# Effects of ozonated water as a sanitizer on quality and safety of fresh baby leaves red chard

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**Summary**
In recent years, an increased demand worldwide for minimally processed products, especially leafy green salads, has been linked to an increased number of outbreaks of human gastroenteritis caused by associated food-borne pathogens, including *Escherichia coli*. In this study, treatment with ozone of simulated contaminations with *E. coli* (6–7 log CFU·g$^{-1}$) on the baby leaves red chard at harvest and in the water used for washing were evaluated. Leaves (inoculated directly on leaves or inoculated in wash water) were washed by immersion in water with various doses of ozone (0 mg·l$^{-1}$, 0.5 mg·l$^{-1}$ and 1.0 mg·l$^{-1}$) for 3 min. Then they were packed and stored at 5 °C for 12 days. On each day of evaluation, *E. coli* counts as well as leaf colour, respiration rate, chlorophyll, total phenolics content and antioxidant capacity were evaluated. The results indicated that simulated *E. coli* contamination on postharvest leaves was more difficult to reduce by ozonated water than contamination that occurred during the washing phase. Baby leaves red chard washed in ozonated water kept the quality parameters such as leaf colour, total phenolics content and antioxidant capacity during storage at 5 °C for 12 days.

**Keywords**
*Beta vulgaris*; fresh cut; ozone; safety; vegetables; phenolics

In recent years, an increased demand worldwide for minimally processed products, especially leafy green salads [1], has been linked to an increased number of outbreaks of human gastroenteritis caused by food-borne pathogens associated with these salads [2], including *Escherichia coli* [3]. Studies investigated potential sources of contamination in the supply chain at preharvest (in the field) and postharvest phases [4].

At the preharvest phase, populations of pathogens can be established during cultivation. Therefore, the risk may be increased after harvest either by direct contamination or proliferation of existing pathogen populations during processing and postharvest handling procedures [5].

During the washing stage of minimally processed vegetables, sanitizers are used in an attempt to reduce their microbiological load. Water is widely used in the washing stage and one of the challenges for the minimally processed vegetable industry is to reduce water and wastewater consumption rates [6]. Most commercial washing processes use recycled water treated with a sanitizer [7]. According to López-Gálvez et al. [8], inoculated fresh-cut lettuce washed with sodium hypochlorite, lactic acid, citric acid and peroxyacetic acid, and using tap water as the control, had little effect on *E. coli* on iceberg lettuce leaves. These results demonstrated the risks associated with recycling of the washing water due to recontamination and/or cross-contamination.

Sodium hypochlorite is widely used in the food industry as it is easy to apply and is economically effective [9, 10]. The agent is a potent oxidant, the efficacy of which depends on pH, temperature and concentration of the organic matter present in the water [11]. However, its safety has been questioned because it is a possible source of

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halogenated compounds such as chloramines or trihalomethanes, which may be harmful to human health [6].

Therefore, efforts have been made to find alternatives to chlorine. Ozonated water is an alternative because it is effective at low concentrations and for short exposure periods, and is not considered toxic for use in food [12]. BELTRÁN et al. [13] and AGUAYO et al. [14] mentioned that it is urgent to find a replacement sanitizer for chlorine and ozonated water can be an alternative for washing minimally processed lettuce and tomato. According to antimicrobial efficacy studies on lettuce, reductions of 1.5 log CFU·g$^{-1}$ to 2.5 log CFU·g$^{-1}$ in the microbiological flora were obtained by ozonated water at 1.5 mg·l$^{-1}$ to 3.0 mg·l$^{-1}$, respectively [5]. In addition, it was reported that effectiveness of ozone treatment agains microbial contamination did not increase above 3 mg·l$^{-1}$ [15].

The background information shows that treatment with $O_3$ can cause loss of antioxidant compounds and chlorophyll content in vegetables due to its strong oxidizing activity [16]. According to BELTRÁN et al. [13], ozonated water can be used as a sanitizer for fresh-cut lettuce due to good retention of organoleptic properties and browning delay with no detrimental reduction in the antioxidant components. For tomato slices, AGUAYO et al. [14] recommended washing with 0.4 mg·l$^{-1}$ ozonated water for no longer than 3 min because extended exposure did not decrease the microbial counts and possibly extra time in ozone could decrease other components of the fruit tissue.

Therefore, in view of the paucity of published data [13, 14, 17], the aim is to ascertain the reaction of these compounds when vegetables are washed in ozonated water. Given that consumers consider red chard leaves very attractive, evaluating the sanitizing effect of $O_3$ on freshness and quality of these leafy vegetables is relevant for the minimally processed food industry. In this study, conditions of contamination with *E. coli* directly on the baby leaves and in water used for washing were simulated and, in both cases, ozone was used as the sanitizer. So the aim of this study was to evaluate the effect of washing with ozonated water on *E. coli* survival and the quality of minimally processed red chard leaves.

## MATERIALS AND METHODS

### Plant material

The red chard (*Beta vulgaris* L. var. cicla cv. SCR 107) was grown using the nutrient film technique (NFT) hydroponic system under clean closed conditions. The baby leaves were harvested manually at commercial maturity, defined as length between 9 cm and 11 cm. Leaves were carefully washed under tap water.

### Bacterial strain and inoculation

*E. coli* ATCC 35218TM (American Type Culture Collection, Manassas, Virginia, USA) was used in this study. Stock cultures were kept at –80 °C in tryptone soy broth (TSB, Merck, Darmstadt, Germany) supplemented with 10% glycerol until use for less than 7 days. To activate the strain, it was incubated for 12–24 h at 37 °C in TSB with agitation to reach 6–7 log CFU·ml$^{-1}$ [18]. Volumes of 100 $\mu$l and 1 ml of broth culture were inoculated onto the upper (adaxial) surface of the red chard leaves (1 kg) and washing water (1 l), respectively. Both were kept at 22 °C for 24 h before processing to allow the growth of the bacteria as an acclimation short period.

### Minimal processing of red chard

Red chard leaves were processed in a cold room at 7 °C as recommended by the fresh cut industry [8]. A cold room was used because lower temperatures improve the stability of ozone in water compared to room temperature. Briefly, inoculated leaves were after 24 h washed by dipping in ozonated water containing various concentrations of ozone: 0 mg·l$^{-1}$ (D0), 0.5 mg·l$^{-1}$ (D05) and 1.0 mg·l$^{-1}$ (D1). The ozonated water was used immediately after the desired ozone concentration was reached, considering a time of 3 min washing per treatment and 0.5 kg red chard per 5 l washing water. These conditions had been optimized in a previous study under constant agitation and enough leaves separation. In the same way, uninoculated leaves were treated with inoculated water administering the same doses of ozone as previously mentioned. The treatments were performed as follows: D0 + *E. coli* leaves (D0L); D0.5 + *E. coli* leaves (D05L); D1 + *E. coli* leaves (D1L); D0 + *E. coli* washing water (D0W); D0.5 + *E. coli* washing water (D05W); D1 + *E. coli* washing water (D1W) (Tab. 1). Ozonated water was prepared using an ozone generator QLO-40G (QLozone, Changsha, Hunan, China) injected by the air stone of 10 cm of diameter located at the bottom of the container. The concentration of ozone in water was measured by a portable $O_3$ analyzer I-2019 (CHEMetrics, Midland, Virginia, USA).

Subsequently, leaves were centrifuged manually (Ilko, Santiago, Chile) and drained on a stainless steel mesh for 5 min. Then, 40 g of

leaves were packed in 0.15 m × 0.25 m perforated polypropylene bags (NY5/NY10/MPE45; Plaspak, Santiago, Chile). The gas permeability of the film was 25 ml $O_2$ $m^{-2}·d^{-1}$ and 71 ml $CO_2$ $m^{-2}·d^{-1}$ (data provided by the supplier). The bags were sealed with an impulse heat sealing machine model 300 (Zhejiang, Wenzhou, China) and stored at 5 °C for 12 days. Each bag with 40 g of leaves corresponded to one repetition following the methodology described by Silveira et al. [19]. All measurements were taken in triplicate.

### Measurements

All measurements were taken on the red chard leaves on day 0 (2–4 h after processing), day 1, day 4, day 8 and day 12 of storage at 5 °C. The samples for the analysis of chlorophyll, total phenolics content and total antioxidant capacity were frozen at –80 °C for approximately 30 days until their measurement.

### *E. coli* survival

*E. coli* survival was determined using a sample of 10 g of leaves per replicate. Serial dilutions were prepared in phosphate buffered saline for plating. *E. coli* were quantified using eosin methylene blue (EMB) agar (Merck) after incubation at 37 °C for 2 days [18]. Also, total aerobic mesophilic bacteria were quantified on plate count agar (PCA, Merck) incubated at 37 °C for 2 days.

### Colour

The colour changes of the red chard leaves were measured using a tristimulus colorimeter Minolta CR-300 (Konica Minolta, Ramsey, New Jersey, USA) following the methodology described by Silveira et al. [19]. The measurements were performed on the adaxial sides of 10 leaves per repetition and the colour parameters were expressed as lightness ($L$), chroma ($C$) and hue angle ($H$).

### Respiration rate

The respiration rate was evaluated according to the methodology described by Char et al. [20]. Red chard leaves (approximately 40 g) were placed in airtight glass containers (approximately 1 000 ml capacity). The increase in $CO_2$ content in the headspace was monitored using a gas chromatograph 5890 Series II equipped with a thermal conductivity detector (Hewlett Packard, Palo Alto, California, USA). The respiration rate was expressed as milligrams of $CO_2$ per kilogram and hour. One glass container was considered a repetition per treatment.

**Tab. 1.** Description of treatments applied to red chard baby leaves.

| Sample | $O_3$ concentration [mg·l$^{-1}$] | Object of inoculation with *Escherichia coli* |
|---|---|---|
| **$O_3$ + *Escherichia coli* on leaves** | | |
| D0L | 0.0 | Leaves |
| D05L | 0.5 | Leaves |
| D1L | 1.0 | Leaves |
| **$O_3$ + *Escherichia coli* in washing water** | | |
| D0W | 0.0 | Water |
| D05W | 0.5 | Water |
| D1W | 1.0 | Water |

### Chlorophyll content

To obtain the extract, 0.4 g of frozen sample from each repetition were homogenized with 5 ml of acetone (80 : 20, v/v) by Ultra-Turrax homogenizer T18 (IKA, Staufen, Germany) at 58 Hz during 45 s and then centrifuged at 5 000 ×$g$ for 15 min. For quantification, the supernatant was used to determine chlorophyll a (Chla) and chlorophyll b (Chlb) content according to Grimalt et al. [21]. The extract absorbance was measured at wavelengths 646 nm and 663 nm employing a UV-Vis spectrophotometer T70 (HCS Scientific and Chemical, Singapore, Singapore). Eq. 1 and Eq. 2 were used to determine the individual levels of chlorophylls *a* and *b*.

$$C_{Chla} = 12.25A_{663} - 2.79A_{646} \quad (1)$$

$$C_{Chlb} = 21.50A_{646} - 5.10A_{663} \quad (2)$$

where $C_{Chla}$ is content of chlorophyll a, $C_{Chlb}$ is content of chlorophyll b, $A_{663}$ is absorbance at 663 nm and $A_{646}$ is absorbance at 646 nm. Results were expressed as grams per kilogram of fresh weight (FW).

### Total phenolics content

The total phenolics content (*TPC*) was determined by the Singleton and Rossi method [22] with some modifications. To extract the sample, 5 g of frozen sample obtained from each repetition were weighed into a 50 ml tube and 20 ml of methanol were added. The sample was homogenized at 58 Hz for 30 s with a homogenizer IKA T18. Next, the supernatant was filtered and centrifuged for 15 min at 5 000 ×$g$ at 4 °C. A volume of 0.5 ml of 0.25 mol·l$^{-1}$ Folin-Ciocalteu reagent and 0.5 mol·l$^{-1}$ $Na_2CO_3$ were added. Absorbance of the sample was measured at a wavelength of 725 nm using a multi-plate spectrophotometer (Biochrom Asys UVM 340; Biochrom, Cambridge, United Kingdom). A calibration curve

was made with gallic acid and *TPC* was expressed as grams of gallic acid equivalents (GAE) per kilogram FW.

### Total antioxidant capacity

Total antioxidant capacity (*TAC*) of the extracts was determined using two methods, namely, by the violet radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [23] with some modifications and the ferric reduction antioxidant power (FRAP) method with modifications [24]. Sample extraction was performed following the same methodology as described for *TPC*.

For the DPPH method, 194 $\mu$l of the DPPH solution was added to 21 $\mu$l of extract and measured at 517 nm after waiting 30 min at room temperature (25 °C) using a multi-plate spectrophotometer Biochrom UVM 340.

Antioxidant activity by the FRAP method was done by adding 198 $\mu$l extract to 6 $\mu$l of the FRAP reagent. The absorbance was measured at 593 nm after waiting 30 min at room temperature (25 °C) using a multi-plate spectrophotometer Biochrom UVM 340.

The results from both methods were expressed as milligrams of Trolox equivalents (TE) per kilogram FW.

### Statistical analysis

The experiment was set up as a two-factor factorial design: $O_3$ doses (0 mg·l$^{-1}$, 0.5 mg·l$^{-1}$ and 1 mg·l$^{-1}$) and inoculation (leaves and washing water). The data were subjected to analysis of variance (ANOVA) to determine the significant differences between means using the Infostat software (2015 version, National University of Córdoba, Córdoba, Argentina). Tukey's multiple range test was conducted for the separation of means at a significance level of $p \leq 0.01$ or $p \leq 0.05$. One bag corresponded to one repetition and three were used for each measurement and day of evaluation.

## RESULTS AND DISCUSSION

### Microbial growth

Inoculated leaves had a load of 4.9 log CFU·g$^{-1}$ of *E. coli*, while inoculated washing water reached an initial load of 4.8 log CFU·g$^{-1}$ (Fig. 1). According to the statistical analysis, interaction was noted between the $O_3$ doses and inoculation factors ($p = 0.0387$). On day 0, ozonated water caused a significant reduction in *E. coli* compared to the control (D0W), by approximately 1 log CFU·g$^{-1}$. The initial load reduction was 1.45 log CFU·g$^{-1}$ for the D1W treatment. Therefore, at the beginning both inoculated leaves and water showed a significant reduction in *E. coli* by $O_3$ treatments.

The same trend was observed on day 1 for inoculated leaves treated with ozonated water. On day 4, D05L reached the lowest count of 3.44 log CFU·g$^{-1}$ followed by D1L. The rest of the treatments had counts above 4 log CFU·g$^{-1}$. On day 8, D0W and D1W showed the lowest counts. At the end of storage (day 12), ozone doses of 0.5 mg·l$^{-1}$ and 1.0 mg·l$^{-1}$ applied in inoculated washing water achieved significantly lower loads of 3.85 log CFU·g$^{-1}$ to 3.87 log CFU·g$^{-1}$ compared to 0 mg·l$^{-1}$ (D0W).

The total mesophilic counts of the raw material were 5.4 log CFU·g$^{-1}$ after inoculation, whereas the microbial load of washing water was

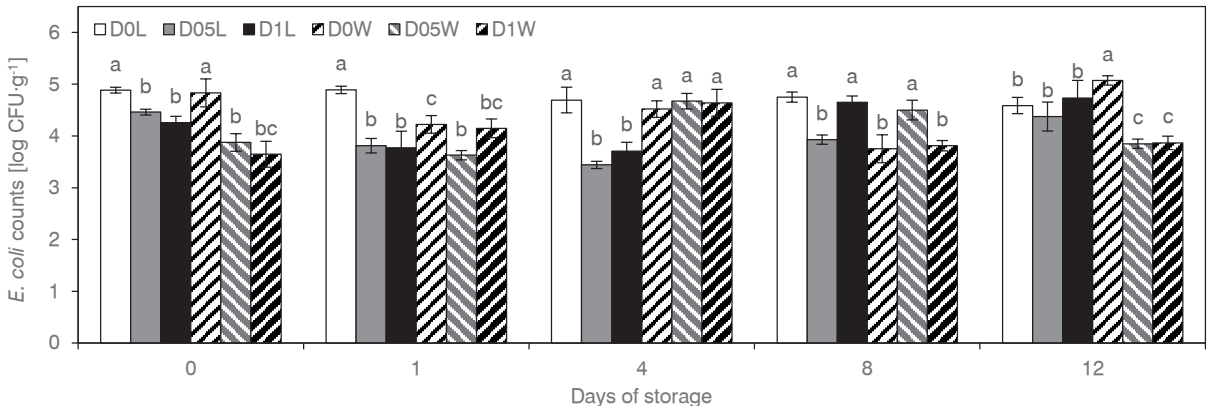


**Fig. 1.** Counts of *Escherichia coli* on red chard baby leaves washed in ozonated water and stored for 12 days at 5 °C.

Bars marked by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).
Designation of samples is explained in Tab. 1.

5.1 log CFU·g$^{-1}$ (data not shown). On day 12, the mesophilic counts increased to 6.3–6.7 log CFU·g$^{-1}$ (data not shown).

The washing step in the minimally processed food industry has economic and environmental implications mainly because of the large amount of water needed. One challenge is to reduce the water consumption and wastewater discharge rates. Depending on the food sector, it is estimated that a 20–50% reduction in water consumption can be achieved by recycling and reusing water [6]. One way is to disinfect the water with a suitable sanitizer. The amount of wastewater generated per mass unit of product is dependent on the decontamination technique used. A technique capable of efficiently decontaminating both the washing water process and minimally processed vegetables will allow a high recycling ratio, thereby reducing the wastewater rates [25].

The application of $O_3$ in inoculated washing water at 0.5 mg·l$^{-1}$ and 1 mg·l$^{-1}$ decreased the *E. coli* load on the red chard leaves by 1.5 log CFU·g$^{-1}$ after 12 days of storage at 5 °C. However, the applications of water with $O_3$ on inoculated leaves decreased the load of *E. coli* by 1 log CFU·g$^{-1}$ after 8 days of cold storage. These results showed that *E. coli* inoculated on the baby leaves was more difficult to reduce than that applied in ozonated water. This difference could be because bacteria can easily attach to the leaf or penetrate the stomata [9]. However, using inoculated water for washing, the contact time between bacteria and plant tissue was shorter, so less *E. coli* were found compared to inoculated leaves as occurred in the field with contaminated vegetables. Therefore, the best recommendation to guarantee the safety of minimally processed red chard baby leaves is to avoid all possible contamination from the field because later it is harder to reduce microbial counts during processing after harvest.

When the fresh product becomes contaminated with *E. coli*, the pathogen may adapt to the processing step and survive during refrigerated transportation [26]. Thus, *E. coli* survived on baby spinach leaves for 7 days of refrigerated storage, with minimum changes in populations [27]. *E. coli* O157:H7 was previously found to attach in greater numbers on cut lettuce than on whole leaf surfaces, whereas another pathogen *Salmonella typhimurium* reached equal counts both on intact and cut tissues [28]. It was reported that *E. coli* O157:H7 cells penetrate cut lettuce surfaces more efficiently at 4 °C than during storage at 7 °C, 25 °C or 37 °C [29]. The same authors noted that cut surfaces inoculated with *E. coli* immersed in 200 000 mg·l$^{-1}$ of free chlorine reduced the microbial counts. According to Sapers [30], *E. coli* becoming internalized in leafy vegetables may represent an additional problem for sanitizers. Takeuchi and Frank [31] indicated that *E. coli* O157:H7 in stomata are protected from washing solutions. These authors showed that penetrated cells in the stomata were more protected from elimination by free chlorine than cells on a damaged tissue surface or intact surfaces of lettuce.

$O_3$ is a strong oxidizer with a high bactericidal potential [12], attractive for decontaminating minimally processed vegetables. In accordance with this current study, Selma et al. [32] applied aqueous ozone to shredded lettuce at 5 mg·l$^{-1}$ for 5 min and achieved a 1.8 log CFU·g$^{-1}$ reduction in *Shigella sonnei*. In the same way, the data shown in this work were also in agreement with those found by Vurma et al. [27], who used $O_3$ (5–10 mg·l$^{-1}$) in its gaseous form to decontaminate spinach leaves, obtaining a 1.8 log CFU·g$^{-1}$ reduction in *E. coli* O157:H7. Similarly, Rosenblum et al. [33] treated processed water contaminated with *Bacillus subtilis* with 2 mg·l$^{-1}$ $O_3$ for 10 min, causing a 1.56 log CFU·ml$^{-1}$ reduction.

### Colour

According to the statistical analysis, on days 0, 1 and 4, values of lightness ($L$) did not show any differences between factors, ranging from 44.4 to 46.6 (Tab. 2). After 8 and 12 days of storage, significant differences were found between samples treated with ozone of different concentrations. $L$ values significantly increased due to $O_3$ treatment. Non-significant differences were found for the inoculation factor.

Chroma was not affected by the $O_3$ concentration or the inoculation factors, reaching values from 23.1 to 31.0 throughout storage (Tab. 2). For hue angle, non-significant differences were found. All data ranged from 117.5° to 124.2° (Tab. 2).

Colour is one of the most important parameters to influence red chard quality and one of the biggest postharvest problems is rapid senescence of these leaves, which is manifested as yellowing caused by the breakdown of chlorophyll [34]. In a previous study, spinach treated with 0.106 mg·ml$^{-1}$ of $O_3$ manifested slight changes in leaf colour. An increase in the $O_3$ inlet concentration to 2.11 mg·ml$^{-1}$ resulted in the formation of light green spots after the first day of treatment and more pronounced leaf bleaching took place as the exposure time increased from 1 to 3 days [35]. However, in this study the colour of the red chard did not change after application of ozonated water.

**Tab. 2.** Colour parameters of red chard baby leaves after treatment.

| Treatment | | Day of storage | | | | |
|---|---|---|---|---|---|---|
| | | Day 0 | Day 1 | Day 4 | Day 8 | Day 12 |
| **Lightness** | | | | | | |
| Ozone concentration | 0.0 mg·l$^{-1}$ | 44.4 ± 1.5 ** | 45.2 ± 2.7 | 44.6 ± 2.3 | 43.9 ± 1.3 $^{c}$ | 45.8 ± 2.3 $^{c}$ |
| | 0.5 mg·l$^{-1}$ | 45.7 ± 2.3 | 44.6 ± 1.4 | 45.3 ± 3.1 | 45.9 ± 2.1 $^{b}$ | 46.3 ± 1.2 $^{b}$ |
| | 1.0 mg·l$^{-1}$ | 45.2 ± 2.4 | 44.6 ± 2.3 | 46.6 ± 2.6 | 48.6 ± 2.1 $^{a}$ | 50.3 ± 2.4 $^{a}$ |
| Inoculation | Leaves | 44.2 ± 2.6 ** | 43.2 ± 3.1 | 43.2 ± 2.4 | 43.4 ± 2.5 | 43.8 ± 2.8 |
| | Water | 45.9 ± 3.2 | 44.4 ± 2.3 | 43.3 ± 2.8 | 44.6 ± 2.4 | 43.1 ± 3.2 |
| **Chroma** | | | | | | |
| Ozone concentration | 0.0 mg·l$^{-1}$ | 28.9 ± 2.8 ** | 26.3 ± 1.4 | 26.3 ± 2.4 | 24.6 ± 2.3 | 23.6 ± 2.8 |
| | 0.5 mg·l$^{-1}$ | 27.7 ± 2.5 | 27.6 ± 2.1 | 26.5 ± 2.7 | 27.5 ± 2.3 | 23.1 ± 2.1 |
| | 1.0 mg·l$^{-1}$ | 27.0 ± 1.7 | 27.3 ± 1.9 | 27.3 ± 1.7 | 28.1 ± 3.1 | 26.6 ± 2.3 |
| Inoculation | Leaves | 29.8 ± 1.5 ** | 28.1 ± 2.8 | 26.8 ± 2.2 | 27.6 ± 1.4 | 25.6 ± 2.7 |
| | Water | 31.0 ± 2.6 | 29.8 ± 2.4 | 28.7 ± 2.7 | 26.5 ± 2.6 | 25.3 ± 2.3 |
| **Hue angle** [°] | | | | | | |
| Ozone concentration | 0.0 mg·l$^{-1}$ | 120.4 ± 1.9 ** | 121.1 ± 2.5 | 118.6 ± 2.3 | 119.9 ± 2.5 | 124.2 ± 2.6 |
| | 0.5 mg·l$^{-1}$ | 120.4 ± 2.1 | 122.8 ± 2.9 | 123.6 ± 2.2 | 120.3 ± 2.1 | 121.4 ± 2.9 |
| | 1.0 mg·l$^{-1}$ | 119.8 ± 2.3 | 121.6 ± 2.1 | 120.3 ± 1.9 | 121.5 ± 2.8 | 123.7 ± 2.6 |
| Inoculation | Leaves | 121.3 ± 3.1 ** | 119.2 ± 2.2 | 122.6 ± 3.2 | 123.5 ± 2.6 | 119.3 ± 2.1 |
| | Water | 120.9 ± 2.6 | 118.3 ± 2.3 | 119.4 ± 2.7 | 121.3 ± 2.4 | 117.5 ± 2.8 |

Red chard baby leaves were washed with ozonated water and stored for 12 days at 5 °C. Values represent means of three repetitions (ten leaves per treatment) ± standard deviation. Table shows the statistical analysis per factor according to the factorial design. No interaction between factors (ozone doses × inoculation) was found. Different letters in superscript compare per day and indicate statistical differences at $p = 0.0315$ for each factor.
** – not significant.

### Respiration rate

The respiration rate of the baby leaves increased after immersion to ozonated water. On day 0, D0L, D05L and D1L reached rates of 25.1 mg·kg$^{-1}$h$^{-1}$, 29.3 mg·kg$^{-1}$h$^{-1}$ and 30.2 mg·kg$^{-1}$h$^{-1}$ of $CO_2$, respectively (Fig. 2). On day 1, the highest rate was found for D05L compared to D0L and D1L. On day 4, D0L and D05L exhibited 36.3 mg·kg$^{-1}$h$^{-1}$ and 41.5 mg·kg$^{-1}$h$^{-1}$ of $CO_2$, respectively, while D1L exhibited the highest values (Fig. 2). At the end of storage, D05L and D1L showed the highest rates.

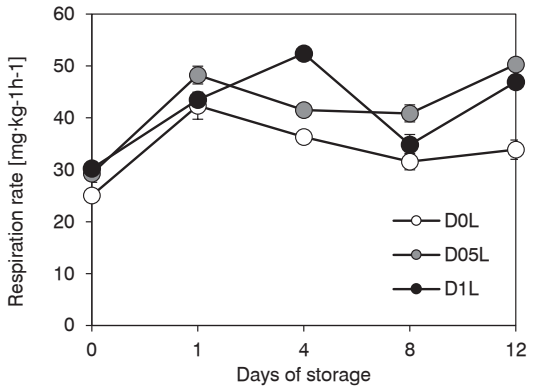


**Fig. 2.** Respiration rate of inoculated red chard baby leaves washed in ozonated water and stored for 12 days at 5 °C.

Values represent means of three repetitions ± standard deviation.
Designation of samples is explained in Tab. 1.

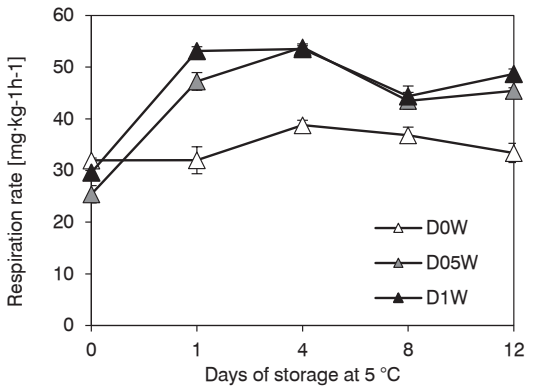


**Fig. 3.** Respiration rate of red chard baby leaves washed in inoculated water after ozonation and stored for 12 days at 5 °C.

Values represent means of three repetitions ± standard deviation.
Designation of samples is explained in Tab. 1.

Similarly to the trend observed for inoculated leaves treated by $O_3$ water showed on day 1 47.4 mg·kg$^{-1}$h$^{-1}$ and 53.2 mg·kg$^{-1}$h$^{-1}$ of $CO_2$ for D05W and D1W, respectively (Fig. 3). At the same time, D0W reached the lowest value compared to the $O_3$ treatments. On day 4, D05W and D1W exhibited significantly higher respiration rates than D0W. On day 8, D05W and D1W showed a slight decrease in respiration. At the end of storage, values for all treatments ranged from 33.9 mg·kg$^{-1}$h$^{-1}$ to 48.3 mg·kg$^{-1}$h$^{-1}$ of $CO_2$ (Fig. 3).

Regarding the metabolic activity, contrary to what was observed in our study, ZHANG et al. [17] reported a decrease in the respiration rate of minimally processed celery washed with ozonated water (0.08 mg·l$^{-1}$ and 0.18 mg·l$^{-1}$). The authors did not find significant difference ($\alpha$ = 0.05) between vitamin C and total sugar of fresh-cut celery treated with ozonated water and the non-treated samples.

### Chlorophyll content

According to the statistical analysis, interaction was found between ozone concentration and inoculation factors ($p$ = 0.042). On day 0, no differences were determined in Chla among treatments with a range 0.86–0.96 g·kg$^{-1}$ FW. On day 1, D05W and D1W had significantly higher contents compared to D0L, D1L and D0W. On day 4, D0W, D05L and D0L treatments had significantly higher contents than D05W, D1L and D1W. At the end of storage (day 12), D1L and D1W differed from the rest of the treatments reaching the lowest values of Chla content (Tab. 3).

From the aspect of treatment, there was a progressive decrease in Chla for all ozone concentrations throughout storage (Tab. 3). D1L and D1W treatments had the lowest contents of Chla with 0.36 ± 0.13 g·kg$^{-1}$ FW and 0.32 ± 0.03 g·kg$^{-1}$ FW, respectively.

For Chlb content, the values obtained were between 1.46 g·kg$^{-1}$ FW and 0.52 g·kg$^{-1}$ FW. On day 0, no differences were found among treatments (Tab. 3). On day 1, D1W was different from the rest of the treatments. On day 4, all treatments applied to inoculated leaves showed significantly higher values of Chlb than those in inoculated water. On day 8, D05W reached the lowest value. On day 12, D05L and D1W treatments had significantly lower Chlb contents than the rest of the treatments.

### Total phenolics content and total antioxidant capacity

Initially, *TPC* was not affected by $O_3$ concentration or the inoculum. On days 1, 4, 8 and 12 no significant differences were found among treatments, ranging from 1.12 g·kg$^{-1}$ FW to 0.93 g·kg$^{-1}$ FW (Fig. 4).

DPPH-based *TAC* after processing (day 0) was similar for all treatments with a range from 0.80 g·kg$^{-1}$ FW to 0.86 g·kg$^{-1}$ FW (Tab. 4). On day 1, D0W exhibited the lowest value of 0.69 g·kg$^{-1}$ FW differing from the rest of the treat-

**Tab. 3.** Chlorophyll content of red chard baby leaves after treatment.

| Treatment | Days of storage | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 4 | 8 | 12 |
| **Chlorophyll a** [g·kg$^{-1}$] | | | | | |
| D0L | 0.94 ± 0.02$^{aAC}$ | 0.66 ± 0.07$^{cC}$ | 0.70 ± 0.03$^{bB}$ | 0.60 ± 0.03$^{cC}$ | 0.55 ± 0.02$^{aD}$ |
| D05L | 0.92 ± 0.08$^{aA}$ | 0.84 ± 0.03$^{abB}$ | 0.73 ± 0.14$^{abC}$ | 0.71 ± 0.03$^{bC}$ | 0.49 ± 0.06$^{abD}$ |
| D1L | 0.88 ± 0.09$^{aA}$ | 0.62 ± 0.03$^{cB}$ | 0.55 ± 0.04$^{cC}$ | 0.59 ± 0.15$^{cC}$ | 0.36 ± 0.13$^{cD}$ |
| D0W | 0.96 ± 0.07$^{aA}$ | 0.81 ± 0.04$^{bB}$ | 0.74 ± 0.16$^{aC}$ | 0.70 ± 0.07$^{bC}$ | 0.44 ± 0.05$^{bD}$ |
| D05W | 0.92 ± 0.02$^{aA}$ | 0.86 ± 0.02$^{abB}$ | 0.65 ± 0.07$^{cC}$ | 0.80 ± 0.03$^{aB}$ | 0.41 ± 0.04$^{aD}$ |
| D1W | 0.86 ± 0.12$^{aB}$ | 0.92 ± 0.16$^{aA}$ | 0.69 ± 0.04$^{cC}$ | 0.72 ± 0.04$^{bC}$ | 0.32 ± 0.03$^{cD}$ |
| **Chlorophyll b** [g·kg$^{-1}$] | | | | | |
| D0L | 1.43 ± 0.06$^{aA}$ | 1.36 ± 0.06$^{aB}$ | 1.28 ± 0.07$^{aB}$ | 1.25 ± 0.17$^{aB}$ | 0.86 ± 0.06$^{bC}$ |
| D05L | 1.42 ± 0.03$^{aA}$ | 1.33 ± 0.18$^{aB}$ | 1.26 ± 0.09$^{aB}$ | 0.88 ± 0.05$^{bC}$ | 0.53 ± 0.03$^{dD}$ |
| D1L | 1.46 ± 0.04$^{aA}$ | 1.34 ± 0.07$^{aB}$ | 1.28 ± 0.11$^{aB}$ | 1.25 ± 0.06$^{aB}$ | 1.39 ± 0.02$^{aD}$ |
| D0W | 1.42 ± 0.14$^{aA}$ | 1.35 ± 0.04$^{aA}$ | 1.04 ± 0.13$^{bB}$ | 1.26 ± 0.14$^{aA}$ | 1.12 ± 0.09$^{aB}$ |
| D05W | 1.42 ± 0.09$^{aA}$ | 1.36 ± 0.03$^{aA}$ | 0.98 ± 0.12$^{bB}$ | 0.75 ± 0.11$^{cB}$ | 0.68 ± 0.03$^{cB}$ |
| D1W | 1.45 ± 0.16$^{aA}$ | 1.20 ± 0.06$^{bB}$ | 1.01 ± 0.09$^{bC}$ | 0.92 ± 0.05$^{bC}$ | 0.52 ± 0.02$^{dD}$ |

Chlorophyll content is expressed in grams per kilogram of fresh weight. Values represent means of three repetitions ± standard deviation. Means followed by the same letter in superscript, lowercase and uppercase in column and row, respectively, are not significantly different according to Tukey's test ($p \leq 0.05$).
Designation of samples is explained in Tab. 1.

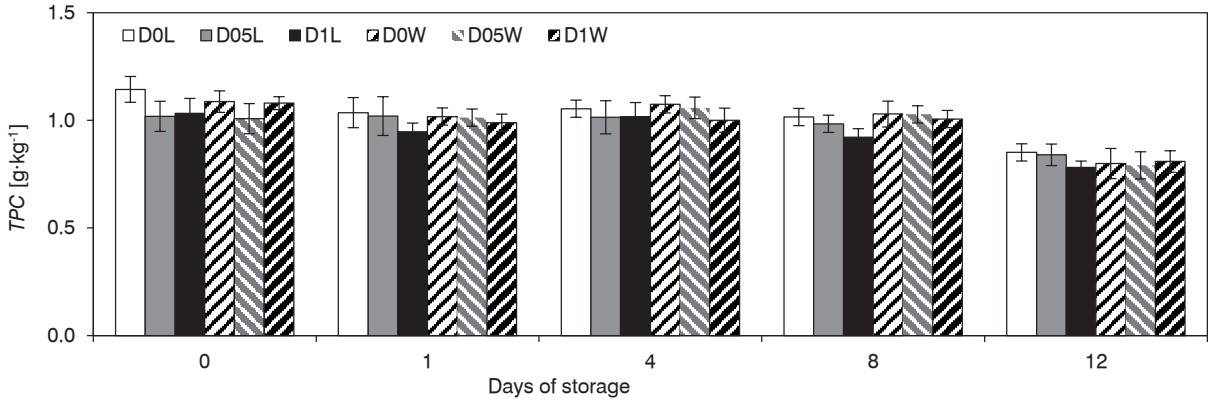


**Fig. 4**. Total phenolics content of red chard baby leaves washed in ozonated water and stored for 12 days at 5 °C.

*TPC* – total phenolics content (expressed as grams of gallic acid equivalents). Values represent means of three repetitions ± standard deviation.
Designation of samples is explained in Tab. 1.

**Tab. 4.** Total antioxidant capacity of red chard baby leaves after treatment.

| Treatment | Days of storage | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 4 | 8 | 12 |
| ***TAC* determined by DPPH method** [g·kg$^{-1}$] | | | | | |
| D0L | 0.86±0.02$^{aA}$ | 0.83±0.04$^{aA}$ | 0.72±0.01$^{aB}$ | 0.74±0.03$^{aB}$ | 0.62±0.02$^{aC}$ |
| D05L | 0.84±0.01$^{aA}$ | 0.71±0.02$^{aB}$ | 0.62±0.04$^{bC}$ | 0.74±0.02$^{aB}$ | 0.65±0.03$^{aC}$ |
| D1L | 0.80±0.02$^{aA}$ | 0.74±0.03$^{aA}$ | 0.71±0.03$^{aA}$ | 0.69±0.02$^{bA}$ | 0.57±0.03$^{aB}$ |
| D0 W | 0.83±0.01$^{aA}$ | 0.69±0.03$^{bC}$ | 0.78±0.04$^{aB}$ | 0.78±0.03$^{aB}$ | 0.53±0.04$^{bD}$ |
| D05W | 0.84±0.02$^{aA}$ | 0.84±0.02$^{aA}$ | 0.81±0.03$^{aA}$ | 0.78±0.01$^{aB}$ | 0.61±0.04$^{aC}$ |
| D1W | 0.85±0.02$^{aA}$ | 0.86±0.01$^{aA}$ | 0.77±0.01$^{aB}$ | 0.69±0.03$^{bC}$ | 0.57±0.03$^{aD}$ |
| ***TAC* determined by FRAP method** [g·kg$^{-1}$] | | | | | |
| D0L | 1.18±0.05** | 1.07±0.13** | 1.17±0.06** | 1.05±0.03$^{a}$ | 1.17±0.02$^{a}$ |
| D05L | 1.15±0.04 | 0.99±0.07 | 1.14±0.05 | 1.06±0.05$^{a}$ | 1.09±0.03$^{a}$ |
| D1L | 1.11±0.14 | 1.02±0.03 | 1.16±0.08 | 1.17±0.04$^{a}$ | 1.07±0.02$^{b}$ |
| D0 W | 1.10±0.03 | 1.04±0.02 | 1.13±0.03 | 1.09±0.02$^{b}$ | 1.07±0.04$^{b}$ |
| D05W | 1.06±0.13 | 1.06±0.03 | 1.15±0.02 | 1.08±0.07$^{b}$ | 1.19±0.03$^{a}$ |
| D1W | 1.15±0.16 | 1.08±0.08 | 1.17±0.02 | 1.15±0.04$^{a}$ | 1.12±0.02$^{a}$ |

Values represent means of three repetitions ± standard deviation. Means followed by the same letter in superscript, lowercase and uppercase in column and row, respectively, are not significantly different according to Tukey's test ($p \leq 0.05$).
*TAC* – total antioxidant capacity is expressed as grams of Trolox equivalents per kilogram of fresh weight, ** – not significant.
Designation of samples is explained in Tab. 1.

ments. On day 4, D05L showed the lowest value of 0.62 g·kg$^{-1}$ FW. On day 8, D1L and D1W, the lowest values were recorded. At the end of storage, D0W exhibited the lowest antioxidant capacity.

Regarding FRAP-based *TAC*, no significant differences among treatments were determined on days 0, 1 and 4 (Tab. 4). On day 8, D05L, D1L and D1W exhibited the highest values. On day 12, D1L and D0W showed 1.07 g·kg$^{-1}$ FW, which were the lowest values on that day.

In previous studies, an increase in phenolic compounds was found in vacuoles of various plant species as a response to $O_3$ [36]. However, in this study *TPC* and *TAC* showed no significant changes compared to the controls. There was also no significant change in *TAC*, determined either by the DPPH or FRAP method, of the red chard treated with ozone compared to the untreated vegetable. These results suggest that the $O_3$ concentrations were enough to reduce the microbial counts but provoking no oxidative stress on the vegetal tissue or, as it was reported previously by Beltrán et al. [13], changes in *TPC* were independent of the ozone washing treatments.

## CONCLUSIONS

When leaves were contaminated by *E. coli*, the sanitation action of ozone was slightly more difficult, so washing with ozonated water only decreased the *E. coli* load by 1 log CFU·g$^{-1}$ after 8 days of cold storage (at 5 °C). However, inoculated water treated by ozone decreased the *E. coli* load by 1.5 log CFU·g$^{-1}$ on red chard leaves after 12 days at 5 °C. Red chard baby leaves washed in ozonated water kept the quality parameters such as leaf colour, polyphenolic compounds and antioxidant capacity for 12 days at 5 °C. In future research, sanitization of the leaves of vegetables infected with other bacteria can be studied, using different concentrations of ozone and different storage duration of the sanitized vegetables.

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