Journal of Nature Studies 18(2): 44-51
Online ISSN: 2244-5226

EFFICACY OF OZONATED WATER AS DISINFECTANT FOR ORGANIC ROMAINE LETTUCE (*Lactuca sativa* L.)

Bryan Rey R. Oliveros[1]* and Ida F. Dalmacio [2]

[1]Institute of Food Science and Technology, College of Agriculture and Food Science, University of the Philippines Los Baños, Los Baños, Laguna, Philippines 4031

[2]Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Los Baños, Laguna, Philippines 4031

*Corresponding author: broliveros@up.edu.ph

ABSTRACT – There is a need to find an alternative disinfectant to chlorine for Filipino organic farmers as the absence of postharvest disinfection may pose food safety risks to ready-to-eat produce. To address this gap, this study evaluated the efficacy of ozonated water in disinfecting uninoculated and artificially-inoculated organic Romaine lettuce through microbiological quality testing and microbiological shelf life determination. Data showed that disinfection of uninoculated, organic Romaine lettuce leaves with 125 ppm ozonated water resulted to heterotrophic plate count reductions (0.77-1.18 $\log_{10}$) equivalent to that of 200 ppm chlorinated water treatment at any exposure times (2, 5, and 10 min) (p>0.05). For the artificially-inoculated samples, both disinfectant solutions (chlorinated and ozonated water) produced equivalent reductions in *E. coli* count (1.31-2.39 $\log_{10}$) at any exposure times (2, 5, and 10 mins) (p>0.05), as well. Moreover, disinfection of organic Romaine lettuce with chlorinated and ozonated water did not exceed the recommended limit for heterotrophic plate count during the entire 6-day storage period at 10°C. Thus, disinfection of Romaine lettuce leaves using ozonated water at 125 ppm for at least 2 min could substitute the FDA-recommended chlorine disinfection for use in organic farming.

Keywords: chlorine, organic Romaine lettuce, ozonated water

All fruits and vegetables, especially organic produce, have microbial load upon harvest. Unfortunately, some produce that require no cooking before consumption (ready-to-eat or RTE) could harbor pathogenic microorganisms. These pathogens may just be on the surface but studies have shown that these could also be internalized (Cooper *et al.*, 2007). If these pathogens are not controlled in RTE produce, this may lead to food poisoning outbreaks. For example, in United Kingdom, consumption of mixed salad resulted to an outbreak that sent more than 150 people in the hospital due to *E. coli* contamination (Meikle, 2016). In addition, in the United States, 36% of all *E. coli* O157 illnesses is attributed to vegetable raw crops (Golden and Boyer, 2015). Fortunately, these outbreaks from consumption of contaminated RTE produce could be prevented or reduced if intervention measures such as disinfection procedures are present. Disinfection involves the use of antimicrobial compounds incorporated in the wash water to reduce or eliminate the contaminating pathogens in the produce.

To cite this paper: Oliveros, B.R.R. & Dalmacio, I.F. 2019. Efficacy of Ozonated Water as Disinfectant for Organic Romaine Lettuce. *Journal of Nature Studies*. 18(2), 44-51.

Today, hypochlorite is one of the widely used produce disinfectant because of its efficacy. Unfortunately, due to the nature and goal of organic farming, use of chlorine is not allowed thereof. In fact, in several European countries including the Netherlands and Germany, the use of chlorine is already banned (Schmid, *et al.,* 2004). With that, an alternative disinfectant to chlorine should be investigated. One promising compound is aqueous ozone.

Ozone gas was first used in treating drinking water because of its efficacy without affecting the taste. Later on, it was used as an antimicrobial agent in the food industry. *In vitro* experiments proved its antimicrobial activity against spoilage and pathogenic microorganisms (Khadre and Yousef, 2001). *In vivo* antimicrobial experiments were also done in apples (Achen and Yousef, 2001) and in other commodities like alfalfa sprouts and potatoes (Bialka and Demirci, 2007).

In the Philippines, however, no studies have been conducted with regards to the use of ozonated water in postharvest disinfection of organic produce. Our organic farmers have only resorted to washing their produce with plain water. In a survey that we conducted among organic farmers, 100% of our respondents stated that they do not use any kind of vegetable disinfectant. This practice is risky as it may lead to food poisoning if the RTE produce is contaminated with pathogens. In order to avoid outbreaks attributed to organic RTE vegetables from happening, an alternative disinfectant to chlorine that is safe and eco-friendly should be explored, hence this study.

This study evaluated the efficacy of ozonated water as a substitute disinfectant to chlorine for RTE organic produce. Its effect on the reduction of the natural microbiota and artificially-inoculated *E. coli* in organic Romaine lettuce were evaluated. In addition, its effect on the microbiological shelf life of the produce was also investigated.

MATERIALS AND METHODS

Bacterial Culture and Culture Conditions

Pure bacterial culture of *E. coli* was obtained from the Microbiology Division of the Institute of Biological Sciences, University of the Philippines Los Baños. The culture was maintained by monthly transfers using tryptic soy agar (TSA) followed by overnight incubation at 37°C and storage at 4°C. Before every use, 50 µL of the bacterial culture was transferred to 5 mL culture broth and was incubated at 37°C for an appropriate time until log phase is reached.

Preparation of Wash Water

Water used for preparing all solutions was at 25°C and all washings were done at the same temperature. Ozonated water was freshly prepared using a commercially available ozonator (Triwin water ozonator) that has an output of 125 ppm. In its preparation, the bubbler of the ozone machine was submerged into the water and the machine was kept running during the whole duration of the disinfection exposure times. For chlorinated water, a commercially available hypochlorite solution (Zonrox, 3.5%) was diluted into tap water to arrive at 200 ppm which is the FDA-recommended concentration. The tap water set-up was prepared by allowing the tap water to stand overnight to dissipate all residual chlorine present.

Preparation of Organic Romaine Lettuce

Samples were obtained from an organic farm in Majayjay, Laguna. Before transporting to the laboratory, lettuce heads were cleaned and sorted. Upon arrival at the laboratory, samples were kept at 4ºC overnight. The next day, lettuce leaves were cut into 3 cm x 3 cm strips and were divided into two batches, uninoculated and artificially-inoculated set-up. The first batch was directly used for the uninoculated set-up experiments while the other batch was disinfected first with 200 ppm chlorine for 2 min thrice before artificial inoculation. Coliform count after this step was <10 CFU/g and was determined using Violet Red Bile Agar (VRBA).

Microbiological Quality of Organic Romaine Lettuce After Disinfection

For the uninoculated set-up, the first lot of lettuce leaves weighing 100 g was washed with 1 L of tap water for 2, 5 and 10 min, respectively. Washing was done by submerging the leaves into the solution placed in beakers while constantly stirring the mixture for the whole duration of each exposure time. The second lot (100 g) was disinfected with freshly prepared, 1 L of 200 ppm chlorine solution and the third lot (100 g) was disinfected by continuous bubbling of 125 ppm ozonated water, both at the same exposure times as with the tap water. After each exposure time, all lettuce lots were spin-dried separately for around 2 min using a sanitized salad spinner. Samples were tested for heterotrophic plate count (HPC) using plate count agar (PCA) and the plates were incubated at 35°C for 48 hr. After the allotted incubation period, log reductions in each sample were computed. All analyses were done in three trials with three replications per trial. Same was done in the artificially-inoculated set-up except that it was plated using Violet Red Bile Agar (VRBA) for *E. coli* count (ECC) and the plates were incubated at 35°C for 24 hr.

Shelf Life of Organic Romaine Lettuce After Disinfection

For this part, 100 g of lettuce leaves was subjected to 1 L each of the wash water; tap water for 2 min, chlorinated water (200 ppm) for 2 min, and with ozonated water (125 ppm) at an exposure time that is comparable to that of chlorine from the previous experiment, which is 2 min. After washing, the leaves were spin-dried, packaged at 10 g each in resealable plastic bags, and were stored at 10ºC. Samples were tested for HPC using PCA at designated sampling days (0, 2, 4, 6 days). Plates were incubated at 35°C for 48 hr and CFU/g of each treatment was computed.

Statistical Analysis

Median log reduction (CFU/g) for HPC and ECC from each treatment were calculated from three replications from three trials of the experiment. Collected data were subjected to Kruskal-Wallis test and Dunn's test to determine if there were significant differences ($p<0.05$) between median values of microbial populations.

RESULTS AND DISCUSSION

Effect of wash water on the microbiological quality of organic Romaine lettuce

The disinfection efficacies of ozonated water, in terms of HPC log reduction, when compared to the other two treatments are shown in Tables 1 and 2. The starting HPC was 5.44 $\log_{10}$.

As can be seen in Table 1, washing of Romaine lettuce with tap water for 2, 5, and 10 min resulted to HPC reductions ranging from 0.13-0.18 $\log_{10}$ but the increase in exposure time did not directly translate to higher log reductions ($p>0.05$). On the other hand, disinfection of lettuce leaves using ozonated water produced HPC reductions ranging from (0.77-1.18 $\log_{10}$) and increase in exposure time also resulted to higher log reductions ($p<0.05$). For the comparison between the tap water and ozonated water disinfection, the latter resulted to higher HPC reduction (1.18 $\log_{10}$) at 10 min of exposure ($p<0.05$). Nonetheless, in both treatments, samples washed with tap water always had the least HPC log reduction.

Table 1. Median log reductions in heterotrophic plate counts (HPC) of organic Romaine lettuce leaves washed with tap water and ozonated water (125 ppm) at different time of exposures.

WASH WATER	MEDIAN LOG REDUCTIONS (log_{10} CFU/g) IN HPC		
	TIME OF EXPOSURE (min)		
	2	5	10
Tap water	0.18^{A}	0.18^{A}	0.13^{AB}
Ozonated water (125 ppm)	0.86^{A}	0.77^{A}	1.18^{C}

(values with the same letter are not significantly different from one another)

In Table 2, disinfection of lettuce leaves with chlorinated and ozonated water resulted to similar HPC log reductions regardless of the time of exposure (p>0.05). Highest log reductions in HPC were obtained after 10 min of exposure in both treatments. On the other hand, exposure of lettuce leaves to ozonated water for 2 min was already enough to produce a log reduction comparable to that of the FDA-recommended chlorination.

Table 2. Median log reductions in heterotrophic plate counts (HPCs) of organic Romaine lettuce leaves washed with tap water and ozonated water at different time of exposures.

WASH WATER	MEDIAN LOG REDUCTIONS (log_{10} CFU/g) IN HPC		
	TIME OF EXPOSURE (min)		
	2	5	10
Chlorinated water	0.82^{AD}	1.10^{ABD}	1.20^{BC}
Ozonated water	0.86^{ABD}	0.77^{A}	1.18^{DC}

(values with the same letter are not significantly different from one another)

In all treatments, lowest HPC reduction (<0.2 log_{10}) was obtained from the tap water set-up. This is because plain water lacks antimicrobial property and any reduction in the HPC can only be attributed to the dislodging of the microorganisms on the leaf surface. Similar findings were reported by Baur, *et al.* (2004) who mentioned that washing shredded lettuce with tap water only produced an approximately 0.5 log_{10} HPC reduction in the initial counts. Same is true with iceberg lettuce in the studies of Delaquis, *et al.* (2004) and Lopez-Galvez, *et al.* (2013) who noted <1 log_{10} and 0.6 log_{10} HPC reduction with tap water, respectively.

The United States Food and Drug Administration (US FDA) recommends the use of 50-200 ppm chlorinated water for 1-2 min as disinfection for fresh-cut produce (Delaquis, *et al.*, 2004). In this study, the upper limit of that recommendation was used. Results of this study indicate that HPC log reductions achieved with the use of ozonated water at 125 ppm for 2-5 min are at par to the recommended USFDA disinfection (Table 2). Both treatments produced an HPC reductions between 0.77-0.86 log_{10}. This antimicrobial activity is attributed to the oxidizing nature of chlorine and ozone. Directly, ozone oxidizes several cellular constituents including proteins, lipids, and cellular enzymes. In vegetative cells, ozone oxidizes the peptidoglycan of the cell envelope which later leads to leakage of cell contents and ultimately to lysis. The same could be said with chlorine. In other studies, similar reductions ranging between 0.48-1.25 log_{10} were reported for aqueous ozone but at a lower concentration ranging between 0.5-7.5 ppm (Sengun, 2013; Garcia, *et al.*, 2003). Similar conclusion on the efficacy between aqueous ozone and chlorine was reported by Ölmez and Akbas (2009) who used 2 ppm aqueous ozone and 100 ppm chlorination, both for 2 min.

With regards to the artificially-inoculated set-up, the disinfection efficacy of ozonated water, in terms of ECC log reduction, when compared to the other two treatments are shown in Tables 3 and 4.

In Table 3, ECC log reduction of lettuce leaves washed with tap water did not increase as time of exposure was increased (p>0.05). All ECC reductions for tap water were <1 $\log_{10}$. As for the ozonated water disinfection, highest reduction (1.41 $\log_{10}$) was observed after 10 min of exposure but there was no increase in log reduction as time of exposure was increased. Nonetheless, ozonated water disinfection resulted to better ECC log reduction than tap water after 10 min (p<0.05). Congruent with the previous experiment, ECC log reduction was very minimal with tap water. Our result is also in agreement with other studies (Ölmez and Akbas, 2009; Wang, *et al.*, 2004).

Table 3. Median log reduction in *E. coli* counts of organic Romaine lettuce washed with tap water and ozonated water (125 ppm) at different time of exposures.

WASH WATER	MEDIAN LOG REDUCTIONS ($\log_{10}$ CFU/g) IN *E. coli*		
	TIME OF EXPOSURE (min)		
	2	5	10
Tap water	0.18^{A}	0.19^{A}	0.19^{A}
Ozonated water	1.31^{AB}	1.39^{AB}	1.41^{B}

(values with the same letter are not significantly different from one another)

Table 4 presents the comparison of the ECC log reduction in Romaine lettuce leaves between chlorinated and ozonated water. The table shows that chlorinated water resulted to same reductions (2.25-2.39 $\log_{10}$) across increasing time of exposure (p>0.05). Similar trend on ECC reduction (1.31-1.41 $\log_{10}$) could be noted for ozonated water treatments (p>0.05). Between the two treatments, it is observed that disinfection of organic produce using ozonated water resulted to log reductions that are equivalent to the efficacy of using chlorinated water at any time of exposure (p>0.05). This means that disinfection with ozonated water performed similarly with industry-known vegetable disinfection (chlorination).

Table 4. Median log reduction in *E. coli* counts of organic Romaine lettuce washed with chlorinated water (200 ppm) and ozonated water (125 ppm) at different time of exposures.

WASH WATER	MEDIAN LOG REDUCTION ($\log_{10}$ CFU/g) IN *E. coli*		
	TIME OF EXPOSURE (min)		
	2	5	10
Chlorinated water	2.25^{ABC}	2.35^{AC}	2.39^{C}
Ozonated water	1.31^{B}	1.39^{AB}	1.41^{BC}

(values with the same letter are not significantly different from one another)

Effect of wash water on the microbiological shelf life of organic Romaine lettuce

Figure 1 presents the changes in the HPC of lettuce leaves washed with three treatments throughout the 6-day storage period at 10 ºC. At day 0, all samples had an HPC range of 5.0-5.57 $\log_{10}$ CFU/g. The same initial microbial load was reported in the study of Baur, *et al*. (2004) where 4-6 $\log_{10}$

CFU/g of aerobic mesophiles were observed. The microbial loads of all treatments were checked against the recommended upper limit for HPC which is 7.7 $\log_{10}$ CFU/g (Baur, *et al*., 2004). By the end of the storage period, the samples washed with tap water exceeded this limit. Samples that were disinfected with chlorinated and ozonated water reached day 6 without exceeding the HPC limit. The aforementioned disinfection treatments preserved the microbial load of samples stored at 10ºC. On the other hand, a different observation in terms of final HPC (<7.7 $\log_{10}$ CFU/g) was reported in an earlier study after storage of lettuce leaves at 4ºC for 12 days (Olmez and Akbas, 2009). In this study, there was an increase of about 3 $\log_{10}$ CFU/g in the HPC at the end of the 6-day storage period. This increase is higher than the increase in HPC reported by Sengun (2013) which was about 1.15-1.79 $\log_{10}$ CFU/g in lettuce stored at 4ºC for 15 days. Differences in the reported final HPCs at the end of the storage period among these studies, including this one, could be attributed to the differences in temperature of storage during the experiments.

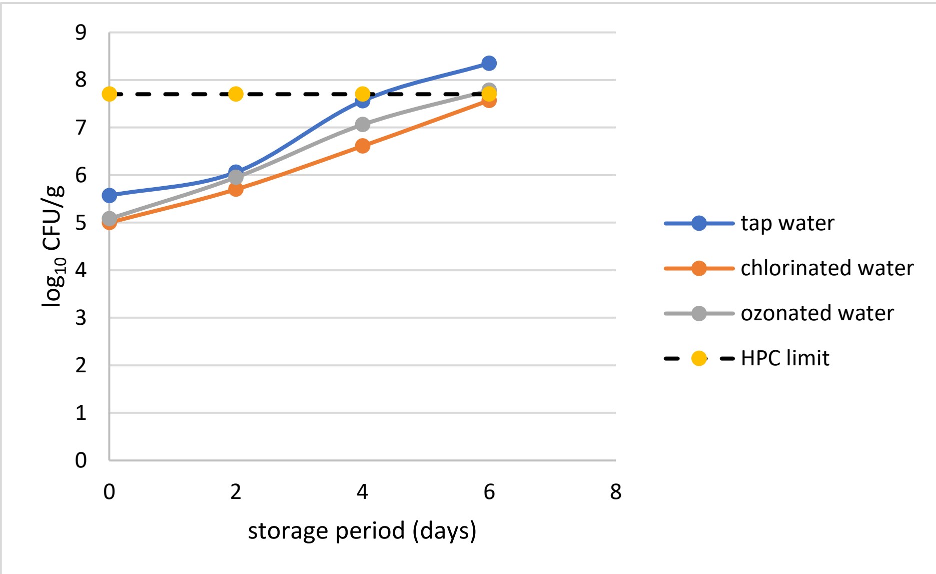

Figure 1. Changes in the heterotrophic plate count (HPC) of organic Romaine lettuce stored at 10ºC for six days and washed with tap water, chlorinated water (200 ppm), and ozonated water (125 ppm) for 2 minutes.

CONCLUSION

The use of ozonated water in the disinfection of organic Romaine lettuce leaves is as efficacious as disinfection using chlorinated water against the natural microbiota of the sample. Moreover, disinfection using ozonated water resulted to reduction of artificially-inoculated *E. coli* that is at par with chlorinated water disinfection. In addition to that, ozonated water disinfection preserved the microbiological quality of the samples stored for six days at 10ºC in as much as the disinfection with chlorinated water did. Thus, the use of ozonated water as fresh produce disinfectant can be a viable alternative to chlorination for organic farmers.

ACKNOWLEDGEMENT

The authors would like to thank Costales Nature Farms, DOST-SEI, and SEARCA-SEAMEO for their support in the conduct of this study.

STATEMENT OF AUTHORSHIP

B.R.R. Oliveros formulated the problem, gathered the primary data, and finalized the manuscript. I.F. Dalmacio gave useful suggestions during the conduct of the experiment and also made an initial draft of the manuscript. Both authors discussed the results and collaborated on the improvement of the manuscript.

REFERENCES

Achen, A. and Yousef, A.E. 2001. Efficacy of ozone against *Escherichia coli* O157:H7 on apples. *Journal of Food Science* 66:1380-1384.

Alexopoulos, A., Plessas, S., Ceciu, S., Lazar, V., Mantzourani, I., Voidarou, C., … Bezirtzoglou, E. 2013. Evaluation of ozone efficacy on the reduction of microbial population of fresh cut lettuce (*Lactuca sativa*) and green bell pepper (*Capsicum annuum*). *Food Control* 30:491–496. http://doi.org/10.1016/j.foodcont.2012.09.018

Baur, S., Klaiber, R., Hammes, W. P., & Carle, R. 2004. Sensory and microbiological quality of shredded, packaged iceberg lettuce as affected by pre-washing procedures with chlorinated and ozonated water. *Innovative Food Science and Emerging Technologies*, 5:45–55. http://doi.org/10.1016/j.ifset.2003.10.002

Bialka, K.L. and Demirci, A. 2007. Decontamination of *Escherichia coli* O157:H7 and *Salmonella enteric* on blueberries using ozone and pulsed UV-light. *Journal of Food Science* 72:M391-M396.

Cooper, J., Niggli, U. and Leifert, C. 2007. Handbook of Organic Food Safety and Quality. England: CRC Press, 443-449 pp.

Delaquis, P. J., Fukumoto, L. R., Toivonen, P. M. A., & Cliff, M. A. 2004. Implications of wash water chlorination and temperature for the microbiological and sensory properties of fresh-cut iceberg lettuce. *Postharvest Biology and Technology* 31:81–91. http://doi.org/10.1016/S0925-5214(03)00134-0

Garcia, A., Mount, J.R., and Davidson, P.M. 2003. Ozone and chlorine treatment of minimally processed lettuce. *Journal of Food Science* 68:2747-2751.

Golden, N., and Boyer, M. 2015. *Foodborne Illness Source Attribution Estimates for Salmonella, Escherichia coli O157 (E. coli O157), Listeria monocytogenes (Lm), and Campylobacter using Outbreak Surveillance Data*. 1-12 pp.

Khadre, M. A., and Yousef, A. E. 2001. Sporicidal action of ozone and hydrogen peroxide: a comparative study. *International Journal of Food Microbiology* 71:131–138. http://doi.org/10.1016/S0168-1605(01)00561-X

Lopez-Galvez, F., Ragaert, P., Palermo, L. A., Eriksson, M., & Devlieghere, F. 2013. Effect of new sanitizing formulations on quality of fresh-cut iceberg lettuce. *Postharvest Biology and Technology* 85:102–108. http://doi.org/10.1016/j.postharvbio.2013.05.005

Meikle, J. 2016. Mixed salad leaves linked to *E. coli* outbreak that has killed two in UK. Retrieved from https://www.theguardian.com/society/2016/jul/18/mixed-salad-leaves-linked-to-e-coli-outbreak-that-has-killed-two-in-uk (October 20, 2017)

Ölmez, H., and Akbas, M. Y. 2009. Optimization of ozone treatment of fresh-cut green leaf lettuce. *Journal of Food Engineering* 90:487–494. http://doi.org/10.1016/j.jfoodeng.2008.07.026

Schmid, O., A. Beck and Kretzschamar, U. (eds). 2004. Underlying principles in organic and "low-input food processing – literature survey. Germany: Research Institute of Organic Agriculture FiBl, 54-59 pp.

Sengun I.Y. 2013. Effects of ozone wash for inactivation of *S. typhimurium* and background microbiota on lettuce and parsley. *Journal of Food Safety* 33:273-281.

Wang, H., Feng, H., and Luo, Y. 2004. Microbial reduction and storage quality of fresh-cut cilantro washed with acidic electrolyzed water and aqueous ozone. *Food Research International*, 37:949–956. http://doi.org/10.1016/j.foodres.2004.06.004