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ULTRASONIC NEBULIZATION AS A NOVEL METHOD OF APPLICATION OF SANITIZERS IN FRESH MANGO

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Abstract: In this study, three application methods (immersion, jet spraying, and ultrasonic nebulization) of sanitizers were investigated on Ataulfo mangoes artificially contaminated with E. *coli and Salmonella*. For this evaluation, the following sanitizers were used: calcium hypochlorite, peracetic acid, and acid electrolyzed water; In addition, the effect of temperature on their physicochemical properties and their antimicrobial capacity was evaluated *in vitro*. Ultrasonic nebulization turned out to be more effective in the action of the three sanitizers studied. The treatments were more lethal when they were applied on the fruit inoculated with E. *coli* with respect to *Salmonella and* the same behavior was presented in the in vitro tests. The temperature had an effect on the pH, the free chlorine and the redox potential; however, these changes were not sufficient to affect antimicrobial properties in in vitro tests. Ultrasonic nebulization improved the bactericidal efficacy of all sanitizers, resulting in complete bacterial inactivation. This research shows that ultrasonic nebulization is a novel sanitizer application method with high potential for the fresh fruit and vegetable industry.

INTRODUCTION

Mexico is the largest mango exporter in the world (FAO, 2022) and its consumption is mostly fresh. Fruits and vegetables are highly recommended in the human diet; however, these foods are often an important vector of microbial disease for the consumer. Pathogens can be of human, animal or environmental origin and can be incorporated into food during preharvest and/or postharvest (Feliziani et al., 2016). Food safety, in microbiological terms, is a permanent concern, both for producers, regulatory agencies, public health institutions, as well as for consumers themselves. This is associated with the increase in cases and epidemiological outbreaks from this origin (Gould et al., 2017). In order to reduce the risk of infections associated with the consumption of fresh fruits and vegetables, the packaging industry implements strict controls on their sanitization, as it is considered one of the most important critical points. In the particular case of Mexico, there is a record of the presence of *Salmonella* and E. coli in mango fruit exports (Ibarra et al., 2015).

There is a variety of physical and chemical treatments to reduce the population of microorganisms in whole fresh products (Ramos et al., 2013), among them, a new generation of chemical agents that has gained interest and that include chlorine dioxide, ozone, organic acids, peroxyacetic acid, electrolyzed oxidizing water and hydrogen peroxide (Joshi et al., 2013). The effectiveness of these sanitizers is greatly influenced by their physicochemical properties, as well as by the application method and exposure time (Gil et al., 2009). There are two conventional methods in the application of sanitizers, immersion and spraying, both with high product consumption and risk of cross contamination; On the other hand, ultrasonic nebulization, as a sanitizer application method, has been poorly evaluated in fresh foods and could be a technically and economically viable alternative for the food industry (Cabanillas-Beltrán et al., 2020). The application of sanitizers by the ultrasonic nebulization method offers important advantages, among which are: significant reduction of the product necessary for its function and low risk of cross contamination.

The use of peracetic acid, as an alternative sanitizer to chlorine in fruits and vegetables, has gained popularity because it does not produce harmful products (Ramos et al., 2013). On the other hand, electrolyzed water has been successfully applied in the sanitization of a variety of foods, including fruits and vegetables (Pinto et al., 2015). Finally, sodium hypochlorite is a sanitizer widely used in fresh produce, such as lettuce and fresh pepper (*Capsicum annuum*) to control E. *coli* with good results (Mendoza and Cantor, 2012). The purpose of this study was to evaluate, in mango, the efficiency of ultrasonic nebulization, as a novel method in the application of sanitizers such as: peracetic acid, acid electrolyzed water and sodium hypochlorite.

MATERIALS AND METHODS

fruits. The mangoes (Ataulfo variety) were harvested in the municipality of "5 de mayo" in the state of Nayarit (21º 30 '0 "N, 104º 54' 0" W), Mexico. The mangoes were in a state of physiological maturity, with no apparent mechanical or insect damage, and were selected according to weight and color. The fruit was washed with soap, then rinsed with sterile distilled water and dried at room temperature (25 ºC) for 10 minutes in a biological safety hood.

Bacterial strains and inoculum preparation. *Salmonella spp*. and E. *coli* were provided by the State Public Health Laboratory of the State of Nayarit, in Mexico. Each pathogen was cultured separately in 100 ml of sterile trypticasein soy broth (TSB) (Bioxon), in 250 ml Erlenmeyer flasks at 37 ºC for 24 h, with shaking at 150 rpm. Subsequently, 10 ml of each strain were centrifuged at 4,000 g for 20 min, washed and resuspended in 10 ml of sterile peptone water (0.1%) at pH 7.1. The inoculum was adjusted to a concentration of 8 log CFU/ml by microscopic counting in a hemocytometer.

Sanitizers Acidic electrolyzed water was generated using a self-developed electrolysis reactor; containing an electrolytic membrane, platinum electrodes and 0.1% sodium chloride solutions (Sigma, USA) as electrolyte. This equipment was operated at 4.5 amps of direct current and 60-75 V. Acidic electrolyzed water was obtained at a rate of 0.5 L/min and collected in polypropylene containers, to be later used in the experiments. pH and ORP (oxidation-reduction potential or redox) readings were taken with the aid of a Hanna Instruments potentiometer (HI991003 Woonsocket, RI, USA); the concentration of available chlorine was measured through the HI 96734 equipment, from Hanna Instruments. Sodium hypochlorite (150 ppm, Maquisa, Mexico) and peroxyacetic acid (80 ppm, Maquisa, Mexico) were obtained directly from the manufacturers.

Physicochemical characterization of sanitizers. A volume of 200 ml of each sanitizer was placed in beakers and brought to temperatures of: 4, 25 and 35 °C for evaluation. In addition to pH and ORP, dissolved oxygen content was evaluated using a portable oximeter (YSI Pro-2030 model, USA).

In vitro antimicrobial test. The test was carried out with the protocol proposed by Venkitanarayanan et al. (1999), with some modifications. Briefly, a 9 mL volume of each sanitizer or sterile distilled water (as control) was transferred into sterile screw-capped tubes. To each tube containing 9 ml of treatments or sterile distilled water, 1 ml (108 CFU/ml) of the strains under study was added separately, and the samples were kept at 4, 25 and 35 °C for 15, 30 and 60 sec. After each incubation, the number of viable cells in each sample was determined; seeding 100 μL directly, or after serial dilutions in water with 0.1% peptone, on MacConkey agar (Bioxon, Mexico) for E. *coli* and *Salmonella* on XLD agar (Bioxon, Mexico) and incubating at 37 °C for 18 h.

In vivo antimicrobial test. The fruits were inoculated with 100 μL of 8.0 log CFU/mL of the Salmonella or E. coli strains and allowed to dry for 45 min in a biosafety hood to allow cell attachment. Then, the sanitizers were conditioned and applied at 4, 25, and 35 ºC to the artificially inoculated fruits. Three different application methods were used: ultrasonic misting, immersion and jet misting. Ultrasonic nebulization was performed using an ultrasonic aerosol generator (Mist Maker, model DK12, China), which has a liquid reservoir for 2.0 L. Separately, two liters of each treatment were placed in the ultrasonic nebulizer, operating at 1.70 MHz and 5.30 L/h. For the immersion application, polypropylene containers for a volume of two liters were used. Finally, the jet fog was applied using a Fogmaster (Tri-Jet® 6208, USA). Treatments were applied in a Novatech biosafety hood (Model CFLH-90, Mexico) for 1 min. All treatments were applied separately. Once the treatments were applied, the E. coli counts were performed as follows: individual fruits were placed in sterile stomacher bags (Seward, BA6041 / CLR, UK) containing 100 ml of sterile peptone water (0.1%), were washed and rubbed manually for 1 min to resuspend the microbial cells. Microbial counts were performed by inoculating 1 mL of the sample using 3M™ Petrifilm™ E. coli/Coliform Count Plates (USA). For Salmonella counts, fruits were individually placed in sterile stomacher bags containing 100 mL of 3M medium, washed, and gently rubbed manually for 1 minute to resuspend microbial cells. Then, the medium was incubated at 41.5 ºC for 18 h. Thereafter, the plate was covered with 2 ml of sterile peptone water (0.1%) for 1 minute. Finally, the sample (10 µl) was inoculated with a sterile inoculation loop into the 3M ™ Petrifilm ™ Salmonella Express System (USA) and incubated at 41.5 ºC for 18 h.

Data analysis. CFU data of bacterial counts were log-transformed prior to ANOVA to improve the homogeneity of variances. The experiments were repeated twice. Analysis of variance (ANOVA) of the data was performed using Statistica version 10. Differences between data means were compared by least significant differences (LSD). Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Physicochemical characterization of sanitizers. All sanitizers had significant changes ($P < 0.05$) in their pH and free chlorine values, as a function of exposure temperature (Table 1). The physicochemical properties decreased significantly as the test temperature increased. Similar findings have been previously reported (Li et al., 2014; Xie et al., 2012). In this same table it can be seen that the value of the redox potential (ORP) remained without significant change and it is important to point out that this property has a high influence on the sanitizing capacity of the substances.

In vitro antimicrobial test. The efficacy of the treatments on the in vitro microbial viability is presented in table 2. In general, E. *coli* was more sensitive to the application of all the sanitizers in the evaluated conditions. On the other hand, *Salmonella* was slightly more resistant to treatment with acidic electrolyzed water, since it took 30 s for a total inactivation of the microorganism with this sanitizer. In this case, the temperature and exposure time play an important role; at 4 ºC the efficacy was higher than at 25 and 35 ºC. In this sense, it coincides with what was stated by Wei et al. (2005), who assert that the temperature factor is important to maintain the bactericidal efficacy of acidic electrolyzed water. Venkitanarayanan et al. (1999) pointed out that acidic electrolyzed water is highly efficient against pathogenic bacteria with the following physicochemical characteristics: low pH (<3.0), high ORP (>1000 mV) and the presence of available chlorine. Even when a decrease in pH and free chlorine ($P < 0.05$) (Table 1) was detected in acidic electrolyzed water exposed to 25 and 35 ºC, such changes

Temperature	calcium hypochlorite			Peracetic acid			Acid electrolyzed water		
	pH	ORP (mV)	Free chlorine	pH	ORP (mV)	Free chlorine	pH	ORP (mV)	Free chlorine
4 °C	9.80± 0.01a	602± 0.01a	144± 0.01a	3.51± 0.01a	344± 0.01a	7.80± 0.02a	2.00± 0.02a	1076± 0.20a	3.90± 0.01a
25 °C	8.94± 0.01b	602± 0.01a	140± 0.01a	3.49± 0.01a	349± 0.01a	5.50± 0.01b	1.77± 0.01b	1069± 0.10a	3.04± 0.02b
35 °C	8.89± 0.01b	588± 0.01a	133± 0.01b	3.32± 0.01b	334± 0.01a	5.20± 0.01b	1.62± 0.02c	1059± 0.20a	2.85± 0.01b

TABLE 1. Physicochemical characterization of experimental sanitizers, exposed to different temperatures. Data in the same column with a different letter are significantly different (P < 0.05).

		Salmonella Lethality (log CFU/mL)			*E. coli* Lethality (log CFU/mL)		
Treatment	Temp. (°C)	15 s	30 s	60 s	15 s	30 s	60 s
Hypochlorite of calcium	4	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	25	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	35	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
Peracetic acid	4	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	25	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	35	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
acid electrolyzed water	4	8.00± 0.0aA	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	25	6.84± 0.21bB	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA
	35	5.60± 0.02cB	8.00±0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA	8.00± 0.00aA

TABLE 2. Efficacy of sanitizers exposed to different temperatures and applied in vitro, on microbial lethality.

For each exposure time, the values not followed by the same capital letter are significantly different (P < 0.05). For each temperature, the mean values that are not followed by the same lowercase letter are significantly different (P < 0.05).

were not low enough to affect the antimicrobial action.

Several mechanisms of action have been proposed for AEW including membrane damage, amino acid decarboxylation, reactions with nucleic acids, and oxidation enzymes (Hricova, et al., 2008; Pinto, et al., 2015; Ramos et al., 2103). The results suggest that the exposure time plays an important role in the bactericidal efficacy of acidic electrolyzed water, as reported by Kalchayanand et al. (2016). The application of calcium hypochlorite or peracetic acid was highly effective against both microorganisms at temperatures tested in a shorter time (15 s). Peracetic acid works by oxidizing the outer cell membrane of bacterial cells, inactivating them (Joshi et al., 2013); while chlorine acts against the cell membrane of the microorganism affecting the permeability, the zeta potential and the oxidative phosphorylation, which affects the vital functions of the bacteria (Venkobachar et al., 1977). The favorable results of the present study, regarding the sanitizers evaluated, coincide with in vitro studies carried out by authors such as: Banach et al., 2017; Cui et al., 2009; González et al., 2004; Kalchayanand et al., 2016 and Liao et al., 2007.

In vivo antimicrobial test. The bactericidal efficacy of sanitizers for the inactivation of *Salmonella* and E. coli was evaluated after inoculation on mango fruits (Figures 1 and 2). The effectiveness of the sanitizer was dependent on: the type of application, the applied sanitizer, and the bacterial strain. The treatments were more efficient against E. coli than Salmonella, which coincides with the in vitro tests developed in the present study. A total inactivation of E. coli was obtained with all treatments regardless of the application method. However, at 4 ºC the inactivation was not complete (6.39 log CFU / fruit) applying peracetic acid by ultrasonic nebulization.

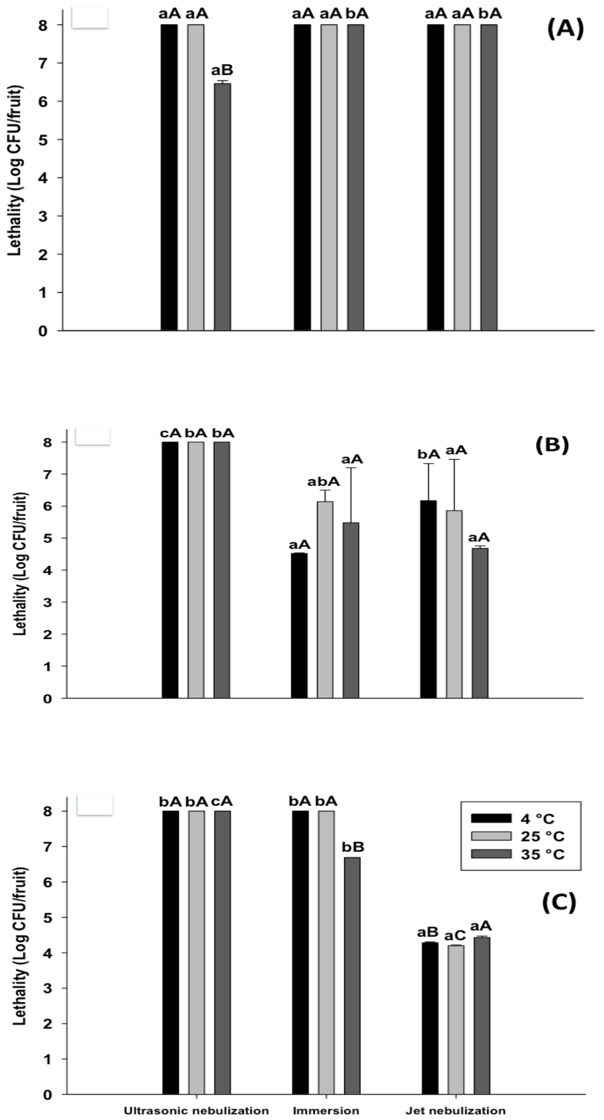

FIGURE 1. Evaluation of the efficacy of different sanitizers against Salmonella on the surface of the mango, after 1 minute of exposure to the sanitizers: A) Calcium hypochlorite; B) Peracetic acid and C) Acid electrolyzed water. For each temperature, mean values followed by a different capital letter are significantly different (P < 0.05). For each application method, mean values followed by a different lowercase letter are significantly different (P < 0.05). The vertical bars indicate the standard error of three replicates.

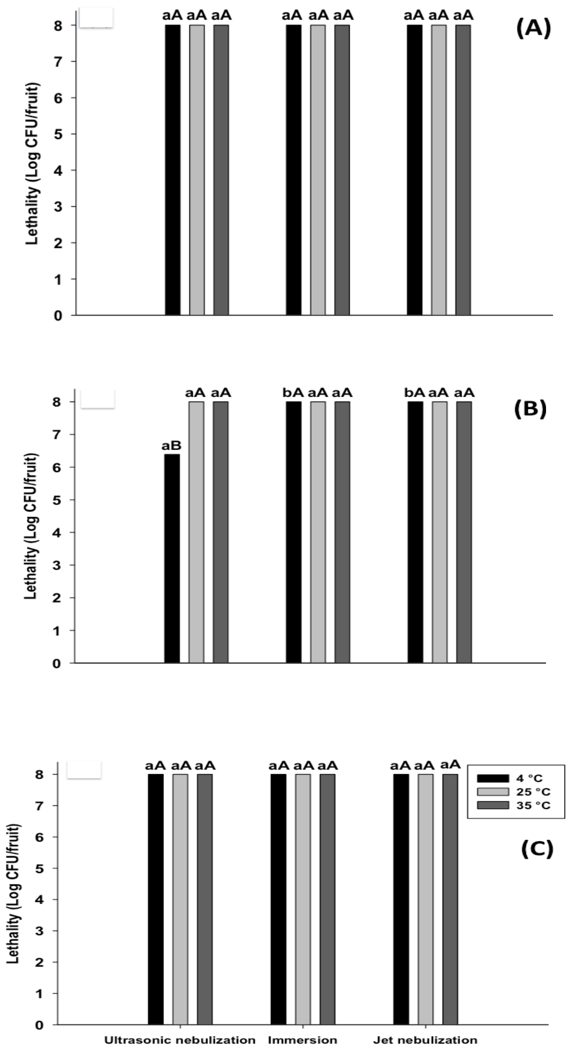

Figure 2. Evaluation of the efficacy of different sanitizers against E. coli on the mango surface, after 1 minute of exposure to the sanitizers: A) Calcium hypochlorite; B) Peracetic acid; C) Acid electrolyzed water. For each temperature, mean values followed by a different capital letter are significantly different ($P < 0.05$). For each application method, mean values followed by a different lowercase letter are significantly different ($P < 0.05$). The vertical bars indicate the standard error of three replicates.

This result suggests that the effectiveness of this sanitizer does not only depend on temperature; but also the method used for its application, due to the total inactivation of Salmonella obtained with the same sanitizer by ultrasonic nebulization. For Salmonella, the best results were obtained by applying calcium hypochlorite, regardless of the application method used, with total inactivation of the strain. Only at 35 ºC a low lethality reduction was obtained (6.46 log CFU / fruit), which suggests that temperature plays an important role in the efficacy of calcium hypochlorite by ultrasonic nebulization. In contrast, the effectiveness of peracetic acid and acid electrolyzed water was strongly affected by application methods, ranging from 4.6 log CFU/fruit (peracetic acid, jet spray) to 6.69 log CFU/fruit (acid electrolyzed water, immersion). In agreement with the results obtained in the present study, Kalchayanand et al. (2016) reported variations in the efficacy of different sanitizers against Salmonella and E. coli. Even though total inactivation of Salmonella was obtained in vitro by exposing the bacteria to sanitizers for 30 s, this exposure time was not enough to completely inactivate it on the surface of the fruit. In this sense, the surfaces of plant tissue can protect microorganisms from sanitizers due to the presence of pores (Beuchat, 2002). In addition, the ability of Salmonella to quickly colonize and adapt to plant surfaces is well known (Steenackers et al., 2012).

On the other hand, the resistance of Salmonella to different sanitizers could be related to the microbial association to form biofilms (Steenackers et al., 2012). Ultrasonic nebulization was found to be more effective against the target strains, compared to the other sanitizer application methods. The effectiveness of this technology could be related to the formation of a mist or aerosol (approximately 5 μm), which manages to effectively cover the surface of the bacteria and allow their inactivation; In addition, the physical condition of the generated fog could enhance the reactive capacity of its components, as is the case of hypochlorous acid. In addition, and particularly with acidic electrolyzed water, it is possible to create an oxidizing atmosphere, above 1,000 mV, which is hostile to all aerobic microorganisms.

CONCLUSIONS

These results show that the sanitizers evaluated are an attractive option to counteract the presence of experimental microorganisms. Ultrasonic fogging, as an application method, enhances the effectiveness of these sanitizers over a wide range of temperatures. This technology can be used on mangos to eliminate the microbial establishment of pathogenic bacteria. In addition, the amount of sanitizer and its costs can be reduced with this technology, due to the low amount that is required to apply to achieve its objective on the fruits and the risk of cross contamination would be significantly lower.

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