

Comparative evaluation between different concentrations of ozonized water on sanitizing of fresh-cut lettuce

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ABSTRACT

Ozone (O₃) is a potent oxidant and sanitizer, utilized in small concentrations and short contact time in order to inactivate bacteria, yeasts and molds, spores, protozoas and viruses. For this reason, its use has been recommended as an alternative treatment in traditional chlorinated compounds to reduce the microbial load. The aim of this article was to evaluate and compare the effectiveness of the ozonized water in the sanitization of fresh-cut lettuce. The samples were harvested in the rural zone of Campinas, SP, Brazil, transported to the laboratory and the toilet operation was carried out, removing the external and dirty leaves and also the heart. In sequence, a portion to constitute treatment 1 (T₁ - without washing) was removed and four other portions of leaves were separated and submitted to the following treatments: T₂) washing in water; T₃) immersion in ozonized water at 1.4 mg L⁻¹; T₄) immersion in ozonized water at 1.6 mg L⁻¹; T₅) immersion in ozonized water at 2.0 mg L⁻¹. The ozone concentration was measured by a commercially available kit (CHEMetrics, Vacu-vials, Ozone K-7402, Calverton, Va., U.S.A.). The efficiency of treatments was evaluated by microbial analysis of: mesophilic and psychrotrophic aerobic bacteria, total coliforms, *Escherichia Coli*, yeasts and molds, besides the presence of *Salmonella* spp. Physical-chemical analysis of pH and soluble solids was also performed. Sanitization was effective to diminish the total coliforms count, reducing initial contamination of >1.1x10³ MPN g⁻¹ to 2.3x10¹ MPN g⁻¹ (1.4 and 1.6 mg L⁻¹), and without detecting the presence of *E.coli* and *Salmonella* spp. To mesophilic aerobic and psychrotrophic microorganisms, the average initial contamination of samples was 1.9x10⁶ CFU g⁻¹ and 2.1x10⁶ CFU g⁻¹, respectively. After the treatment (1.6 mg L⁻¹), they were reduced to 6.3x10³ CFU g⁻¹ and to 2.8x10³ CFU g⁻¹, respectively. The largest reductions in microbial load were obtained when ozonized water at 1.6 mg L⁻¹ was used.

Keywords: Microbiology, food safety, treatment, Brazil

1. INTRODUCTION

The consumption of fresh-cut products has been increasing for their practicality, security and convenience. These products can be defined as fruits or vegetables, or combination thereof, which have been physically altered but remain in fresh state, with quality and safety for consumption for a long time and without changing the nutritional quality (Moretti, 2007). Then, the demand for more healthy diet with fresh food has been answered by the market of these

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products. Fresh-cut products are more perishable than the whole one (Brackett, 1987) and tissues injuries, depending of the handling and cutting, can decrease its quality and shelf-life by accelerating the respiration process. Moreover, handling promotes the contamination by microorganisms and the release of exudates cell provides nutrients for microbial activity. Therefore, security and microbiological quality of these products must be ensured, associated to maintenance of nutritional and sensorial qualities (Vanetti, 2007).

Concern about microbiological risks becomes pronounced in most of fresh-cut products which are consumed raw, in the form of salads, and human handling in some operations of minimum processing, included steps of washing, drying, sorting and packaging, increase the risk of contamination by pathogenic and borne microorganisms. The contamination of raw products consumed in Brazil was verified by several authors. This data emphasize the need for health surveillance in products and food establishments avoiding potential foodbornes (Oliveira *et al.*, 2006; Panza *et al.*, 2006; Souza *et al.*, 2005; Tancredi *et al.*, 2005; Takayanagui *et al.*, 2001; Veiga *et al.*, 2006). In Brazil, the knowledge about fecal contamination in vegetables began in 1945 when researchers detected presence of *Escherichia coli* in 29.3% of several vegetables in São Paulo city. Since then until the present day, food as leaves, parsley, tomatoes and juices are incriminated in world as causes of diseases transmitted by foods (DTF's) (Berbari *et al.*, 2001; Beuchat, 2002).

Wishing to control the production and marketing, and reduce the cases of hospitalization for DTF's, the Resolution RDC n° 12 of 01/02/2001, became a landmark in the country by ensuring microbiological standards for raw products (Brasil, 2001). Although, there is no specific legislation for some bacteria, as psychrotrophic and mesophilic, yeasts and molds, counts above 10^5 colony-forming units per gram of product (CFU g⁻¹) are undesirable due to potential risk of deterioration, loss of sensorial characteristics and compromising the food quality (Vitti *et al.*, 2004). The sanitizing with chorine-based products is widely recommended to delay or eliminate microbiological growth in fresh-cut vegetables. In economic matters, chlorine compounds are most often used (Bari *et al.*, 2003; Berbari *et al.*, 2001; Cardoso *et al.*, 2003; Lima *et al.*, 2001; Silva *et al.*, 2006; Vieites *et al.*, 2005), although the formation of trihalomethanes, carcinogenic compounds, is a disadvantage. Health surveys comparing trihalomethanes concentration in morbidity and mortality from cancer showed positive associations in some cases of carcinomas (Santos, 1989). Thus, interest for others techniques of disinfection which may replace chlorine has emerged. In some countries, sodium hypochlorite has been prohibited by strict laws and the trend is to extend this to others places, then it is necessary some alternatives to reduce or replace hypochlorite (Rico *et al.*, 2007). The regulation 21 C.F.R. § 173.315 of Food Drug Administration, approved the use of ozone in addition to sodium hypochlorite and others sanitizers to fresh and fresh-cut fruits and vegetables (FDA, 2007).

Ozone is a potent oxidizing and sterilizing method which was used for many years to water disinfection in units of treatment. In small concentrations and time of contact, it is enough to inactivate bacteria, yeasts, molds, spores, viruses and protozoa. Results about its effectiveness are so promising, but the susceptibility to microorganisms and action depends on type of product, dosage, application method (ozonated water or gas), temperature, pH, relative humidity and presence of organic substances (Prestes, 2007). Studies developed by Cavalcante (2007), with previously contaminated American lettuce showed that 1.0 mg L⁻¹ of ozonated water in 1 minute in the absence of organic matter is sufficient to reduce at least 6,57 and 5,27 logarithmic cycles of *E. coli* O157:H7 and spores of *B. subtilis*, respectively. According to Kim *et al.* (1999) the use of ozone in the process or storage extends the shelf-life of products such as fruits and vegetables,

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while preserving sensory attributes and do not produce toxic wastes to environment after treatment.

This study performed comparative assessment of ozonated water effectiveness on the sanitization of fresh-cut American lettuce.

2. MATERIAL AND METHODS

2.1 Raw material and processing

American lettuce acquired from a producer of agricultural region of Campinas, SP, Brazil, was used and the samples had been harvested manually and carried to the Laboratory of Postharvest Technology of Agricultural Engineering Faculty of the University of Campinas, where the experiments were realized. In the reception it had the operation of toilet, where the external and dirty leaves were removed, beyond the heart. Leaves had been separated one by one with a sharp knife and the parcel of treatment 1 (T_1 - without washing) was removed. After that, the following treatments were applied: T_2) washing in tap water; T_3) immersion in ozonated water at 1.4 mg L^{-1} ; T_4) immersion in ozonated water at 1.6 mg L^{-1} ; T_5) immersion in ozonated water at 2.0 mg L^{-1} . For the removal of the excess water of the previous step, 20 seconds of centrifugation was carried out, in domestic centrifugal machine, with angular speed average of 2200rpm, equivalent to 800g. Due to instability of ozone, which it hinders the storage; its generation becomes necessary *in situ*. The pilot equipment used can be visualized in the Figure 1.



Figure 1. Equipment for production of ozonated water.

Ozone was generated from an electric discharge of pure oxygen (99% of pureness) which made possible concentrations up to 2 mg L^{-1} . For the mixture of gas in liquid, a diffusing pipe was used, thus ozone gas was bubbled in water generating ozonated water for the tubing (ozonated water exit). The gas excess was destroyed of the external side for a filter with activated coal. Ozone concentration was measured by a commercially available kit (CHEMetrics, Vacu-vials, Ozone K-7402, Calverton, Va., U.S.A.). All stages of processing were followed for Good

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Manufacturing Practices. Room processing was maintained at 10 ± 2 °C and washing water at 7 ± 2 °C, except to treatment T₂ which water at ambient temperature was used. The efficiency of treatments was evaluated by counting of the following microorganisms: mesophilic and psychrotrophic aerobic bacteria, total coliforms, *Escherichia Coli*, yeasts and molds, besides the presence of *Salmonella* spp. Physical-chemical analysis of pH, soluble solids content was also performed.

The experiment was conducted in completely randomized design with 3 replications. Data were submitted to analysis of variance (ANOVA) and the averages compared by Tukey's test (5% probability), using the statistical package *Statistical Analysis System* (SAS Institute Inc., North Carolina, USA, 1989).

2.2 Analysis

2.2.1 Microbiological

Microbial analysis were executed according to methodology described by Silva *et al.* (2007).

- a) *Salmonella ssp*: traditional technique of detection by classical cultural method of presence/absence, ISO 6579 (2007) method, using a sample of 25g mixed manually with 225ml of peptone water, incubated at $37^{\circ}\text{C}\pm 1^{\circ}\text{C}/18\pm 2\text{h}$;
- b) Total count of psychrotrophic and mesophilic aerobic bacteria: performed by plating on the surface method, using Plate Count Agar (PCA). The samples were incubated at $35^{\circ}\text{C}\pm 1^{\circ}\text{C}/48\pm 2\text{h}$ for aerobic mesophilic and $7^{\circ}\text{C}\pm 1^{\circ}\text{C}/10$ days to total count of psychrotrophic aerobic. The reading was done in machine manual colony counter. The results were expressed as colony-forming units per gram of sample (CFU g⁻¹);
- c) Total count of yeasts and molds: by the standard count method in spread plating, using Dichloran Rose Bengal Chloramphenicol (DRBC) Agar. The samples were incubated at 22-25° C for five days. The results were expressed as colony-forming units per gram of sample (CFU g⁻¹);
- d) Total coliforms and *Escherichia coli*: were performed by Most Probable Number method (MPN). The inoculated tubes of Lauryl Sulphate Tryptose (LST) were incubated at $35\pm 0,5^{\circ}\text{C} / 24\pm 2\text{h}$ and the growth with gas production was observed. The confirmation test was performed with tubes of Billiant Green broth (VB) and *E.Coli* broth (EC).

2.2.2 Physical-chemical

Analysis were carried out according to methodology described by AOAC (1995).

- a) Total soluble solid content: direct reading in digital refractometer, using the homogenate pulp. The results were expressed as °Brix;
- b) pH: direct measurement by potentiometry, which is the immersion of digital pHmeter in the homogenized and crushed sample. The results were expressed as pH units;

3. RESULTS AND DISCUSSION

The samples of lettuce evaluated only the removal of leaves more external and damaged had total count of aerobic mesophilic of 1.9×10^6 CFU g⁻¹, which was compatible with the expected to natural microflora of vegetables (ICMFS, 1995). For yeasts and molds averages were higher than Mara Bachelli, Rívia Amaral, Benedito Benedetti. "Comparative evaluation between different concentrations of ozonized water on sanitizing of fresh-cut lettuce". International Commission of Agricultural and Biological Engineers, Section V. Conference "Technology and Management to Increase the Efficiency in Sustainable Agricultural Systems", Rosario, Argentina, 1-4 September 2009.

1.1×10^3 CFU g⁻¹. For total coliforms averages were higher than 10^3 MPN g⁻¹, denoting that may not have been observed good practices of the product chain (Table 1).

Table 1. Microbial load of fresh-cut lettuce after the treatments with and without ozonated water. Averages of 3 replications. Campinas, 2009.

Microorganism	Treatment				
	T ₁	T ₂	T ₃	T ₄	T ₅
Mesophilic*	1.9×10^6	4.1×10^4	7.1×10^4	6.3×10^3	1.3×10^4
Psychrotrophic*	2.1×10^6	1.4×10^4	4.8×10^4	2.8×10^3	<10 est
Yeasts and molds*	$>1.1 \times 10^3$	2.4×10^2	2.3×10^1	2.3×10^1	<1.8
Total coliforms**	$>1.1 \times 10^3$	2.4×10^2	2.3×10^1	2.3×10^1	<1.8

T₁= without washing; T₂= washed in tap water; T₃= 1.4mg L⁻¹; T₄= 1.6 mg L⁻¹; T₅= 2.0 mg L⁻¹

*Values in CFU g⁻¹;

**Values in MPN g⁻¹

Washing in tap water (T₂) contributed not only to remove dirtiness, but also to reduce microbial load from soil. The same results were found by Lund *et al.* (2005) that analyzed the use of sanitizers on microbial load reducing of fresh-cut cassava. For this study, total average of aerobic mesophilic was 4.1×10^4 CFU g⁻¹, to total coliforms, yeasts and molds the average was 2.4×10^3 MPN g⁻¹ showed that only the washing with tap water contributes to microbial reduction.

For the treatments applied on different concentrations of ozone was observed the reduction of initial count of total coliforms of $>1.1 \times 10^3$ MPN g⁻¹ to 2.3×10^1 MPN g⁻¹ at concentrations of 1.4 and 1.6 mg L⁻¹, and to <1.8 MPN g⁻¹ at 2.0 mg L⁻¹. These results are in accordance with the limit established by Brazilian legislation of 10^2 MPN g⁻¹ to vegetables (fresh, sanitized, refrigerated or frozen for direct consumption) (Brasil, 2001) that also recommend absence of *Salmonella* in 25g of sample. In this study none of the analyzed samples showed contamination by *Salmonella*. Average values found for yeasts and molds were $>1.1 \times 10^3$ CFU g⁻¹ initially. The application of ozone had passed to 2.3×10^1 CFU g⁻¹ to 1.4 and 1.6 mg L⁻¹ concentrations and <1.8 CFU g⁻¹ to 2.0 mg L⁻¹ concentration. Although not regulated by Law in Brazil, these values are within the limits related by Brackett (1994) and Berbari *et al.* (2001).

To aerobic psychrotrophic microorganisms, the initial average was of 2.1×10^6 CFU g⁻¹ and changed to 2.8×10^3 CFU g⁻¹ at 1.6 mg L⁻¹ concentration (T₄).

Flavor of fresh vegetables arises from volatile compounds with sugars and acids, according classification of Chitarra and Chitarra (2005), and the lettuce is not considered an acid product which indicates to be more favorable to the presence and development of pathogenic microorganisms during storage. Therefore, it is necessary to measure the sugar content to indentify the availability of these sugars to the microorganisms.

Results of soluble solids and pH are in Table 2. For soluble solids, the sample without treatment (T₁) presented values of 2.35° Brix and was statistically different from product washed in tap water (T₂). All concentrations of ozonated water (1.4, 1.6 and 2.0 mg L⁻¹) had not showed significant changes between them.

The initial value of the pH was 5.84 (T₁) and after the washing process with water or ozone, it arisen up to a maximum of 6.02 (T₅). These values are close to the ones found by Freire Jr (1999)

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and Bolin and Huxsoll (1991) that were 5.9 and 6.0, respectively. The samples treated with concentrations of 1.6 (T₄) and 2.0 mg L⁻¹ (T₅) were statistically equal to pH, however differ from the others treatments and are expected to plants tissues which is between 5-7, according Chitarra and Chitarra (2005). This range is very suitable to bacteria and fungi growth.

Table 2. Results of pH and soluble solids according with the different treatments with and without ozonated water. Average values of 3 replications. Campinas, 2009.

Analysis	Treatment				
	T ₁	T ₂	T ₃	T ₄	T ₅
pH	5.84 B	5.88 B	5.85 B	5.98 A	6.02 A
Soluble solids (°Brix)	2.35 A	1.56 B	2.06 AB	2.13 AB	1.75 AB

T₁= without washing; T₂= washed with tap water; T₃= 1.4mg L⁻¹; T₄= 1.6 mg L⁻¹; T₅= 2.0 mg L⁻¹
Averages followed by distinct capital letters in the same line differ between itself to the level of 5% of probability, for Tukey's test.

4. CONCLUSION

The treatment with ozonated water at 1.6 mg L⁻¹ was the most effective to promote the greatest reductions in microbial load of minimally processed lettuce. Washing with tap water was an important and beneficial step on reducing of microorganisms in fresh-cut lettuce. The disinfection process adopting ozonated water showed capable to benefit in the maintenance of sanitary quality of fresh-cut American lettuce.

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