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Romolo Laurita [a,b]*, Giorgia Gozzi [c]*, Silvia Tappi [c,d] **, Filippo Capelli [a], Alina Bisag [a], Giulia Laghi [a], Matteo Gherardi [a,b], Beatrice Cellini [c], Doaa Abouelenein [e,f], Sauro Vittori [e], Vittorio Colombo [a,b,d], Pietro Rocculi [c,d], Marco Dalla Rosa [c,d], Lucia Vannini [c,d]

[a] *Department of Industrial Engineering (DIN), University of Bologna, Via Saragozza 8, Bologna, Italy*

[b] *Interdepartmental Center for Industrial Research for Advanced Mechanics and Materials, University of Bologna, Via Saragozza 8 Bologna Italy*

[c] *Department of Agricultural and Food Sciences, University of Bologna, Piazza Goidanich, 60, Cesena FC, Italy*

[d] *Interdepartmental Centre for Agri-Food Industrial Research, University of Bologna, via Q. Bucci 336, Cesena, FC, Italy*

[e] *School of Pharmacy, University of Camerino, Italy*

[f] *Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, 44519 Zagazig, Egypt.*

* These two authors contributed in equal measure to this research

**Corresponding author. Mail: silvia.tappi2@unibo.it

Abstract

Plasma Activated Water (PAW) obtained by exposing water to cold atmospheric pressure plasma, has recently emerged as a promising alternative for food decontamination, compared to the use of traditional chemical sanitizers. The aim of the study was to evaluate the efficacy of PAW treatments for rocket salad decontamination. Washing with PAW for 2, 5, 10 and 20 min was assessed against different endogenous spoilage microorganisms and compared to untreated water and hypochlorite solution. The chemical composition of PAW as a function of treatment and delay time was characterized and the effect on product quality and nutritional parameters was evaluated.

Results showed that PAW allowed an average reduction of 1.7-3 Log CFU/g for total mesophilic and psychrotrophic bacteria and *Enterobacteriaceae* following 2-5 min washing with minimal variation of qualitative and nutritional parameters. Overall, experimental results highlighted the potentiality of PAW treatments as a promising alternative to chlorine having the advantage of a minor adverse impact on environment and consumers' health.

Industrial relevance

To meet consumers demand, the minimally processed fruit and vegetable industry needs to find sustainable solutions as alternative to the use of traditional chemical sanitizers that allow to increase product shelf-life and preserve safety, qualitative and nutritional characteristics.

Plasma activated water represents a promising strategy for food decontamination, but its effects on foods have been only limitedly investigated. The present research is the first study on the use of plasma activated water on fresh rocket leaves, providing new and important information on microbial inactivation and quality of the fresh cut product.

Keywords: emerging technologies; plasma activated water; microbial decontamination; food quality; food spoilage

1. Introduction

The market of fresh and minimally processed vegetables has rapidly grown in the last decades as these products are very attractive to consumers looking for healthy and convenient meals. However, they are characterized by a very short shelf-life that limit their market opportunity and that leads to high amount of wasted product. The main limiting factor for these products shelf-life is surely microbial spoilage. Mesophilic aerobic bacteria, coliforms, *Pseudomonas* spp., *Bacillus* spp., lactic acid bacteria, as well as yeast and moulds are considered to be the main microorganisms found in fresh and ready-to-eat salads with loads up to 10^7 Log CFU/g. Decontamination of fresh products from pathogenic and spoilage microorganisms is usually performed through washing with chemical sanitizers, being sodium hypochlorite the most commonly used one. However, there are several concerns related to human health and environmental pollution that have led to the search for alternative methods. Moreover, wide variability in disinfection efficacy has been reported in numerous studies due to several factors, e.g. microbial protection by internalization within the vegetable tissue or biofilm formation and depletion of free-chlorine during washing (Yoon & Lee, 2018).

On one side, microorganisms are becoming more resistant to existent traditional technologies, and they are able to survive under various stress conditions, resulting in potential risk for human health (Zhao, Patange, Sun, & Tiwari, 2020). On the other side, the necessity to preserve the fresh-like characteristics and the content of bioactive compounds requires a mild technology in the decontamination process. Hence, the combination of effective sanitizing measures, while preserving quality and nutritional features, is the main issues for producers to improve the fresh products' shelf-life. Moreover, the environmental sustainability of the overall process has to be taken into account.

The use of cold atmospheric pressure plasma (CAP) represents a novel approach for sanitation of food products that involves the application of an ionised gas mixture composed by charged particles, electric fields, ultraviolet (UV) photons, and reactive species exploiting its strong oxidative power. Previous research has demonstrated that CAP can efficiently inactivate a wide range of microorganisms, including bacteria, fungi, viruses, bacterial spores and biofilms (Ikawa, Tani, Nakashima, & Kitano, 2016; Kolb et al., 2008; Machala et al., 2013; Pan et al., 2013). Reactive oxygen and nitrogen species (RONS) are the major bactericidal agents, which cause damage to DNA and proteins in microbial cells (Joshi et al., 2011; Laurita, Barbieri, Gherardi, Colombo, & Lukes, 2015).

Plasma-activated water (PAW) is generated by exposing water to a plasma discharge above or underneath water surface. The plasma-generated reactive species interact with water molecules and trigger several chemical reactions, which produce a unique mixture of biochemical reactive chemistries. In recent years, PAW has also been confirmed to possess outstanding biological activity in biomedical and agricultural sectors (Kaushik et al., 2018; Sajib et al., 2020).

PAW can represent a promising alternative to washing in chlorinated water for fresh fruit and vegetables, since it does not leave any residual chemicals in the products and it is considered more environmentally friendly compared with some traditional chemical sanitizers (Zhao et al., 2020).

There are few studies (Baier et al., 2013; Giannoglou et al., 2020; Hertrich, Boyd, Sites, & Niemira, 2017; Silvetti et al., 2021) that report the efficacy of cold plasma on the microbial inactivation of fresh cut salads, assessing endogenous microbiota or inoculated pathogens load reduction. However, in all of these studies plasma in the gaseous form was employed, while, to our knowledge, no reports are available on the use of PAW.

On the contrary, several authors have reported on the efficiency of PAW in inactivating natural microbiota and pathogens deliberately contaminated on other types of fresh fruits and vegetables, such as strawberries, button mushrooms, Chinese bayberries, and fresh-cut apples (Liu et al., 2020; Ma et al., 2016; Ma et al., 2015; Xu, Tian, Ma, Liu, & Zhang, 2016), but no study on the effect of PAW on background spoilage microbiota of rocket leaves is available in literature. Moreover, the effect on microbial load has been investigated by many authors, while the effect on products quality and nutritional value is often scarcely considered.

Fresh fruit and vegetables are rich in bioactive compounds that exert a pronounced effect on human health, due to their antioxidant properties. Hence, the preservation of these compounds is fundamental for product quality. A previous research showed that exposure to RONS produced by CAP treatment reactive species could lead to the oxidation of some phenolic compounds in leafy vegetables such as lams lettuce, but mainly on model systems, while the matrix protected these compounds

(Grzegorzewski, Ehlbeck, Schlüter, Kroh, & Rohn, 2011). Nevertheless, no differences in the antioxidant activity of fresh cut apples (Liu et al., 2020), pears (Chen et al., 2019) and button mushrooms (Xu et al., 2016) were observed after washing with PAW.

The aim of the present research was to investigate the decontamination of endogenous spoilage microbiota of rocket leaves induced by PAW washing and compare it to the effect of hypochlorite solution washing, used as traditional reference method. The effect on product quality was evaluated by measuring pH, color parameters and some bioactive compounds content.

2. Materials and Methods

2.1 Raw materials

Fresh rocket (*Rucola sativa*) leaves were purchased at the local market in Cesena, Italy and stored in a refrigerated cell at 2±1 °C for maximum 24 h before processing.

2.2 Corona discharge for the production of PAW: electrical analysis and chemical characterization of RONS in PAW.

PAW was produced treating distilled water with a corona discharge, as reported in Laurita et al. (2021). Briefly, the plasma source consists of a stainless-steel pin-electrode connected to a high voltage microsecond pulsed generator (AlmaPulse, AlmaPlasma s.r.l.) at a distance of 5 mm from the liquid surface. A volume of 450 ml of distilled water, in a borosilicate Erlenmeyer flask on a stirrer (IKA Magnetic Stirrers RCT basic), was grounded. The CAP is generated in the air gap between the tip of the high voltage electrode and the liquid surface. The generator was operated at a peak voltage of 9 kV, with a fixed frequency of 5 kHz. The voltage (V) and the current (i) were measured using a high voltage probe (Tektronix P6015A) and a current probe (Pearson 6585) connected to a digital oscilloscope (Tektronix DPO4034, 350 MHz, 2.5 GSa s^{-1}). The average power (P) dissipated in the discharge was determined applying the following formula:

$$P = \frac{1}{T}\int_0^T i(t)\,V(t)\,dt, \quad (1)$$

where T is the applied voltage period.

The concentrations of H_2O_2, NO_2^- and dissolved O_3 produced in PAW by the CAP treatment were measured. The Amplex® Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the Nitrate/Nitrite colorimetric assay (ROCHE, Baesel, Switzerland) were used for the measurement of H_2O_2 and NO_2^-, respectively. The analysis of their concentrations was performed photometrically according to the manufacturer's protocols using a microplate reader (Rayto, P.R.

China). The O_3 concentration produced in the liquid phase was evaluated according to the manufacturer's protocols using Chematest 20 (SWAN Analytics). Moreover, the pH and the conductivity were evaluated by the means of inoLab® pH 7110 and Oakton Instrument: Con 6+ Meter, respectively. The RONS concentrations were assessed for four different treatment times (1, 2, 3 and 4 min) and four different delay times (2, 5, 10 and 20 min). All measurements were performed at least 3 times.

2.3 PAW treatment

PAW was produced exposing distilled water the aforementioned corona discharge for 4 min. Immediately after the treatment, rocket samples were immersed in PAW for 2, 5, 10 and 20 min in the ratio product:liquid of 1:20 (w:v) at room temperature. 20 g of rocket leaves were treated each time. During immersion, samples were constantly agitated in an orbital agitator.

After dipping, rocket leaves were removed from PAW and blotted with adsorbent paper to remove excess liquid. For each treatment time, two independent treatments were carried out.

Untreated rocket leaves were considered as control. Moreover, in order to evaluate the effect of PAW treatment, washing in untreated water was tested and a dipping in sodium hypochlorite (NaClO) 100 ppm for 2, 5, 10 and 20 min was used as common reference washing procedure.

For analysis of bioactive compounds, after each treatment time, rocket samples from both washing replicates were immediately freeze dried after treatment, the obtained samples were stored at -20°C until analysis.

2.4 Analytical determinations

2.4.1 Microbiological analysis

Immediately after each washing treatment, rocket samples were aseptically transferred to sterile saline solution (0.9 %, w/v, 1:10 w/v) and homogenized in a Stomacher 400 apparatus (Seward Ltd., Worthing, West Sussex, UK) for 2 min. Ten-fold serial dilutions of the homogenates were then performed and inoculated on suitable media for counting viable cells of various microbial groups. Total aerobic mesophilic and psychrotrophic microorganisms were evaluated onto Standard Plate Count Agar (Oxoid, Basingstoke, UK) plates incubated at 30 °C for 48 h and at 4 °C for 10 d, respectively. *Enterobacteriaceae* were counted on Violet Red Bile Glucose agar (Oxoid, Basingstoke, UK) incubated at 37 °C for 24 h. For each washing replicate (n=2), microbiological analyses were carried out at least in duplicate and the data were expressed as the mean of log colony forming units (CFU) per gram ±standard deviation.

2.4.2 Physico-chemical parameters

pH was measured in triplicate with a pH-meter (Crison, Barcellona, Spain) on the juice obtained by 10 g of treated or untreated sample. Water content was determined in triplicate by gravimetric method, drying samples in an oven at 70°C until constant weight was achieved. Results were expressed as g/100g.

Image analysis was used to determine samples color. Digital images were acquired placing the samples inside a black box and illuminated using four parallel lamps (mod. TL-D deluxe, natural daylight, 18W/965, Philips, NY, USA) with a colour temperature of 6500 K. The Colour Digital Camera (mod. D7000, Nikon, Shinjuku, Japan) used was equipped with a 60 mm lens (mod. AF-S micro, Nikkor) and located vertically over the sample at a fixed distance of 23 cm and at an angle with the lightning source of approximately 45°. The pre-processing of RGB images, segmentation and colour quantification were performed with ImageJ analysis software (NIH, USA). The average value of the segmented pixels was registered as L, R, G and B values. For each sample, images of 10 leaves were acquired and analysed.

2.4.3 Nutritional quality

Total Phenolic Content (TPC) was determined spectrophotometrically according to Mustafa et al. (2016) method with some modifications. Briefly, 0.5 mL of extracts solution (100 mg freeze dried sample extracted with 10 mL ethanol 70% with Formic acid (1.5%, v/v) was introduced into test tubes, then 2.5 mL of Folin-Ciocalteu reagent solution and 7 mL of Na_2CO_3 (7.5% w/w in water) solution were added. The reaction mixture allowed to stand at room temperature in the dark for 2 h and absorption was measured at 765 nm. The TPC was expressed as mg of gallic acid equivalents (GAE) per 100 g of dry weight of the rocket samples using gallic acid calibration curve.

Total Flavonoid Content (TFC) was determined following a method described by (Chen, Chen, Xiao, & Fu, 2018) with slight variations. Briefly, 0.5 mL of extract solution (10 mg/mL), 0.15 mL of $NaNO_2$ (0.5 M), 3.2 mL of methanol (30% v/v) and 0.15 mL of $AlCl_3 \cdot 6H_2O$ (0.3 M) were mixed. After 5 min, 1 mL of NaOH (1 M) was added. The solution was mixed well, and the absorbance was measured against the blank reagent at 506 nm. The standard calibration curve for TFC was made using quercetin standard solution (0 to 100 mg/L) under the same procedure as described above. TFC was expressed as mg of quercetin equivalents (QE) per 100g of dried extract.

Analytical determinations of TPC and TFC were carried out in triplicate for each freeze-dried sample.

2.5 Statistical analysis

To investigate statistical significance of observed differences in microbial, qualitative and nutritional data, a one-way analysis of variance (ANOVA) followed by the Tukey's HSD test (multiple range test) was used ($p < 0.05$). Analysis was conducted using Microsoft Excel 365 and Minitab ver. 19.0.

3. Results and Discussion

3.1 Electrical analysis and chemical characterization of RONS in PAW

Figure 1 shows current and voltage waveforms for the corona discharge operated at a peak voltage of 9 kV and a frequency of 5 kHz, referring to two applied voltage periods representative of the discharge electrical behavior during the treatment of the distilled water. Each applied voltage period (200 µs) exhibited four voltage peaks, whose intensities reached maximum values up to 9.24 kV. The average discharge dissipated power, which can be calculated directly from the measured current and voltage reported in **Figure 1**, is 480.95 ± 33.42 W.

pH and conductivity of PAW as a function of treatment time are shown in **Figure 2**. As previously reported, the plasma treatment induced a reduction of pH (Laurita et al., 2015) up to 3.3 (dotted black line) after 4 min of plasma treatment, and an increase of conductivity (dotted purple line) up to 200 mS/cm.

Figures 3 and **4** report the behaviors of the concentration of the investigated RONS as a function of the treatment time and of the delay time. H_2O_2, NO_2^- and O_3 concentrations increased with the increasing of the treatment times up to 4.5 ± 0.1 mg/L, 30.4 ± 0.9 mg/L and 0.3±0.1 mg/L respectively. Focusing on the post-discharge kinetic of reactive species reported in **Figure 3b**, the decrease in concentration of both H_2O_2 and NO_2^- is related to the post-discharge processes in acid environment mediated by peroxynitrite (ONOOH) (Laurita et al., 2015; Lukes, Dolezalova, Sisrova, & Clupek, 2014). Finally, it is worth to note that the O_3 decay reported in **Figure 4b** is in accordance with the half-life values reported by Gardoni, Vailati, & Canziani (2012).

3.2 Effect of PAW on microbial load

The effect of PAW on the microbial loads of the target microbial groups of the rocket leaves was evaluated after washing for 2, 5, 10, and 20 min and compared to untreated water and hypochlorite solution (**Figure 5**).

Initial contamination levels of the untreated samples were about 5.74 ± 0.49, 6.17 ± 0.70 and 3.67 ± 0.49 Log CFU/g for total mesophilic and psychrotrophic bacteria and *Enterobacteriaceae*,

respectively. These data are consistent with literature reporting rather high contamination levels for rocket leaves with counts for the aerobic mesophilic and psychrotrophic microorganisms up to 7.5 Log CFU/g, yeasts and moulds about 5.8 Log CFU/g, and the range for *Enterobacteriaceae* 4.3–5.9 Log CFU/g (Abadias, M., Usall, Anguera, Solsona, & Viñas, 2008).

The results presented in **Figure 5** clearly show that untreated water cannot be used to effectively decrease surface contamination of fresh rocket leaves since mean reductions for the target spoilage bacteria did not exceed 0.6 Log units regardless washing time. By contrast, all the considered microbial populations were significantly ($p < 0.05$) affected by PAW treatments depending on washing time. Psychrotrophic bacterial population was the most sensible one and it was reduced by 2 Log CFU/g after a 2-min washing. By extending treatments up to 5 min, the decontaminating effect of PAW was enhanced resulting in a 3 Log CFU/g reduction. However, no further significant improvement was observed following the longest treatments. On the other hand, a 5-min washing was effective in decreasing counts of mesophilic bacteria and *Enterobacteriaceae* by 1.7-2.5 Log CFU/g. Similarly to the psychrotrophic population, PAW decontamination activity against mesophiles and enterobacteria did not significantly increase by prolonging the treatment time.

Overall, PAW had a stronger effect on the spoilage-related microorganisms with respect to the reference method (NaClO, 100 ppm) indicating that it can be an alternative to conventional washing with a chlorine-based sanitiser. More precisely, no significant influence on the presence of total mesophilic bacteria was detected over 20 min treatment with NaClO. Moreover, longer treatments were necessary with NaClO than PAW to have significant differences compared to untreated samples for enterobacteria and total psychrophilic population. In particular, this was observed only after 10 min with maximum reductions of about 1.4-1.7 Log CFU/g. However, it was less effective than PAW since resulted in lower population decreases for both microbial groups which did not further improve by lengthening the treatment to 20 min.

It is not surprising that the chlorine treatments achieved rather limited reductions and its use for sanitizing fresh produce is controversial due to several issues which limit its effectiveness (Yoon & Lee, 2018). Besides the possibility for indigenous microbiota to internalize within tissues of fresh produce and to produce biofilm, depletion of free chlorine due to the presence of organic matters is well known to affect the disinfection capacity of the treatment, thus making processing time little relevant. On the other hand, the bactericidal action of PAW relies on the RONS content and its low pH. An important feature of PAW used in the present study is that only limited changes occurred in the content of nitrite anion by increasing washing time being its content about 25 ppm even after 20 min (**Figure 3**).

Several studies showed that PAW can efficiently inactivate a wide variety of microorganisms from different fresh fruit and vegetables, but no study on rocket has been performed yet. It should however be considered that effectiveness of washing with PAW depends on several aspects related to the mode and conditions of PAW generation, which affect its chemico-physical properties, as well as on the different sizes, shapes, surface-to-volume ratio, and initial microbial contamination level of various fruits and vegetables. Despite differences in these factors, our data are in agreement with previous studies. Choi et al. (2019) reported reductions of 2.0, 2.2, 1.8, and 0.9 Log CFU/g in mesophilic aerobic bacteria, lactic acid bacteria, yeast and moulds, and coliforms, respectively of shredded salted Chinese cabbages. While such values are consistent with those obtained in this study, it is important to point out that the PAW they used was generated following a 120 min discharge resulting in NO_2^- and NO_3^- concentrations up to 88 and 394 ppm, respectively, while H_2O_2 was negligible. Xiang et al. (2019) showed significant reductions in populations of total aerobic bacteria and yeasts and moulds (2.32- and 2.84-Log CFU/g, respectively) of mung bean sprouts after washing for 30 min with PAW produced by 30 sec (APPJ; 5 kV, 40 kHz) and characterized for a content in H_2O_2, NO_3^- and NO_2^- of about 17, 571 and 531 μmol/L, respectively. Reductions up to 1.7 Log CFU/ g for mung bean seeds inoculated with *Escherichia coli* O154 were achieved by Machado-Moreira, Tiwari, Richards, Abram, & Burgess (2021) but only after 3h of treatment with PAW. A shorter washing time of 5 min, which is consistent with our study, was demonstrated to have a significant effect in decreasing initial counts of aerobic bacteria and mould and yeast of fresh-cut 'Fuji' apples (Liu et al., 2020). Not only PAW treatment reduced by approximately 1-2 Log CFU/g the indigenous spoilage microbiota, but also limited their proliferation over refrigerated storage while reducing superficial browning and maintaining quality features thus confirming its efficacy in prolonging the product shelf-life.

Compared to Liu et al. (2020), our results indicate that PAW can be a suitable treatment to reduce bacterial population in rocket salad being active even after the shortest treatment. To successfully extend the produce shelf-life it would be however important to test also the fate of the surviving cells during storage to check whether bacteria were lethally or sub-lethally damaged by PAW and hence could recover or develop stress resistance responses.

3.3. Effect on quality and nutritional parameters

Water content and pH of rocket leaves did not show any significant variation during washing in PAW compared to the untreated sample (data not reported). Although the pH of PAW is significantly lower compared to rocket leaves one (6.17 ± 0.05), washing up to 20 min did not promote any change, in agreement with Xu et al. (2016).

Table 1 reports the colorimetric parameters measured by the image analysis. A significant decrease was observed for the parameters of L (luminosity), R (red components) and G (green parameter) in all samples, but variations do not appear to be proportional to treatment time.

According to literature, the effect of plasma exposure on colour of fresh produce is generally quite minimal but depends on the specific matrix (Baier, Ehlbeck, Knorr, Herppich, & Schlüter, 2015). In a previous study, Ramazzina et al. (2015) observed an immediate reduction of chlorophyll content in kiwifruit subjected to gaseous plasma treatment that was attributed to the Type II chlorophylls degradation induced by reactive species. However, the author also observed a better retaining of colour during the following storage, that was probably due to degradative enzymes inactivation.

Changes in colour of fresh products could be due to pigment degradation but also to changes in the tissue microstructure. However, to our knowledge there are no reports on the use of PAW on rocket leaves, so the observed effect on colour should be further clarified. Moreover, it is important to determine if these changes are perceptible by consumers and how they evolve during the storage of the product.

Consumption of bioactive compounds, especially polyphenols, plays a central role in the protection of human organs against inflammatory and neurodegenerative disorders and oxidative stress. However, the preservation of natural bioactive compounds in food products is a major challenge for the food industry. In this sense, cold plasma has been proposed as one of the most promising solutions to maximize the retention of polyphenol in food products.

Table 2 shows the time dependent changes in the total phenolic content (TPC) and total flavonoid content (TFC) of PAW treated rocket samples. According to the obtained results, all PAW treated samples showed non-significant differences in the TPC ($p > 0.05$) compared to the control samples, except for the 5 min treatment which induced a slight reduction. These results are in good accordance with the previous findings reported by Xiang et al. (2019) in which the PAW treatment did not induce any significant change in the total phenolic content of mung bean sprouts.

A significant variation in total flavonoid content was observed for PAW treated rocket samples with higher values for 10 and 20 min (3098.85 ± 53.20 and 3046.88 ± 38.58 mg gallic acid/ 100g, respectively) compared to the control samples (2524.88 ± 39.53 mg quercetin/ 100g).

The increase in the content of some specific phenolic compounds was observed after a short exposition to plasma in apples (Tappi et al., 2018) and blueberries (Sarangapani, O'Toole, Cullen, & Bourke, 2017) and after PAW treatment on mung bean sprouts (Fan, Liu, Ma, & Xiang, 2020). This effect was attributed to a physiological response of the tissue to the stress promoted by the reactive species. However, the increase of exposure time generally promoted oxidative reactions and the progressive decrease in the phenolic content and antioxidant activity. In the present research, TPC

was not affected even at the longer treatment time, while flavonoids accumulation occurred. A deeper analysis of the single phenolic compounds could help to clarify the response of this specific matrix to the exposure to plasma reactive species.

4. Conclusions

In summary, this study demonstrates PAW has the potential to decontaminate rocket salad with limited changes in quality parameters. Compared to hypochlorite, which was chosen as a reference sanitizer due to its widespred use in the food industry, PAW treatments resulted to be more effective against the target background microbiota. In fact, shorter treatments were necessary to significantly reduce *Enterobacteriaceae* and psychrotrophic bacterial populations, and greater inactivations were achieved for all the spoilage microbial groups after 2 min.

However, the observed slight changes in color and bioactive compounds content should be further clarified. Moreover, the behavior of the surviving microbiota and quality parameters should be evaluated during storage of the packed product in order to determine the effect of PAW on the shelf-life in real distribution and storage conditions.

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Figure captions

Figure 1. Voltage and current waveforms during distilled water treatment

Figure 2. pH (black) and conductivity (purple) values of not treated solution (NT) and 1, 2, 3 and 4 min of plasma treatment.

Figure 3. (a) Chemical analysis of the concentrations of H_2O_2 and NO_2^- after 1, 2, 3 and 4 min of plasma treatment and (b) their decay kinetic at room temperature.

Figure 4. (a) Chemical analysis of the concentrations of O_3 after 1, 2, 3 and 4 min of plasma treatment and (b) its decay kinetic at room temperature.

Figure 5. Mean counts of (a) total aerobic mesophilic and (b) psychrotrophic bacteria, and (c) *Enterobacteriaceae* on rocket leaves collected before and after the washing with PAW, untreated water or sodium hypochlorite (NaClO, 100 ppm) at different times. Mean values with different letters significantly differ among treatment times and washing solutions ($p < 0.05$).