potential varied with experimental conditions but as great as a 5-log reduction (999.99% kill) was observed, as compared to non-treated controls. To properly define CT values for disinfection, as well as to assess the potential for phytotoxicity with various formulations and release-rates, further experiments should be done at a both optimal and sub-optimal storage and shipping temperatures.

- Evidence was observed that treating stone fruit with the essential oils cinnamaldehyde and thymol may marginally reduce human pathogen populations. However, effective disinfection, as has been reported in other studies with fruit, will likely require longer contact times than evaluated in these studies.

Sub-Project ID: Assessment of sanitizer efficiency in postharvest fruit disinfection

Immediate Objectives: To determine any synergistic effects of chlorine and lactic acid on the reduction of human pathogens on stone fruit.

OVERVIEW OF APPROACH:

In order to preliminarily assess if the addition of lactic acid has the potential to reduce the amount of chorine needed be effective in the reduction of pathogens on stone fruit, small scale studies were done in the lab where peaches, plums and nectarines (PPN) were spot-inoculated onto the surface of non-wounded fruit with either a cocktail of *Salmonella montevideo* and *S. typhimurium* or a cocktail of *E. coli* O157:H7 strains and then washed in a solution containing different combinations of chlorine and lactic acid. For brevity, details of strains used, inoculum development and inoculation methods are available on request.

Peaches, plums, and nectarines were inoculated with a high concentration of bacteria (log8 cfu/ml) and air dried for approximately 6 hours. Two experiments were done with 6 treatments in each to evaluate two different combinations of lactic acid and chlorine.

Experiment 1: 0.5% Lactic acid and 100ppm chlorine

1. No wash (control).
2. Washed in water (control)
3. Washed in 100ppm chlorine and 0.5% lactic acid (pH approximately 2.5)
4. Washed in 100ppm chlorine and 0.5% lactic acid with pH adjusted to neutral (pH 7.0).
5. Washed in 100ppm chlorine. (pH 7.0)
6. Washed in 100ppm chlorine with pH adjusted to approximately 2.7.

Experiment 2: 0.5% lactic acid and 25ppm chlorine

1. No wash (control).
2. Washed in water (control)
3. Washed in 25ppm chlorine and 0.5% lactic acid (pH approximately 2.5)
4. Washed in 25ppm chlorine and 0.5% lactic acid with pH adjusted to neutral (pH 7.0).
5. Washed in 25ppm chlorine. (pH 7.0)
6. Washed in 25ppm chlorine with pH adjusted to approximately 2.8.

The addition of lactic acid to chlorine in treatment 3 decreased the pH to approximately 2.5-2.7. The subsequent treatments in each experiment were pH adjusted to analyze the effects of pH compared to lactic acid itself. Fruit was washed in each treatment solution (except treatment 1) for 2 minutes.

Following treatment, the inoculated area of the fruit was excised with a sterile razor and placed in a sterile stomacher bag with neutralizing buffer. The samples were then

stomached with a Seward Stomacher 80 and plated on selective agar, for *E. coli* O157:H7 CHROMagar O157+ pyruvate and for *Salmonella* xylose lysine deoxycholate (XLD) agar + pyruvate. Pyruvate is added to media to encourage recovery and resuscitation of sub-lethal injured cells for more accurate enumeration. After incubation, plates were observed for characteristic colonies.

RESULTS

Experiment 1: 0.5% lactic acid and 100ppm chlorine

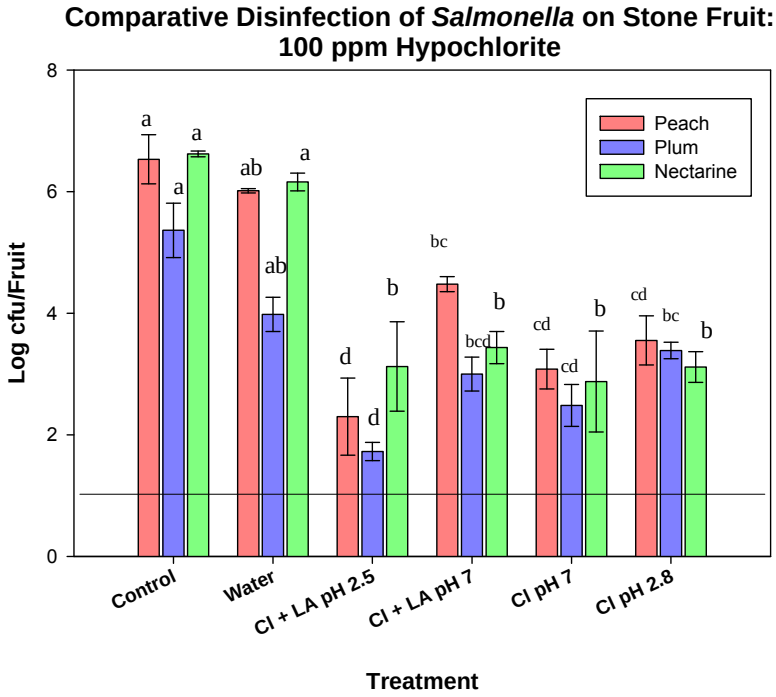


Fig 1: Comparative recovery of *Salmonella* on peaches, plums, and nectarines following various wash methods. The letters represent mean comparisons using Tukey's statistical test. Means with the same letter are not significantly different. The black line is the limit of detection.

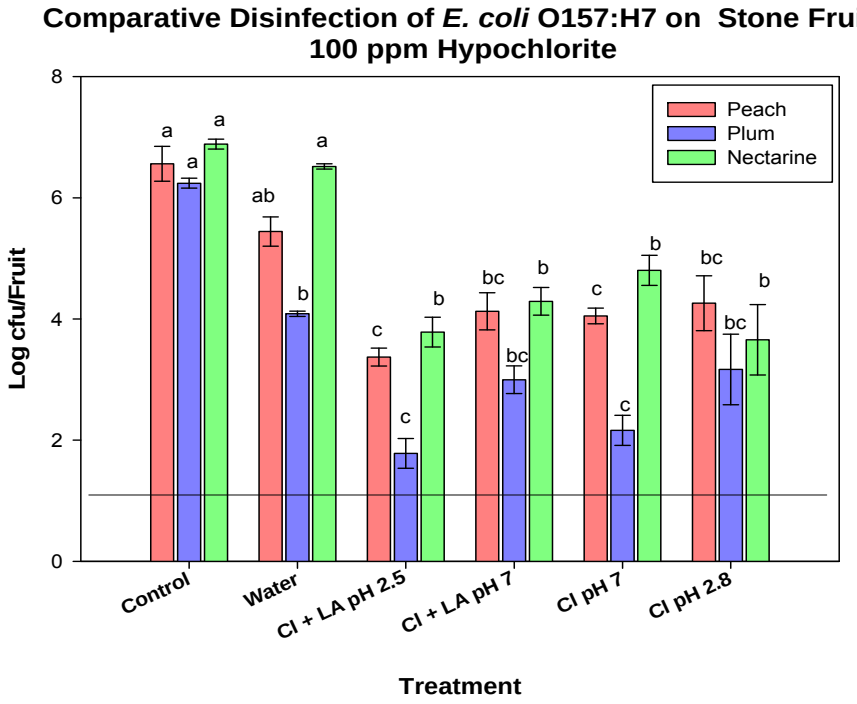


Fig 2: Comparative recovery of *E. coli* O157:H7 on peaches, plums, and nectarines following various wash methods. The letters represent mean comparisons using Tukey's statistical test. Means with the same letter are not significantly different. The black line is the limit of detection.

Peach All chlorine and lactic acid treatments reduced *Salmonella* and *E. coli* O157:H7 significantly compared to the water treatment on peach. However, there was no significant difference between the treatments indicating that the addition of 0.5% lactic acid did not improve the efficiency of chlorine. The pH adjustment also did not change the efficiency of the treatments. *E. coli* O157:H7 was reduced approximately 2.5-3 logs and *Salmonella* was reduced approximately 2-4 logs by the chlorine and lactic acid treatments.

Plum The chlorine + 0.5% lactic acid treatment and chlorine without lactic acid treatments were significantly better than water treatment at reducing *E. coli* and *Salmonella* on plums. However, most of the lactic acid and chlorine treatments were not significantly better than each other with the exception being chlorine + 0.5% lactic acid at low pH compared to chlorine at low pH on *E. coli* O157:H7. *E. coli* O157:H7 was reduced 2.5-4.5 logs and *Salmonella* was reduced 1.2-3.8 logs by all treatments.

Nectarine All chlorine and lactic acid treatments worked better than the water control on nectarine at removing *E. coli* O157:H7 and *Salmonella* although there was no significant difference between the treatments. The addition of 0.5% lactic acid did not seem to significantly improve the use of chlorine by itself. *E. coli* O157:H7 was reduced 2-3 logs, and *Salmonella* was reduced by about 3.5 logs by the lactic acid and chlorine treatments.

Experiment 2: 0.5% lactic acid and 25ppm chlorine.

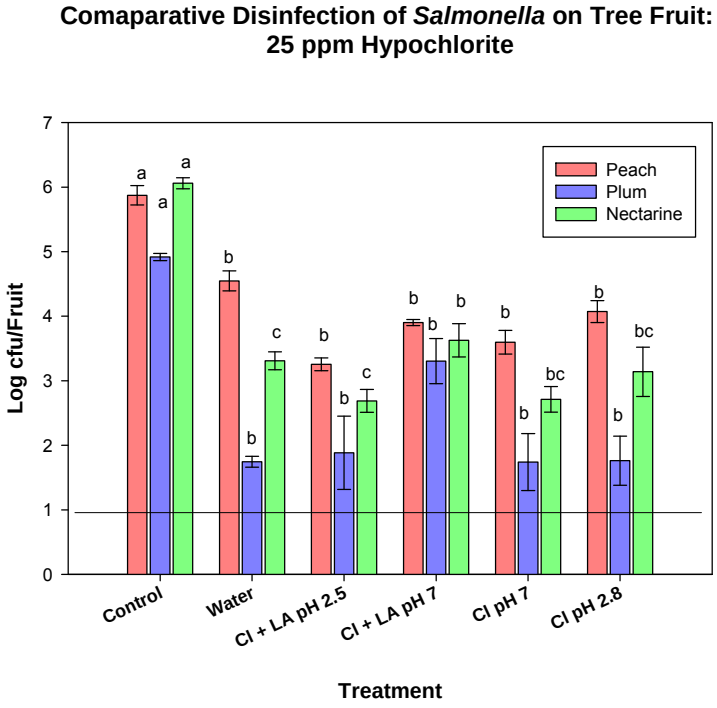


Fig 3: Comparative recovery of *Salmonella* on peaches, plums, and nectarine following various wash methods. The letters represent mean comparisons using Tukey's statistical test. Means with the same letter are not significantly different. The black line is the limit of detection.

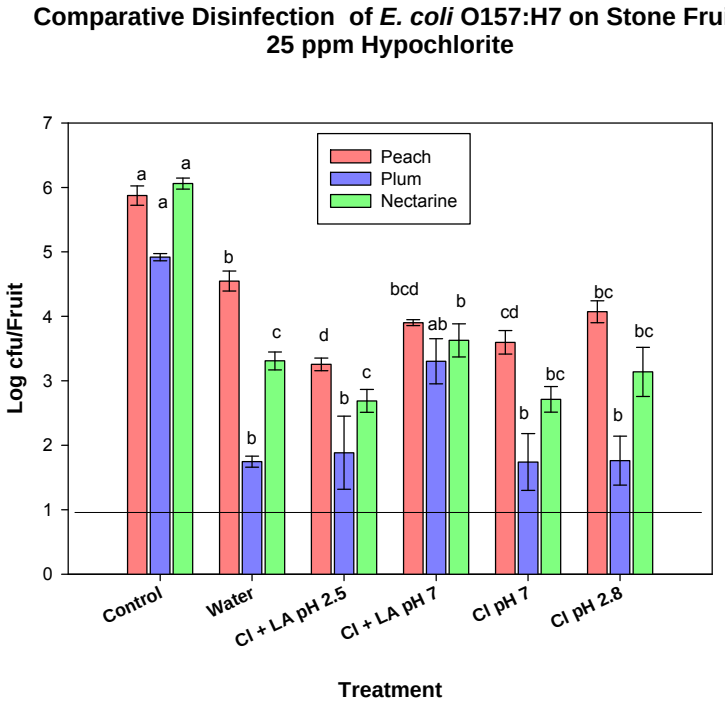


Fig 4: Recovery of *E. coli* O157:H7 on peaches, plums, and nectarines following various wash methods. The letters represent mean comparisons using Tukey's statistical test. Means with the same letter are not significantly different. The black line is the limit of detection.

Peach There was no significant difference between the chlorine and lactic acid treatments compared to the water treatment in the reduction of *Salmonella* on peach. The chlorine + 0.5% lactic acid at low pH was and the chlorine at neutral pH treatments were significantly better than the water treatment at reducing *E. coli* O157:H7. The chlorine + lactic acid at low pH treatment was significantly better than chlorine at low pH. Both *Salmonella* and *E. coli* O157:H7 were reduced more than 2 logs by the chlorine and lactic acid treatments.

Plum There was no significant difference between any of the treatments compared to water in the reduction of *Salmonella* and *E. coli* O157:H7 on plums. However, all treatments, including water, reduced *Salmonella* by more than 2.5-4 logs and reduced

E. coli O157:H7 1.5-3 logs. The differences in the water wash control performance in pathogen removal between Exp. 1 and Exp 2 are not apparent based on fruit quality or sourcing.

Nectarine There was no significant difference between any of the treatments compared to the water treatment in the reduction of *Salmonella* and *E. coli* O157:H7 on nectarine. The chlorine + lactic acid at neutral pH did not work as well as water in reducing *Salmonella* and *E. coli* O157:H7, which may likely be a function of variability in fruit surface properties at the point of inoculation, such as microwounding . Both pathogens were reduced 2.5-3.5 logs by all the treatments including water.

OVERALL CONCLUSIONS

The addition of 0.5% lactic acid does not seem to significantly improve the action of chlorine alone in the reduction of human pathogenic bacteria under these treatment conditions. In the first experiment, the use of 100ppm chlorine and 0.5% lactic acid appeared to have the most impact in log reduction, but the Tukey's statistical test was unable to separate the mean values to substantiate this as significant. In some cases, the chlorine + 0.5% lactic acid pH 2.5 treatment worked significantly better than the chlorine by itself at pH 2.5 indicating that the improved effectiveness of the first treatment was not due to the lower pH or elevated oxidation reduction potential (ORP), alone.

The 25ppm chlorine treatments also did not appear to work any better than washing with water alone in most cases. This is, in part a reflection of the greater degree of removal, overall, by washing alone in Exp 2 fruit as compared to Exp 1 fruit. Overall, the ability for either pathogen to survive on plums was much less than on peaches or nectarines, even on the non-treated control. This may indicate that the surface of plums is, in general, less conducive to pathogen survival than peaches or nectarines and appear to be more "cleanable".

Sub-Project ID: Assessment of chlorine dioxide gas as a sanitizer for stone fruit.

OVERVIEW OF APPROACH

In order to preliminary determine the ability of chlorine dioxide gas to reduce microbial load on the surface of stone fruit, small scale experiments were done using slow release chlorine dioxide precursors and sachets provided by ICA TriNova, LLC within glass treatment chambers. Peaches, plums, and nectarines were inoculated with a high concentration (log 8 CFU/ml), of either a cocktail of *E. coli* O157:H7 strains genetically-tagged with antibiotic resistance (kanamycin) and green fluorescent protein (GFP) to facilitate recovery and identification, or a cocktail of *Salmonella poona* and *S. montevideo* tagged with kan:GFP, and allowed to air dry overnight at 20^{o}C. Sachets containing chlorine dioxide precursors were placed in glass treatment chambers with water for activation. The control chamber contained water, but no chlorine dioxide precursors. Chamber RH was determined to be ≥ 90% in previous related studies. Fruit

was placed inside the treatment chambers and all treatment chambers were stored at 2.5^{O}C for 7 days.

The concentration of chlorine dioxide gas that fruit was exposed to was determined by consulting a ClO_2 formulation release-curve graph provided by the company that defines the amount of chlorine dioxide precursor used by time. In this experiment, three amounts of chlorine dioxide precursors were used for the 7 day treatment, resulting in a cumulative exposure of approximately 9.2 mg/L, 18.4 mg/L, and 27.5 mg/L of chlorine dioxide gas.

Following treatment, the inoculated area of the fruit was excised with a sterile razor blade and placed in a sterile stomacher bag with neutralizing buffer. Samples were rubbed-shake-rubbed to remove any surviving bacteria and plated on selective media, TSA + kanamycin + pyruvate for *E. coli* O157:H7 and XLD + pyruvate for *Salmonella*. After incubation, plates were observed for characteristic colonies.

RESULTS

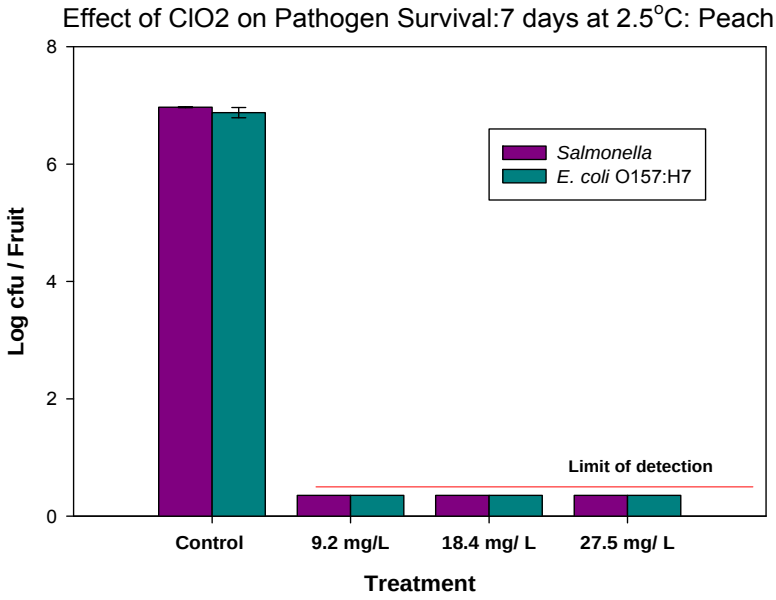


Fig 5: Comparative recovery of *Salmonella* and *E. coli* O157:H7 on peach after 7 days storage at 2.5^{O}C with and without chlorine dioxide exposure.

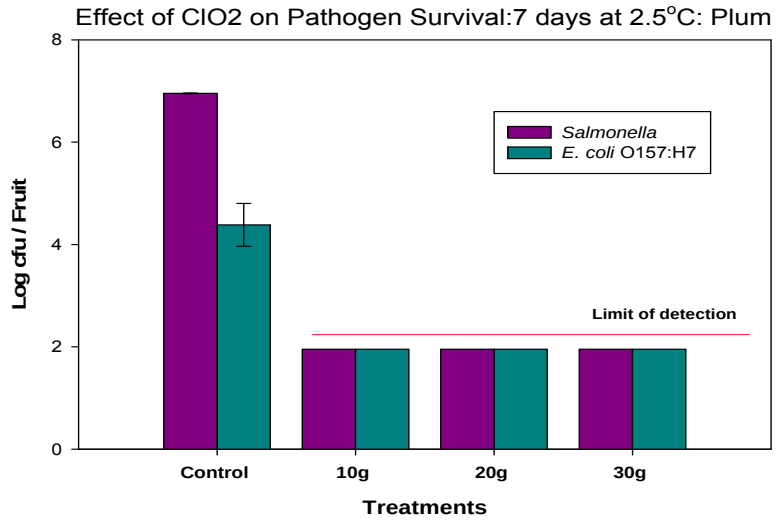


Fig 6: Comparative recovery of *Salmonella* and *E. coli* O157:H7 on plum after 7 days storage at 2.5°C with and without chlorine dioxide exposure.

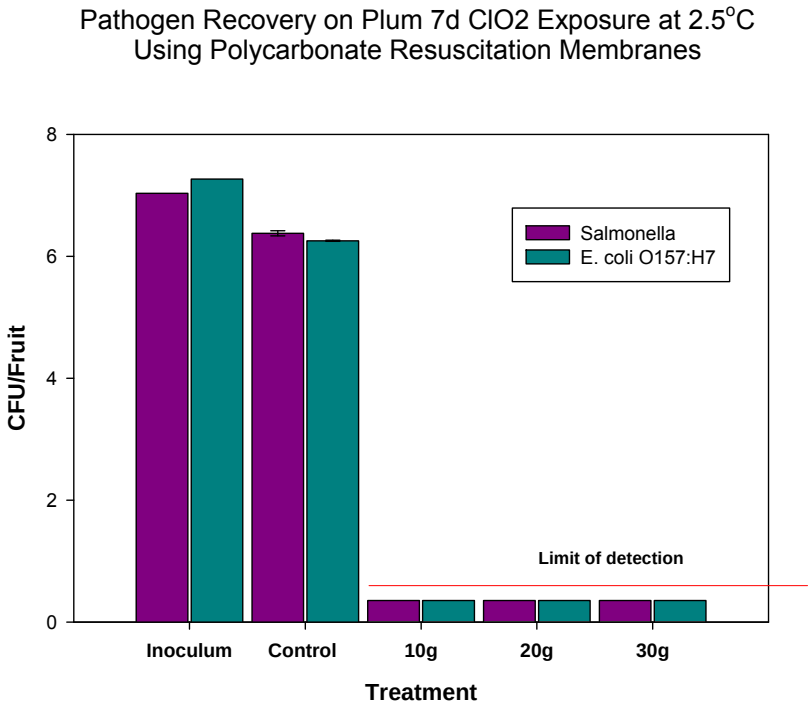


Fig 7: Comparative recovery of *Salmonella* and *E. coli* O157:H7 on plum after 7 days storage at 2.5°C with and without chlorine dioxide exposure and with the use of polycarbonate membranes to facilitate the recovery of sub-lethally injured cells.

After 7 days at 2.5°C, while viable pathogens were readily revered from non-treated fruit at low temperature and high RH, no pathogens were detected under the same storage conditions following exposure to ClO2 by standard enumeration methodology on agar culture. The limit of detection (sensitivity) is denoted by the drawn line for each experiment. The limit of detection for peach was lower than plums due to adjustment in the experiment based on initial results to improve recovery sensitivity using standard techniques. An assessment of PCR-based detection, using the Biocontrol Assurance GDS™ rapid pathogen detection kit system results was also conducted. All samples were positive for *E. coli* O157:H7 indicating that DNA from washed fruit could still be amplified and detected. It appeared unlikely that pathogen DNA came from viable bacteria, however, as the enrichment growth culture used for GDS™ testing was not turbid indicating the absence of actively growing bacteria. The high dose challenge, more than 1,000,000 initial cells used in these lab tests could result in a positive detection if cells are inactivated or severely injured but had not lysed prior to processing for detection.

This experiment was repeated on plums with the use of polycarbonate membranes to aid in the recovery of sub-lethally injured cells. Results were similar to the first experiment with no recovery of pathogens on ClO_2 exposed fruit but substantial survival on non-treated controls under the low temperature and high RH conditions. GDS pathogen detection assessments resulted in 1 out of 3 positive for *E. coli* O157:H7 and no amplification-detection for *Salmonella*. However, plating the enrichment broth failed to recover viable pathogens indicating that the DNA amplified by GDS was most likely from non-viable cells.

CONCLUSION

Chlorine dioxide is a very good bactericide under treatment conditions that may be encountered in short-term storage and distribution of PPN. The storage temperature, in retrospect, was within a temperature range that induces chilling injury and would not be optimal in commercial applications. To properly define CT values for disinfection, as well as to assess the potential for phytotoxicity with various formulations and release-rates, further experiments should be done at a both optimal and a broader range of typical storage and shipping temperatures.

Sub-Objective ID: To determine the critical dose of chlorine dioxide gas needed for effective disinfection of damaged and undamaged tree fruit.

OVERVIEW OF APPROACH

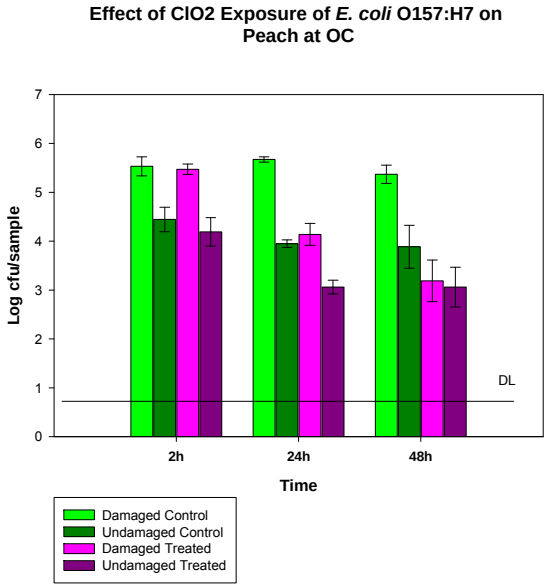
In order to determine the critical dose of chlorine dioxide gas needed for fruit disinfection, small scale experiments were done in the lab using slow release chlorine dioxide precursors and sachets provided by ICA TriNova, LLC within glass treatment chambers, as described above. Peaches, plums, and nectarines either lightly rubbed with a fine sand paper to mimic superficial damage during harvest or handling, or left undamaged were inoculated with a high challenge dose of bacteria (log 8 CFU/ml), then

allowed to air dry at 20-22°C for approximately 6 hours. Fruit was then stored at 0°C overnight.

Sachets containing the same amount of chlorine dioxide precursors were placed in glass treatment chambers with water for activation. Control treatment chambers contained only water. Fruit was placed in the treatment chambers and then stored at 0°C for varying amounts of time. The storage time was terminated at 2 hours, 24 hours, and 48 hours. This resulted in cumulative fruit exposure to chlorine dioxide gas concentrations of 0.5 mg/ L, 2.1 mg/L, and 3.7 mg/L respectively, according to the release-curve graphs provided by TriNova.
Following treatment, the inoculated area of the fruit was excised with a sterile razor blade and placed in a sterile stomacher bag with neutralizing buffer. Samples were rubbed-shake-rubbed to remove any surviving bacteria and plated on trypticase soy agar (TSA) overlaid with polycarbonate membranes to facilitate recovery of sub-lethally injured cells, followed by aseptic transfer to selective media, TSA + kanamycin + pyruvate for *E. coli* O157:H7 and XLD + pyruvate for *Salmonella*. After incubation, plates were observed for characteristic colonies.

RESULTS

Peach The chlorine dioxide gas treatment reduced *E. coli* O157:H7 recovery by about 2 logs on damaged fruit by 48 hours. *E. coli* O157:H7 was only reduced by about 0.8 logs after 48 hours in the absence of ClO_2 exposure. *Salmonella* was reduced by about 1.8-2 logs on damaged and undamaged fruit by 24h, however, either fruit to fruit variability or potential evidence of recovery was seen by 48h. *E. coli* O157:H7 survival on undamaged untreated fruit was slightly reduced as compared to abrasion- damaged fruit. *Salmonella* survival was also slightly greater on damaged untreated fruit.



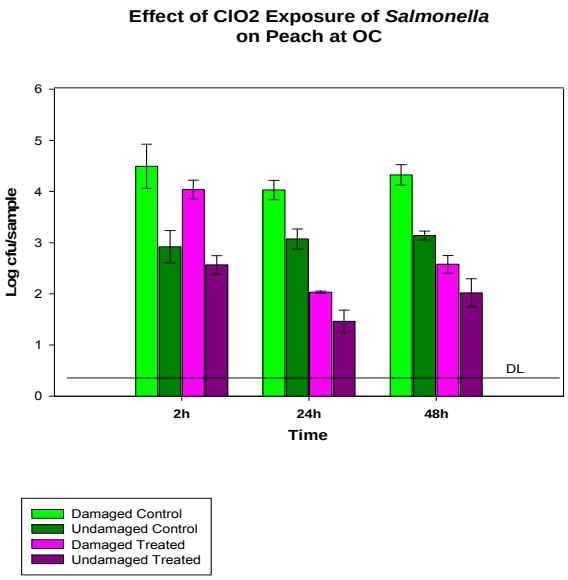


Fig 8 and 9: Average reduction in survival of pathogens on untreated and ClO2 treated fruit at 0C for 2-48h.

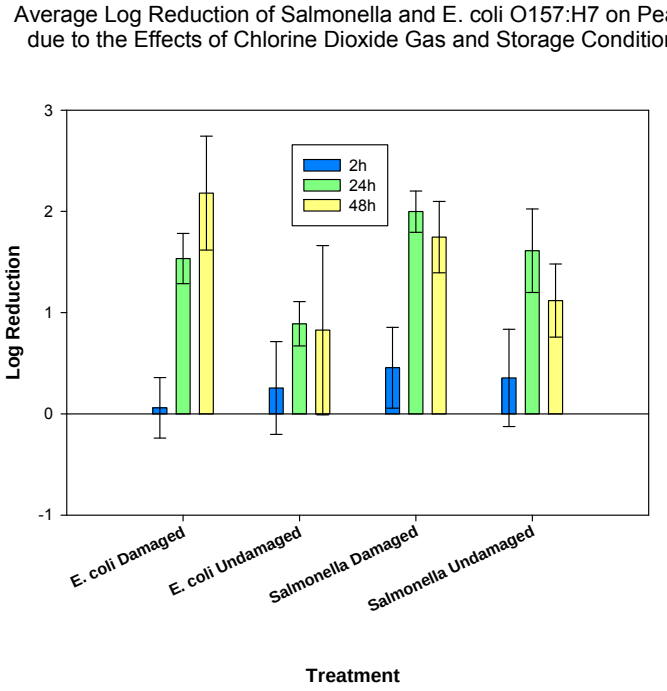


Fig 10: Average log-reduction relative to non-treated controls

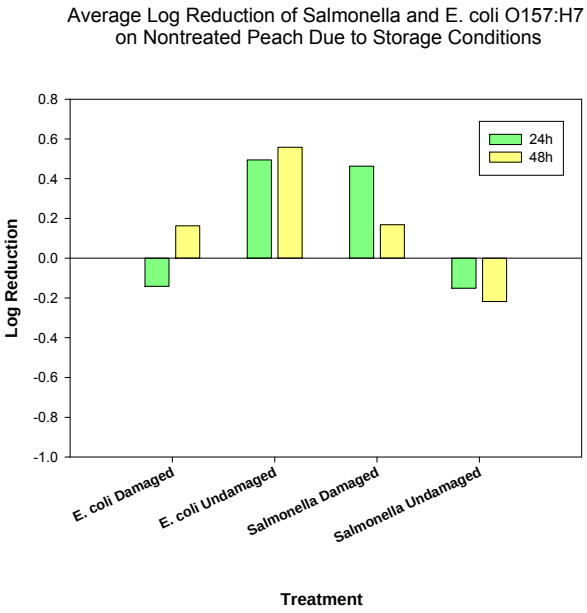


Fig 11: Average log-reduction of non-treated controls over 48h period at OC and high RH in sealed treatment chambers.

Plum The recovery of *E. coli* O157:H7 and *Salmonella* was reduced on damaged and undamaged fruit by 24h on plums. However, the source of variability over time has not been identified. Whether pathogens can recover from sub-lethal injury at 0C after 48h, thereby increasing recovery is unknown. The effect by the experimental system storage conditions alone appeared to have very little effect on the reduction of pathogens on plums. A change in about 0.5 logs was seen for *Salmonella* on damaged fruit between 24h and 48h. The opposite was seen for *E. coli* O157:H7 with greater loss of viability on damaged as compared to undamaged plums. These experiments should be repeated to determine whether compounds released from superficially wounded plums are differentially inhibitory to *E. coli* and *Salmonella*.

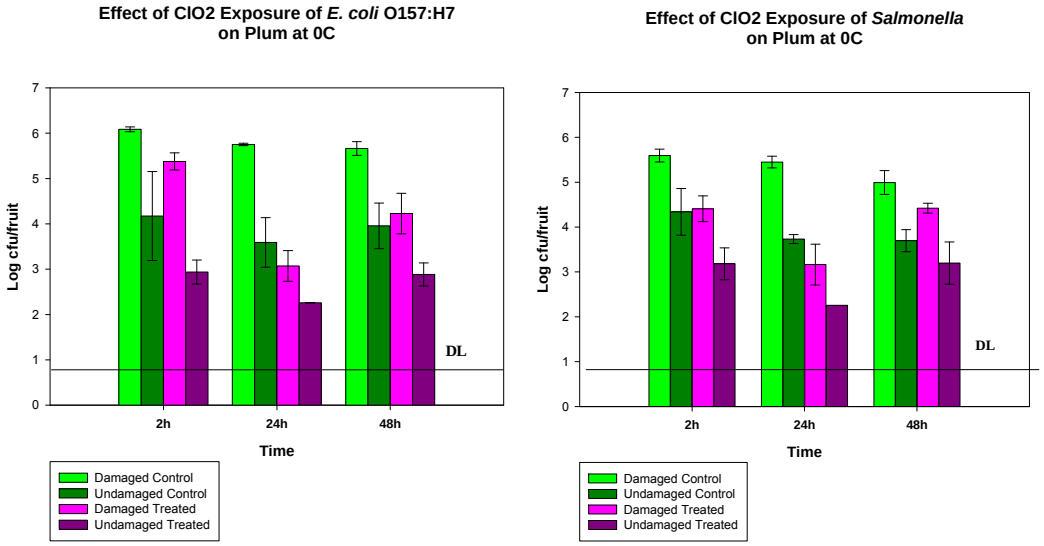


Fig 12 and 13: Average reduction in survival of pathogens on untreated and ClO2 treated fruit at 0C for 2-48h.

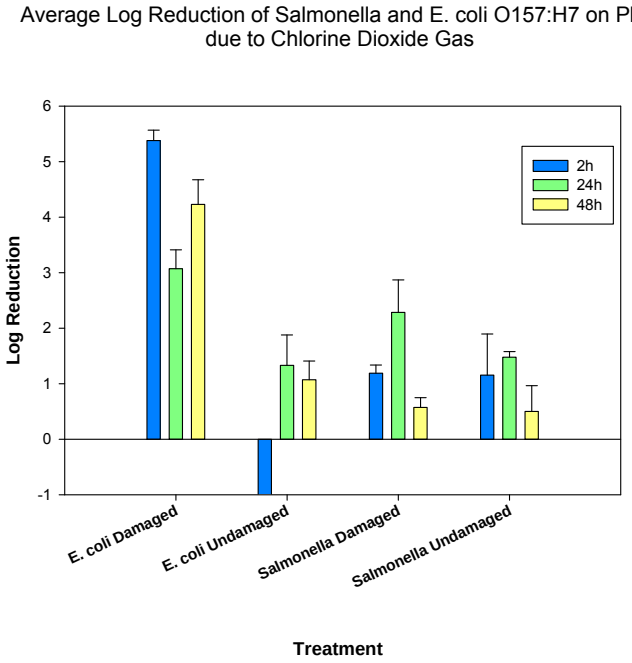


Fig 14: Average log-reduction relative to non-treated controls.

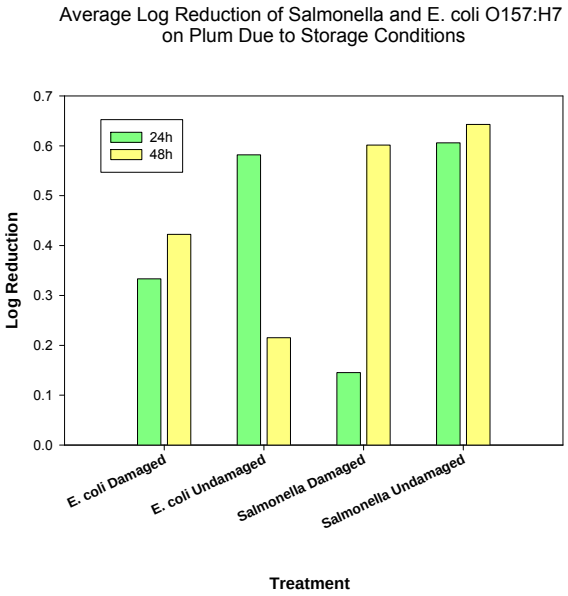


Fig 15: Average log-reduction of non-treated controls over 48h period at OC and high RH in sealed treatment chambers

Nectarine The recovery of *E. coli* O157:H7 and *Salmonella* was reduced on both damaged and undamaged fruit, at least one log at 24h, but disinfection was less effective in this experiment than with either peaches or plums. Again, fruit variability, variation in formulation stability over time, or other factors may influence the outcome in these small-scale experiments. The storage conditions, alone, had very little effect on the reduction of pathogens on nectarines expect for *Salmonella* on damaged fruit where there was about 1 log in reduction.

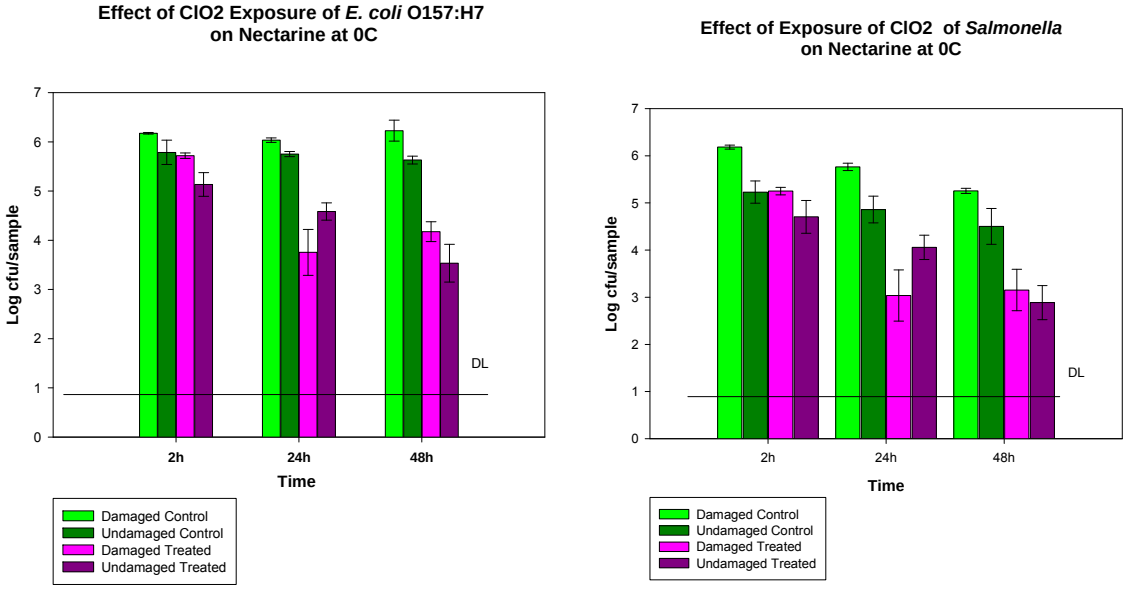


Fig 16 and 17: Average reduction in survival of pathogens on untreated and ClO2 treated fruit at 0C for 2-48h.

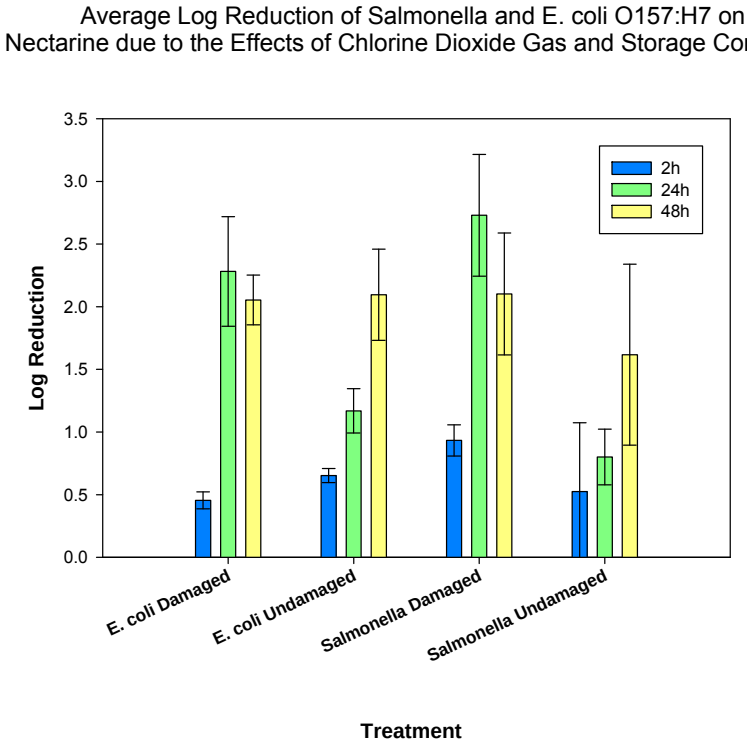


Fig 18: Average log-reduction relative to non-treated controls.

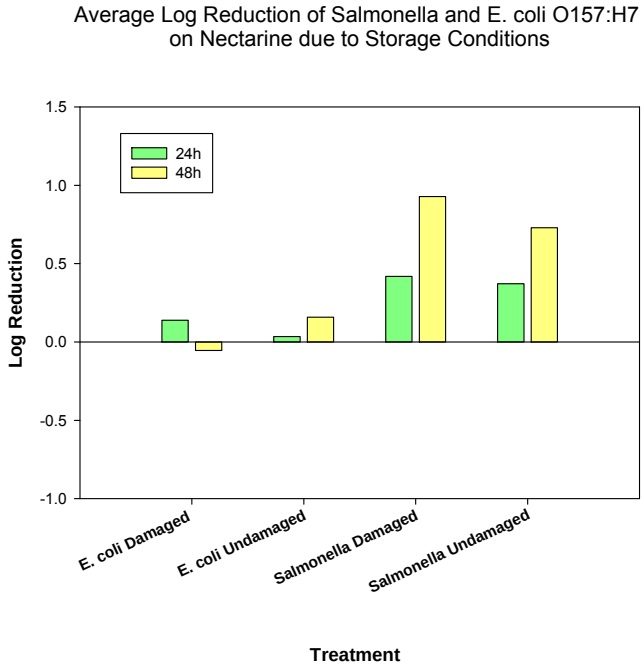


Fig 19: Average log-reduction of non-treated controls over 48h period at OC and high RH in sealed treatment chambers

CONCLUSION

Chlorine dioxide gas was effective at reducing human pathogens under these treatment conditions, though total disinfection potential within 48h at 0C was less than previous experiments conducted over longer periods of exposure and a higher cumulative dose of chlorine dioxide gas,. The chlorine dioxide treatment was able to reduce the recovery of viable bacteria by almost 1000-fold (99.9% kill) in some cases. There remains some uncertainty as to the cause of those outcomes where disinfection levels were greatest at 24h with apparent evidence of recovery at 48h. Further experiments should be done to optimize treatment at 0^{O}C. Further experiments should
also be done for extended time points with multiple sampling.

Sub-Objective ID: To evaluate the efficacy of the essential oils cinnamaldehyde and thymol to reduce surface microbial load on tree fruit.

OVERVIEW OF APPROACH

Peaches, plums, and nectarines were inoculated with a high concentration (log 8 CFU/ml), of either a cocktail of *E. coli* O157:H7 strains genetically-tagged with antibiotic resistance (kanamycin) and green fluorescent protein (GFP) to facilitate recovery and identification, or a cocktail of *Salmonella poona* and *S. montevideo* tagged with kan:GFP, and allowed to air dry for approximately 6 hours. The fruit was stored at 15°C until treatment to aid in attachment of bacteria to the fruit surface. Cinnamaldehyde + thymol solutions were prepared at 1mM, 5mM and 10mM concentrations. Fruit was treated at 3 different time points followed immediately by recovery of any surviving bacteria. Time zero recovery was done immediately after spot inoculations had visibly dried and recovery repeated at 24 and 72 hour time points. The delayed-recovery time points were done to determine the effectiveness of essential oils on bacteria that had a longer time to attach to the surface of the fruit and potentially aggregate in 'biofilms'.

The fruit was treated by *brush-washing* the inoculated area with a sponge that had been dipped in the essential oil solutions. A treatment with water was done as a control. The treated area of the fruit was excised with a sterile razor and place in a sterile stomacher bag. The essential oil solution was allowed a contact time of approximately 5 minutes before neutralizing buffer was added to the bag. The samples were stomached for 30 seconds on normal setting with a Seward Stomacher 80 and plated on selective media, TSA + kanamycin+ pyruvate for *E. coli* O157:H7 and XLD + pyruvate for *Salmonella*. After incubation, plates were observed for characteristic colonies.

RESULTS

None of the treatments appeared to be significantly better than water at reducing *E. coli* O157:H7 on PPN. There did not seem to be a significant difference in effectiveness of treatments in reducing *Salmonella* on peaches or nectarines. There is some evidence, as seen in Figure 24 that essential oils may be effective at reducing *Salmonella* on plums, but more testing should be done to confirm these findings. Overall, the effectiveness of all the treatments appears to remain the same for the reduction of *E. coli* O157:H7 after 72 hours.

CONCLUSIONS

There is some evidence that treating stone fruit with the essential oils cinnamaldehyde and thymol may help reduce human pathogen populations. However, effective disinfection, as has been reported in other studies with fruit, will likely require longer contact times.

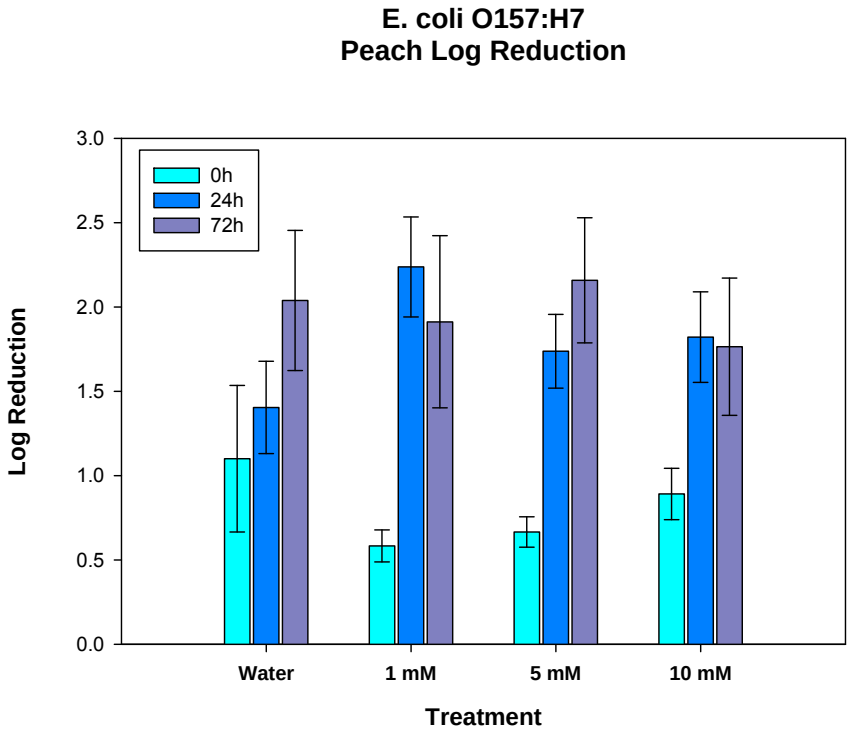


Fig 20: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

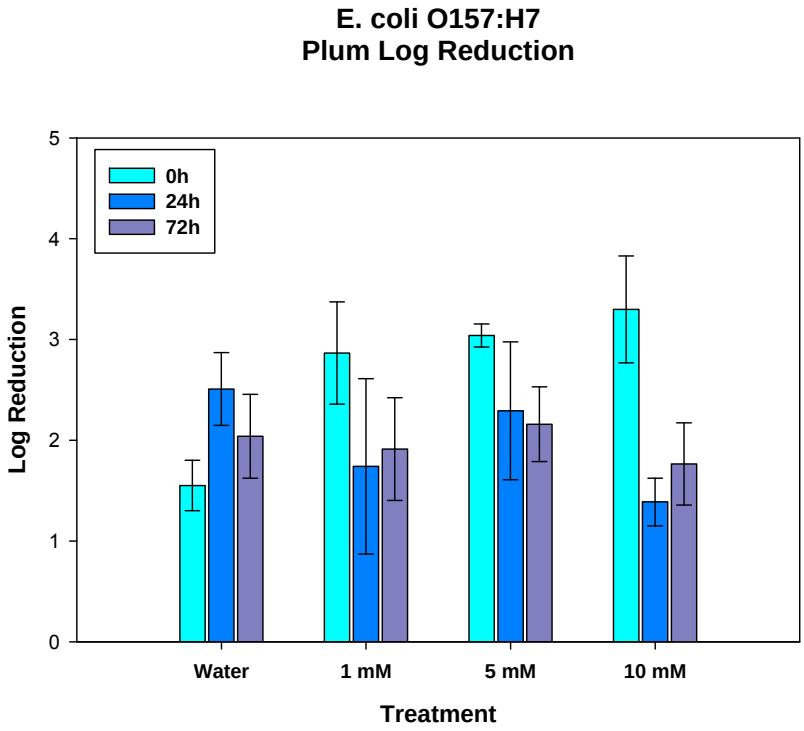


Fig 21: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

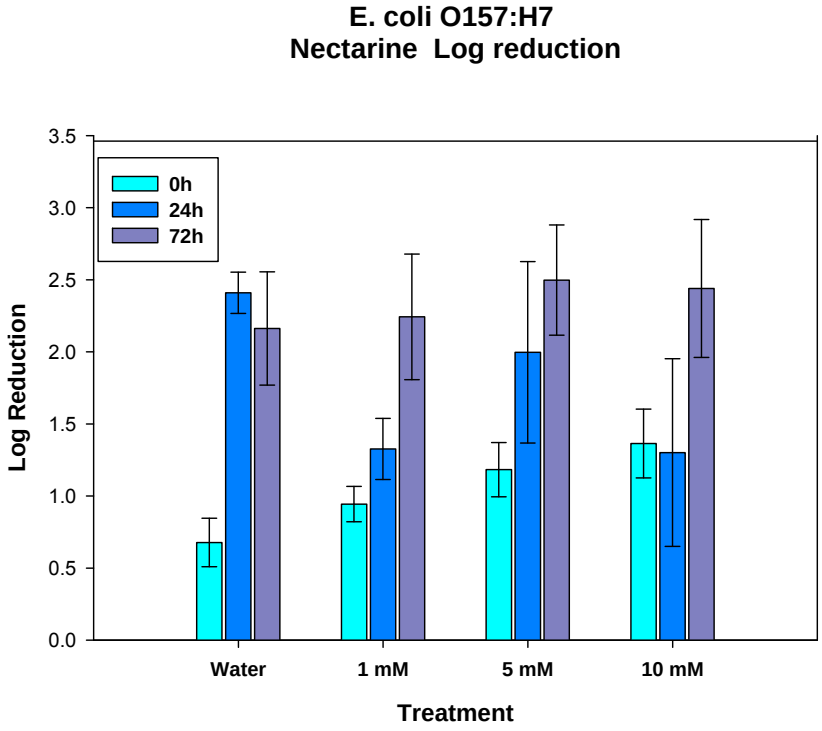


Fig 22: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

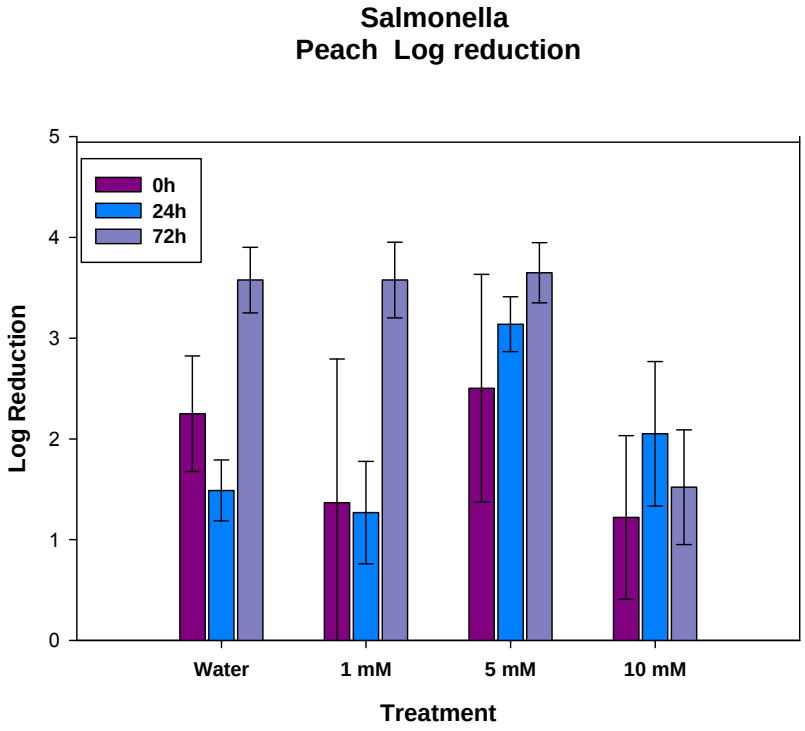


Fig 23: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

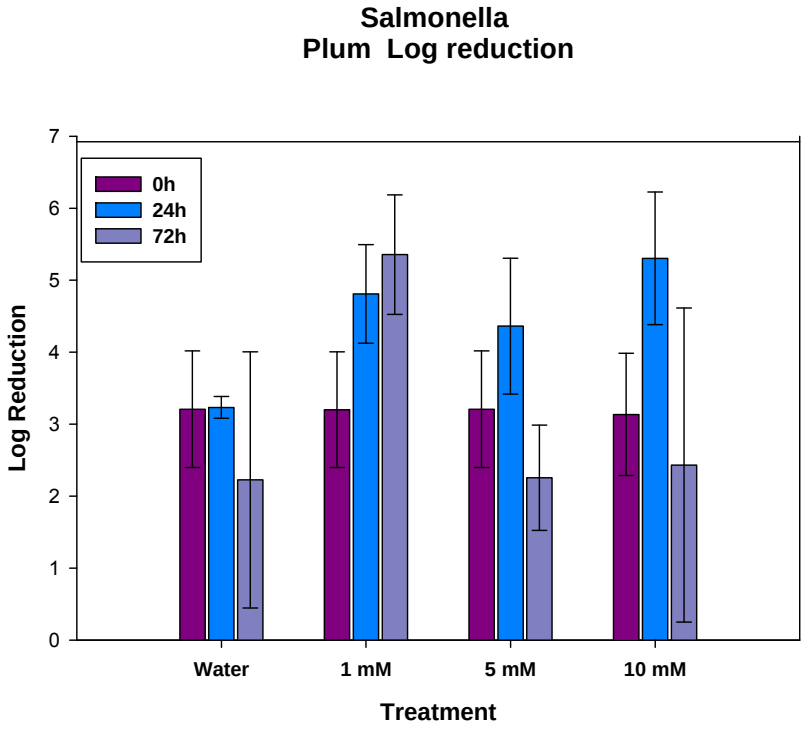


Fig 24: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

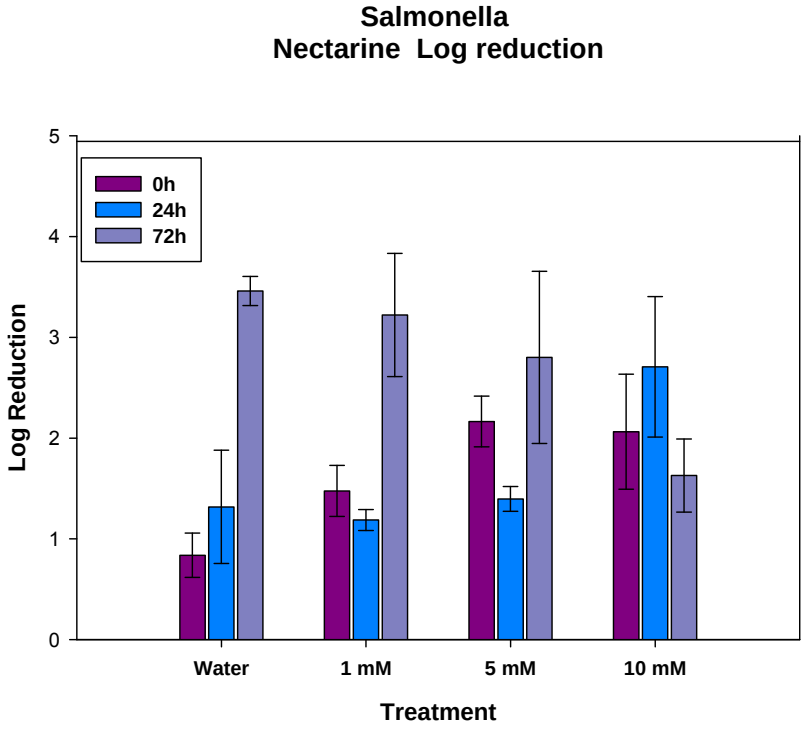


Fig 25: Comparative log-reduction of fruit washes with water or a mixture of cinnamaldehyde + thymol solutions

NEXT STEPS

Based on the outcomes of our research project we feel the priority areas for further investigation include;

1. Complete assessment of chlorine dioxide gas as a postharvest disinfectant for postharvest plant pathogens, *E. coli* O157:H7, and *Salmonella* on peaches, plums, and nectarines (PPN).
2. Evaluate the efficacy of a GRAS formulation as a postharvest disinfectant for postharvest plant pathogens, *E. coli* O157:H7 and *Salmonella* on peaches, plums, and nectarines (PPN).
3. Evaluate the survival of surrogate, nonpathogenic *E. coli* on PPN fruit in experimental farm orchards as affected by tree morphology and position on the tree.