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Research article

Ozone washing decreases strawberry susceptibility to *Botrytis cinerea* while maintaining antioxidant, optical and sensory quality

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ABSTRACT

This work aimed to evaluate the effect of ozone washing (maximum concentration 3.5 mgL^{-1}- 5 and 15 min) on *Botrytis cinerea* decay, physicochemical parameters, bioactive compounds, *in vitro* and *in vivo* antioxidant activity, and sensory properties of strawberries cv. Albion throughout refrigerated storage at 5 ± 1 °C. A 5 min long ozonation delayed the onset of *B. cinerea* infection by 4 days, and significantly reduced its incidence as storage progressed (~17 % lesser than in control at day 8), without impairing physicochemical parameters or sensory quality. This treatment did not affect the antioxidant activity of strawberry extracts neither in *in vitro* (ORAC and ABTS assays) or *in vivo* assays using *Caenorhabditis elegans* as a model organism. Higher ozone doses did not achieve greater reduction of *B. cinerea* decay throughout the storage period. This study demonstrated that exposing strawberries to a 5 min long aqueous ozone treatment could extend their storability at 5 °C.

1. Introduction

Strawberries are a rich source of health promoting compounds such as vitamin C and phenols, including flavonoids and phenolic acids. These compounds are associated with its high antioxidant potential that, in addition to flavour attributes, make the strawberry one of the most desired and consumed crops globally (Tulipani et al., 2014). However, it is a very perishable fruit due to fast softening, fast moisture loss, and its high susceptibility to fungal infection (Prusky et al., 2010). Strawberry decay can be caused by a multitude of fungi, being *Botrytis cinerea* the major pre- and postharvest plant pathogen (Prusky et al., 2010). Hence, the development of postharvest strategies to extend the strawberry's storage life whilst preserving its quality attributes is essential. In this context, different preservation techniques such as natural antimicrobials, UV-C and pulsed light, ultrasound, and ozone, among others, have been studied (Birmpa et al., 2013; Misra et al., 2014; Romanazzi et al., 2016).

Ozone is an effective sanitizer with a wide antimicrobial spectrum, its application for surface decontamination of harvested crops has been studied in either gaseous or aqueous treatments (Glowacz et al., 2015; Jaramillo-Sánchez et al., 2019; Ong et al., 2012; Perry and Yousef, 2011). Aqueous ozone treatments in strawberries have been evaluated from several perspectives, including their impact on microbiological load (native flora or artificially inoculated human pathogens) and certain quality attributes (firmness, colour, anthocyanin content, etc.) (Aday et al., 2014; Alexandre et al., 2012). However, contradictory data has been reported on the impact of ozone washing on fruit quality. This could be attributed to differences in critical variables of the process (i.e. ozone concentration, exposure time, storage conditions) and to different responses that depend on the fruit cultivar (Keutgen and Pawelzik, 2008).

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In a previous work, the effect aqueous ozone washing had on delaying and reducing the naturally occurring fungal incidence and weight loss of strawberry cv. Albion without significantly affecting mechanical properties was demonstrated. However, the effect of ozone could vary according to the harvest year (Contigiani et al., 2018), which could be related to differences in fungal load or in the fungal communities present in the fruit. Therefore, the evaluation of ozone's efficacy to reduce *Botrytis cinerea* decay during storage could be determinant to select the most convenient treatment in a more accurate way. In addition, studies related to the effect of ozone on the antioxidant properties rely on the use of *in vitro* assays, and *in vivo* studies have not been reported yet. Unlike *in vivo* assays, *in vitro* measurements have the drawback of not taking into account the bioavailability, uptake, and metabolism of the antioxidant compounds (Braga et al., 2018).

Nowadays, the nematode *Caenorhabditis elegans* is a model organism that has been increasingly used to study the effect of beneficial or toxic substances on biological processes (Hunt et al., 2020; Kim and Lee, 2019). One of the great advantages of this biological model is the strong conservation of physiological processes and stress responses between the nematode and mammals, including humans (Kenyon, 2010). Moreover, unlike cell free studies and cell culture systems, *C. elegans* allows for the examination within the context of a whole organism with many different organs and tissues. Particularly, oxidative stress seems to be a major factor limiting lifespan of both *C. elegans* and humans (Finkel and Holbrook, 2000; Larsen, 1993). Therefore, this nematode can be useful for identifying bioactive compounds and evaluating the effect of processing in antioxidant-rich foods. To the best of our knowledge, there are no studies performed in *C. elegans* for evaluating the oxidative stress resistance properties of strawberry extracts.

The objective of this work was to test the hypothesis that ozone washing could be used as an alternative to extend the postharvest life of strawberry fruit with minimum effect on its quality attributes. Thus, this work evaluated the effect of aqueous ozone treatments followed by refrigerated storage at 5 ± 1 °C on strawberry quality, analysing their impact on *B. cinerea* growth, physicochemical parameters, bioactive compounds, *in vitro* and *in vivo* antioxidant activity, and sensory properties of strawberries cv. Albion.

2. Materials and methods

2.1. Instrumentation, chemicals and culture medium

Colour measurements were made with a hand-held tristimulus reflectance spectrocolorimeter (Minolta Co., Model CM-508-d, Japan). For total anthocyanin and phenolic compound contents, and antioxidant capacity (TEAC assay), a UV- VIS spectrophotometer (model V-630, JASCO, Japan) was used. For the determination of antioxidant capacity evaluated by ORAC assay, fluorescence measurements were made by means of a Multilabel Microplate Reader (Perkin Elmer, Victor^3 model, USA). Fluorescence measurements in *Caenorhabditis elegans* assays were performed in a Spark 20M Multimode Microplate Reader (Tecan, NC, USA).

AAPH (2,2′-Azobis (2-methylpropionamidine) dihydrochloride, ABTS (2,2 -azinobis (3-ethylbenzothiazoline)-6-sulfonate), fluorescein sodium salt, gallic acid (GA), Trolox ($C_{14}H_{18}O_4$) and 2,7 – dichlorodihydrofluorescein diacetate (H2DCF-DA) were purchased from Sigma-Aldrich (St. Louis, USA). Ethyl alcohol, sodium acetate, sodium carbonate, and Tween 80 were from Biopack (Buenos Aires, Argentina). Folin Ciocalteau reagent, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, disodium hydrogen phosphate, hydrochloric acid, potassium chloride, sodium chloride, sodium hydroxide, manganese sulphate, and malt extract agar (MEA) were purchased from Merck (Darmstadt, Germany).

2.2. Fruit samples

Strawberries (*Fragaria x annanassa* Duch., cv. Albion) with 100 % red colour were acquired from an orchard (Pilar, Buenos Aires province, Argentina). Fruit was immediately transported to the laboratory and those that presented fungal infection, mechanical injuries or showed physiological disorders were discarded. Uniform-in-size fruit was randomly distributed in trays, and kept at 5 ± 1 °C until processed within a day. A total of 600 strawberries were processed. To test for microbiological issues, 90 strawberries were inoculated (60 were subjected to ozone treatments and 30 used as control). For physicochemical, antioxidant, optical, and sensory evaluations 510 strawberries were used (280 strawberries were subjected to ozone treatment and 230 were used as controls).

2.3. Ozone treatments

Aqueous ozone treatments were performed in a bubble column (diameter: 0.1 m, height: 0.24 m) with an inbuilt diffuser to sparge the gas into the liquid phase. Ozone was generated with a corona discharge ozone generator (UTK-O-4/8, Unitek S.A., Argentina) using oxygen at 0.62 bar and a flow rate of 5 L min^{-1} as the feeding gas. Ozone content at the inlet gas supply of the column was 18 ± 2 mg.L^{-1}. Ten strawberries (~200 g) were immersed into the bubble column filled with 1.25 L of distilled water at 20 ± 1 °C, and ozonized for 5 and 15 min. After the treatment, the fruit was dried by rolling them over paper towel, packed in closed polypropylene trays permeable to air (26 cm × 19 cm x 6 cm), and stored at 5 ± 1 °C for 8 days (5 min at maximum were needed for the drying and packing process). Each tray contained 10 strawberries. Non–treated (unwashed) fruit was used as control group. For microbiological evaluations, unwashed fruit inoculated with *B.cinerea* was considered as control. For the determination of bioactive compound contents and antioxidant activity, control and treated samples were taken at selected days of storage, frozen at -80 °C, and kept in an ultra-freezer MDF-U55V (Panasonic, Japan) until analyses. Table 1 summarizes the treatment and storage conditions of the fruit used for quality evaluations.

The concentration of aqueous ozone used in the treatments was determined and reported in a previous work (Contigiani et al., 2018). Measurements were performed both with and without strawberries immersed into the column. Ozone concentration increased during the first 5 min and then remained constant at 3.5 ± 0.5 mg.L^{-1} in water with strawberries, and at 7.0 ± 0.3 mg.L^{-1} in water without them (Contigiani et al., 2018).

2.4. Inoculation with Botrytis cinerea and decay evaluation

B. cinerea BAFC 3003 was provided by BAFC Culture Collection (Faculty of Exact and Natural Sciences, University of Buenos Aires) (http://www.inmibo.exactas.uba.ar). Fungal strain was cultivated on MEA for 14 days at 25 °C. Conidia were harvested by washing with sterile peptone water (0.1 g.100 mL^{-1}) containing Tween 80 (0.05 mL.100 mL^{-1}) and shaking gently on a vortex mixer. The conidia suspension (approximately 10^4–10^5conidia.mL^{-1}) was immediately used for subsequent experiments.

Strawberries were immersed in a hypochlorite solution (200 mg.L^{-1}) for 2 min and rinsed three times with distilled water to decontaminate their surface. A spot-inoculation method was used to inoculate *B. cinerea* in strawberries. Two small and superficial injuries were made on the fruit skin, approximately midway between the calyx and the cap, and 10 μL of inoculum suspension were applied in each injury. The inoculated strawberries were kept in a biosafety hood at 20 ± 2 °C for 2 h to allow attachment of the fungus.

B. cinerea incidence in treated and control fruit was evaluated daily along 8 days of storage. Strawberries with visible fungal development

Table 1. Treatment and storage of strawberries for each quality aspect evaluated.

Quality factor	Treatment	Storage evaluation periods
Botrytis cinerea growth	Aqueous ozone - 5 min	8 days at 5 ± 1 °C (Daily visually inspected)
	Aqueous ozone - 15 min	
	Control (inoculated unwashed fruit)	
Total soluble solids, titratable acidity, pH	Aqueous ozone - 5 min	8 days at 5 ± 1 °C (Evaluated at 0, 4 and 8 days)
	Aqueous ozone - 15 min	
	Control (unwashed fruit)	
Bioactive compounds content Antioxidant capacity (*in vitro*)	Aqueous ozone - 5 min	8 days at 5 ± 1 °C (After each storage period: 0, 4 and 8 days, frozen and kept at -80 °C until analyses)
	Aqueous ozone - 15 min	
	Control (unwashed fruit)	
Antioxidant capacity (*in vivo*)	Aqueous ozone - 5 min	4 days at 5 ± 1 °C (After each storage period: 0, 4, frozen and kept at -80 °C until analyses)
	Aqueous ozone - 15 min	
	Control (unwashed fruit)	
Sensory evaluation	Aqueous ozone - 5 min	1 and 3 days of storage at 5 ± 1 °C
	Control	

were considered decayed. Results were presented as percentage of infected fruit. Thirty berries were analysed for each condition.

2.5. Total soluble solids, titratable acidity and pH

Fruit was homogenized using a pestle and mortar. Total soluble solids (TSS) were measured with a digital refractometer model PR-1 (Atago, Tokyo, Japan) calibrated with distilled water at 20 °C, and the reading was taken with an accuracy of 0.1. The pH of the samples was measured using a digital pHmeter (model 310, Orion, TX, USA) calibrated with commercial buffer solutions of pH 4.0 and 7.0. To determine titratable acidity (TA), a potentiometric titration with 0.1N NaOH was performed; 5 g of homogenized fruit pulp was suspended in 50 mL hot distilled water (80 °C) for 30 min and then filtered through a Whatman (#1) filter paper. The remaining solution was titrated against standardized 0.1N NaOH, using the pHmeter (model 310, Orion, TX, USA) until a pH of 8.2 ± 0.1 was reached (corresponding to the phenolphthalein end point). Results were expressed as percentage of citric acid (Grobelna, Kalisz & Kieliszek, 2019a). Ten strawberries taken from four repetitions of treatments were analysed for each condition and measurements were performed in duplicate.

2.6. Colour measurements

Surface colour of the samples was measured with a hand-held tristimulus reflectance spectrocolorimeter (Minolta Co., Model CM-508-d, Japan) using a 1.1 cm measuring aperture. Readings were obtained using the D65 illuminant and a 2° observer. A standard white tile was used to calibrate the instrument. L*(lightness), a*(green-red chromaticity) and b* (blue-yellow chromaticity) coordinates of the CIELab colour space were recorded (Grobelna, Kalisz & Kieliszek, 2019a). Chroma (C) and hue (h) colour functions were calculated. Thirty strawberries were measured for each condition and two readings were taken at different positions of the sample surface. Measurements were made on the same fruit along storage.

2.7. Extraction of bioactive compounds

Ten strawberries (from four repetitions of treatment) were used to obtain ten extracts for each treatment condition (control, 5 min O_3 and 15 min O_3) and day of storage (0, 4 and 8 days). Frozen (-80 °C) strawberries were thawed for 30 min at room temperature (20 ± 1 °C) and ground using a pestle and mortar; 10 g of the purée were homogenized with 80 ml of a mixture of ethanol and 0.1 N HCl (85:15 v/v). The mixture was poured into a double-walled cylindrical vessel (diameter: 7 cm; height: 10 cm) and sonicated (Vibracell^{R}, net power output: 600 W, Sonic Materials Inc., Newton CT, USA) for 15 min at 20 kHz and 60 % of wave amplitude (71.4 μm) using a 13 mm diameter solid probe. The vessel was connected to a thermostatically controlled water bath (Haake, Model Rotovisco RV 12, Germany) to avoid the overheating of the sample. The extract was later vacuum-filtered through a Whatman (#1) filter paper and stored at -80 °C until analysis. The extracts were used for the measurement of anthocyanins, phenolic compounds, and antioxidant activity.

2.8. Total monomeric anthocyanin content

Total monomeric anthocyanin content was determined according to the pH differential method (Giusti and Wrolstad, 2001). Briefly, the extracts were diluted (1/10) in potassium chloride (0.025 M, pH 1.0) and sodium acetate (0.4 M, pH 4.5) buffers. Samples were kept at room temperature in the dark for 15 min. The absorbance was measured at 510 nm and 700 nm by means of a UV- VIS spectrophotometer (model V-630, JASCO, Japan). The anthocyanin concentration in the samples was calculated using Eq. (1):

$$\text{Anthocyanin content} = \frac{\left[(A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}\right] \times MW \times DF \times 1000}{\varepsilon \times L} \quad (1)$$

where A_{510} and A_{700} are the absorbances measured at 510 and 700 nm, respectively; MW (433 g/mol) is the molecular weight of pelargonidin 3-glucoside; DF is the dilution factor; ε is the molar absorptivity (15600 $L.mol^{-1}.cm^{-1}$ according to Giusti et al., 1999) and L (cm) the cell length. Results were expressed as mg pelargonidin 3- glucoside per 100 g of fruit on a dry weight (DW) basis. Ten extracts were measured for each

condition and absorbance measurements in each extract were made in triplicate.

2.9. Total phenolic content

The total phenolic content (TPC) was determined according to a modified Folin-Ciocalteu colorimetric method (Slinkard and Singleton, 1977; Grobelna, Kalisz & Kieliszek, 2019b). Briefly, 80 μL of the fruit extracts were blended with 2.72 mL of bidistilled water and 0.2 mL of Folin - Ciocalteu phenol reagent. The mixture was kept at room temperature for 6 min in the dark. Then, 2 mL of Na_2CO_3 (7 % w/v) were added and after incubating for 90 min at room temperature, the absorbance was measured at 760 nm. Solutions of gallic acid (1–8 $mg.L^{-1}$) were used to construct the calibration curve ($r^2 = 0.9975$) and the results were expressed as mg gallic acid equivalents (GAE) per 100 g of fruit on a dry weight (DW) basis. Ten extracts were measured for each group of samples and the absorbance in each extract was measured in duplicate.

2.10. Antioxidant activity

2.10.1. In vitro assays

The antioxidant activity of the fruit extracts was determined using the oxygen radical absorbance capacity (ORAC) and the Trolox equivalent antioxidant capacity (TEAC) assays. The ORAC assay was conducted according to the modified method described by Dávalos et al. (2004) with slight modifications. A potassium phosphate buffer solution was used as solvent (75 mM, pH 7.4). Briefly, 25 μL of diluted extracts (1/160 and 1/240) were poured in a 96-well black polystyrene plate (Corning Inc., USA) and mixed with 8.16 10^{-5} $mmol.L^{-1}$ fluorescein (115 μL/well). The mixture was incubated at 37 °C for 10 min, before adding 153 $mmol.L^{-1}$AAPH (60 μL/well). The fluorescence intensity was recorded for 99 cycles every 1.15 min at an excitation wavelength of 485 nm, and an emission wavelength of 520 nm by a Multilabel Microplate Reader (Perkin Elmer, Victor^3 model, USA). Solutions of Trolox (3.125–50 μM) were used to construct the calibration curve (r^2 = 0.9871).

The TEAC assay was performed according to the method described by Re et al. (1999) with slight modifications (Kalisz et al., 2020). The ABTS radical cation was generated by mixing ABTS solution (7 mM) and potassium persulphate (2.45 mM final concentration) in a ratio 2:1, and allowing them to react for 16–18 h at room temperature in the dark. The solution was then diluted in a phosphate buffer (5 mM, pH 7.40) to obtain an absorbance of 1.00 ± 0.01 at 734 nm. A volume of 7.92 mL of the resulting $ABTS^{\bullet+}$ solution was mixed with 80 μL diluted extracts (1/6). The absorbance of the solution was immediately measured at 734 nm, and after 2 h of incubation at 30 °C in the dark. Solutions of Trolox (6–17 μM) were used to construct the calibration curve ($r^2 = 0.9923$).

Antioxidant capacity (TEAC and ORAC) results were expressed as millimoles of Trolox equivalents (TE) per 100 g of fruit on a dry weight (DW) basis. Ten extracts were analysed for each group of samples and measurements in each extract were made in duplicate.

2.10.2. In vivo assay

The antioxidant activity of the strawberry extracts was evaluated *in vivo* by the measurement of intracellular reactive oxygen species (ROS) accumulation in *C. elegans* under thermal stressful conditions, previously fed with *E. coli* (control worms) or *E. coli* plus strawberry extracts: control (untreated fruit), 5 min O_3, and 15 min O_3 at day 0 and 4 of storage. A total of four strawberry extracts were evaluated for control and ozonized fruit.

C. elegans var. Bristol (strain N2) was used throughout the experiment. The strain was obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota (Minneapolis, MN, USA) (https://cgc.umn.edu) and was routinely propagated on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 at 20 °C as described by Brenner (1974). Synchronization of worm culture was achieved by treating gravid hermaphrodites with an alkaline bleach solution as described by Stiernagle (2006) and recovery of hatched L1 larvae on NGM plates seeded with *E. coli*.

The ethanol in the strawberry extracts used for the assay with *C. elegans* was removed by evaporating an aliquot of each extract (10 mL) under vacuum using a rotary evaporator (Decalab, Argentina) at 40 ± 2 °C. Afterwards, the extracts were diluted to a final volume of 20 ml by adding phosphate buffer (5 mM, pH 7.40) and used to feed the worms. Treatments were performed in 24-well sterile tissue culture plates according to the following procedure. In each well, 100 age-synchronized L4-staged nematodes were incubated with 10% of fruit extract in M9 buffer (6 $g.L^{-1}$ Na_2HPO_4, 3$g.L^{-1}KH_2PO_4$, 5$g.L^{-1}$NaCl, 3 $g.L^{-1}$ $MgSO_4{\cdot}7H_2O$, pH 7.2) supplemented with freeze/thaw inactivated *E. coli*OP50 as food source (DO_{600nm} = 2.5) at 20 °C for 48 h. Worms exposed to the M9 buffer without any extract were assayed as a control group and defined as control worms. No toxic effects of any strawberry extracts were observed at the assay concentration.

To quantify intracellular ROS accumulation induced by thermal stress at 37 °C in living nematodes, 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) was used. H2DCF-DA is a membrane-permeable substance which enters the worm's cells where it is converted to H2DCF. Subsequently, this non-fluorescent probe can be oxidized by ROS to yield the fluorescent dye dichlorofluorescein (DCF); thus, changes in fluorescence indicate the accumulation of ROS in worms. After the exposure to the strawberry extracts, nematodes were harvested, washed thrice with buffer PBS with 0.1% Tween 20 (PBST), and then 10 animals were transferred to wells of a 96-well black polystyrene plate (Corning Inc., USA) in 25 μL of PBST buffer. Afterwards, 25 μL of fresh 100 μM H2DCF-DA solution in PBS buffer were added to each well. In all the experiments, control wells containing nematodes without H2DCF-DA and wells containing H2DCF-DA without animals were prepared in parallel. The fluorescence from each well was measured at 37 °C in a Spark 20M Multimode Microplate Reader (Tecan, NC, USA) immediately after the incorporation of the probe. The fluorescence intensity was recorded every 15 min during 3 h at an excitation and emission wavelengths of 485 and 535 nm, respectively. The fluorescence signals of the control wells and the initial fluorescence of each sample were subtracted from the corresponding signals of each well after every measurement. ROS measurements were made in quadruplicate in control worms and in worms exposed to the different strawberry extracts.

2.11. Sensory studies

Difference from control test was used to determine whether global sensory differences existed between ozone-treated and control samples. Sensory analysis was carried out by an untrained consumer panel. Frequent consumers of strawberries (60 persons aged between 20 and 50-year-old) were randomly recruited at the Buenos Aires University. Three samples were provided to the panellists. One of them was identified as control, and the other two (blind control and 5-min ozone-treated fruit) were identified by a three digits number. The blind control was identical to the control sample, and was used as a placebo to screen the panellists' performance. The panellists were instructed to globally evaluate the control sample first (colour, texture, aroma, taste) and then the coded samples. In case of finding differences among samples, consumers were asked to score them in a 5-point scale where 1 and 5 indicated "no difference" and "extremely different", respectively (Supplementary material 1) (Meilgaard et al., 2006). Control and treated fruit stored for 1and 3 days were evaluated. Longer periods of storage were not considered since untreated fruit could present risks to the consumers. For this experiment, informed consent was obtained from all the panellists.

2.12. Statistical analyses

SPSS software v. 19 (SPSS Inc., Chicago, USA) and Infostat software v.2009 (Universidad Nacional de Córdoba, Argentina) were used. Data of

B. cinerea infection, physicochemical and *in vitro* antioxidant properties were analysed by a two-way ANOVA considering treatment (control, 5 min O_3, and 15 min O_3) and storage time (0, 4, and 8 days) as sources of variation. *In vivo* antioxidant properties were analysed by a one-way ANOVA considering seven treatments: control worms, control at day 0 and 4, 5 min O_3 at day 0 and 4, and 15 min O_3 at day 0 and 4 of storage. Duncan's multiple range test was used to compare the mean values. Findings from the sensory evaluation were analysed by a two-way ANOVA (samples and assessors as sources of variation) followed by Dunnett's test. Colour data was analysed by a two-way multivariate analysis of variance (MANOVA) followed by post-hoc Hotelling test with Bonferroni correction. Prior to multivariate analyses, outliers were identified and removed using the Mahalanobis distance. To explore the association between anthocyanin and total phenolic contents or ORAC and TEAC values, Pearson correlation coefficients were calculated with the "correlation" procedure. In statistical analyses the significant level was set at $\alpha < 0.05$ (Quinn and Keough, 2002).

3. Results and discussion

3.1. Strawberry decay caused by Botrytis cinerea

The efficacy of aqueous ozone treatments (maximum concentration 3.5 $mg.L^{-1}$for 5 and 15 min) to control *B. cinerea* infection in inoculated strawberries stored at 5 °C is shown in Figure 1. No significant interactions between treatment and storage time were observed ($F_{4,\ 18} = 0.79$, $p = 0.5457$), whereas main effects of each factor were statistically significant ($p < 0.05$). Although treated and untreated samples exhibited an increase in the percentage of infected fruit during storage, *B. cinerea* incidence was significantly lower in ozonized fruit. Despite the fact that there were no significant differences between both ozone doses assayed, the delay on the onset of infection in relation to the control was 4 days for samples ozonized for 5 min, and 2 days for 15 min treated ones. The percentage of infected fruit in both ozone doses showed similar values from day 6 until the end of storage. Moreover, the infection rates for control and treated samples were almost the same between day 6 and 8 of storage. However, at day 8, ozonized samples reduced by ~17 % the *B. cinerea* incidence when compared to non-treated fruit.

For comparative purposes, the results previously reported by Contigiani et al. (2018) on the reduction of native mycobiota in strawberries cv Albion due to the application of aqueous ozone treatments under the same operative conditions, were also included in Figure 1 (dotted lines).

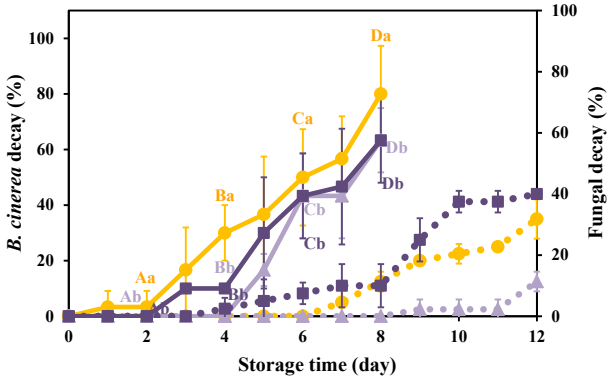

Figure 1. *Botrytis cinerea* incidence (%) on artificially inoculated strawberries exposed to aqueous ozone for 5 and 15 min and stored at 5 ± 1 °C. Vertical bars represent the standard deviation of the means.(●) Control, (▲) 5 min O_3 and (■) 15 min O_3. Different capital letter indicates significant differences among storage day and different lowercase letter indicates differences among treatments, according to the two-way Anova followed by Duncan's test. Dotted lines correspond to previous published data on the effect of aqueous ozone treatments for 5 and 15 min on naturally occurring fungal incidence. (...■...) Control, (...▲...) 5 min O_3, and (...■...) 15 min O_3 (adapted from Contigiani et al., 2018).

Ozonation for 5 min reduced and delayed naturally occurring fungal incidence. After 12 days of storage, the percentage of infected fruit in relation to stored control was reduced by 22% and, as in the case of *B. cinerea* decay, a 2-day delay on the onset of infection was observed. Contrary, ozone treatment for 15 min presented earlier signs of infection, and showed higher percentage of infected fruit than control during storage. In addition, in the previous work it was observed that ozone washing for 5 min reduced the weight loss with respect to control along cold storage. These samples also showed thicker and more electron dense cuticular membranes than control and 15 min treated fruit, which could partially support a protective effect induced by this ozone dose against water loss and fungal incidence (Contigiani et al., 2018).

Aday et al. (2014) analysed the effect of ozonized water treatments (0.075, 0.15 and 0.25 $mg.L^{-1}$ for 2 and 5 min) on fungal growth in strawberries cv. Camarosa and found a complete inhibition of mycobiota development during three weeks of storage at 4 °C. However, the ratio of fruit to ozonized water, and the number of berries evaluated were not reported, hindering the comparison of results. In treatments carried out with ozone in gaseous phase, the *per se* effect of ozone in inhibiting *B. cinerea* mycelial growth and sporulation have been demonstrated by several authors in experiments performed *in vitro* (Minas et al., 2010; Nadas et al., 2003). However, it has been reported that the antimicrobial activity of ozone could be attributed not only to the oxidative damage of vital cell components leading to a microbial growth inhibition, but also to an indirect effect through the induction of plant defence mechanisms. The resistance response to pathogen attack has been shown to be dose-dependant and related to the induction of anti-oxidative systems, the accumulation of phenolic compounds with antimicrobial activity (e.g. stilbenephytoalexins) and pathogenesis-related proteins, and the reinforcement of cell walls (Kangasjärvi et al., 1994; Sandermann et al., 1998). Therefore, the dose-dependant response observed in the present study for the reduction of *B. cinerea* growth and its correlation with naturally occurring fungal incidence could be related partly to the induction of defence responses in the fruit.

Minas et al. (2010) demonstrated that *B. cinerea* inhibition in kiwifruit could be associated with the induction of plant defence mechanisms by exposing the fruit to gaseous ozone (0.3 μL/L for 2–144 h at 0 °C) prior to the inoculation with the pathogen. Moreover, these authors found a strong negative correlation between the accumulation of total phenols and disease incidence during storage at 20 °C.

3.2. Total soluble solids, titratable acidity and pH

Mean values for total soluble solids, titratable acidity, and pH of control and treated samples are listed in Table 2. Initial values of the physicochemical parameters were similar to those reported in the literature for ripe strawberries (Ornelas-Paz et al., 2013). No significant differences ($p > 0.05$) were found in TSS and TA values neither due to the effect of storage nor to ozone washing. Treatment and storage time had a significant effect on the pH of samples ($p < 0.01$), but their interaction did not ($p > 0.1$). Ozonized fruit showed slightly higher pH values than the control, which proved statistically significant in the 15-min treated fruit. Furthermore, the pH of the samples ozonized for 15 min showed a little but significant increase after 8 days of storage.

Contradictory results were reported in the literature on physicochemical parameters of strawberries as affected by different ozone doses in aqueous phase and/or storage conditions. Ferreira, de Alencar, Alvez, Ribeiro & da Silva (2017) found that ozonisation in distilled water at pH 3.0 and pH 8.7 for 5 min (0.11 and 0.04 $mg.L^{-1}$, respectively) maintained TSS values of strawberries cv. Portola throughout refrigerated storage while control fruit showed decreasing values. In addition, Aday et al. (2014) observed that ozone washing in low doses (0.075 and 0.15 $mg.L^{-1}$ for 2 and 5 min) helped in maintaining higher concentration of TSS in strawberries cv. Camarosa when compared to control samples along storage at 4 °C. These treatments also significantly slowed the increase in pH along storage. In contrast, Alexandre et al. (2012) did not

Table 2. Effect of aqueous ozone treatments on physicochemical parameters (total soluble solids, titratable acidity and pH) of strawberries throughout storage at 5 ± 1 °C.

Parameter	Treatment time (min)	Storage period (day)		
		0	4	8
Total soluble solids (ºBrix)	0	$9.1 \pm 0.7^{n.s}$	$8.8 \pm 0.7^{n.s.}$	$8.3 \pm 0.3^{n.s.}$
	5	$8.6 \pm 0.6^{n.s.}$	$10.0 \pm 1.2^{n.s.}$	$9.1 \pm 0.1^{n.s.}$
	15	$9.0 \pm 0.5^{n.s.}$	$8.2 \pm 0.2^{n.s.}$	$9.9 \pm 1.4^{n.s.}$
Titratable acidity (% citric acid)	0	$0.89 \pm 0.06^{n.s.}$	$0.87 \pm 0.07^{n.s.}$	$0.98 \pm 0.06^{n.s.}$
	5	$0.90 \pm 0.09^{n.s.}$	$0.89 \pm 0.06^{n.s.}$	$0.88 \pm 0.09^{n.s.}$
	15	$0.86 \pm 0.02^{n.s.}$	$0.81 \pm 0.11^{n.s.}$	$0.93 \pm 0.01^{n.s.}$
pH	0	3.4 ± 0.1^{Aa}	3.3 ± 0.1^{Aa}	3.5 ± 0.1^{Ba}
	5	3.4 ± 0.1^{Aa}	3.4 ± 0.1^{Aa}	3.5 ± 0.1^{Ba}
	15	3.6 ± 0.1^{Ab}	3.4 ± 0.1^{Ab}	3.6 ± 0.1^{Bb}

Mean values ±standard deviation of physicochemical parameters. n.s. indicates no-significant ($p > 0.05$). Means followed by different lowercase letters (a-b) within a column are significantly different ($p < 0.05$). Different uppercase letter (A-B) within a row are significantly different according to the two-way ANOVA followed by Duncan's test ($p < 0.05$) ($n = 3$).

Table 3. Effect of aqueous ozone treatment on colorimetric parameters and functions of strawberries throughout storage at 5 ± 1 °C.

Treatment time (min)	Storage time (day)	L*	a*	b*	C	h	
0	0	35.0 ± 3.1	39.6 ± 2.5	26.2 ± 4.2	47.6 ± 4.3	33.2 ± 3.0	a,b
	4	36.2 ± 2.8	39.3 ± 2.9	25.5 ± 3.8	47.0 ± 4.3	32.6 ± 2.6	b, c
	8	35.9 ± 2.9	39.6 ± 3.3	26.1 ± 4.2	47.5 ± 4.9	33.1 ± 2.7	a, b
5	0	33.9 ± 2.8	38.9 ± 3.3	25.0 ± 4.3	46.4 ± 4.8	32.4 ± 3.1	a
	4	35.0 ± 2.9	36.0 ± 3.8	22.2 ± 4.9	42.4 ± 5.7	31.3 ± 3.6	c
	8	35.2 ± 2.7	38.4 ± 4.2	24.7 ± 4.8	45.8 ± 5.8	32.4 ± 3.2	a, b, c
15	0	35.1 ± 2.2	40.0 ± 2.9	25.5 ± 3.7	47.2 ± 4.2	32.5 ± 2.7	a, b
	4	35.5 ± 2.4	37.1 ± 3.9	23.0 ± 4.1	43.8 ± 5.3	31.4 ± 2.8	c
	8	35.8 ± 2.3	38.2 ± 2.9	24.1 ± 3.3	45.2 ± 3.9	32.0 ± 2.6	b, c

Mean values ± standard deviations. Different letters indicate significant differences according to two-way MANOVA and post-hoc comparisons using Hotelling-Bonferroni test ($p < 0.05$) ($n = 30$).

find a significant effect of aqueous ozone treatment (0.3 $mg.L^{-1}$-2 min; 80 g fruit per 30 L water) and storage time on the pH values of strawberries cv. Camarosa stored at 4 °C and 15 °C for 13 days.

3.3. Colour

The colorimetric parameters and functions of control and ozonized strawberries during storage are shown in Table 3. Interaction between treatment and storage time was significant ($F_{4,\ 211} = 2.17$, $p < 0.0024$). Control fruit showed no significant changes in colour throughout the storage period. Immediately after ozonisation, samples treated for 5 and 15 min did not exhibit significant variation in colour when compared to control. However, after 4 days of storage, ozonized samples showed slight but significant changes in colour compared to day 0, associated mainly with a slight reduction in a*, b* and chroma values, indicating a decrease in colour saturation or vividness. As storage progressed, a*, b*

Table 4. Effect of aqueous ozone treatment on total monomeric anthocyanins (TAC) and phenolic compounds (TPC), and antioxidant capacity evaluated by ORAC and ABTS assays of strawberries during storage at 5 ± 1 °C.

Parameter	Treatment time (min)	Storage period (day)		
		0	4	8
TAC (mg/100g DW)	0	224.7 ± 68.5^{a}	309.1 ± 49.9^{ab}	350.7 ± 88.7^{b}
	5	285.6 ± 43.9^{ab}	240.2 ± 72.9^{a}	271.4 ± 66.2^{ab}
	15	274.2 ± 75.6^{ab}	302.9 ± 75.0^{ab}	230.6 ± 46.8^{a}
TPC (mg GAE/100g DW)	0	1655.5 ± 234.2^{a}	1725.3 ± 174.0^{a}	2417.8 ± 297.9^{cd}
	5	1902.0 ± 187.0^{ab}	1649.3 ± 205.6^{a}	2589.6 ± 374.2^{d}
	15	1917.2 ± 399.6^{ab}	1689.5 ± 211.6^{a}	2201.9 ± 185.7^{bc}
TEAC (mmol TE/100 g DW)	0	32.8 ± 3.1^{A}	31.8 ± 5.7^{B}	33.8 ± 5.1^{AB}
	5	36.1 ± 4.0^{A}	31.2 ± 5.6^{B}	36.6 ± 6.8^{AB}
	15	36.9 ± 4.9^{A}	32.1 ± 5.1^{B}	31.5 ± 4.0^{AB}
ORAC (mmol TE/100 g DW)	0	27.2 ± 5.9^{A}	42.5 ± 5.9^{B}	48.0 ± 4.2^{C}
	5	31.5 ± 7.5^{A}	36.1 ± 6.7^{B}	45.3 ± 4.5^{C}
	15	31.8 ± 1.9^{A}	43.5 ± 5.7^{B}	47.2 ± 10.9^{C}

Mean values ± standard deviations. Means followed by different lowercase letters (a-b) within a column are significantly different and by different uppercase letter (A-B) within a row are significantly different according to two-way ANOVA followed by Duncan's test ($p < 0.05$) ($n = 10$).

and chroma values in these samples tended to increase and did not show significant differences in comparison with untreated fruit.

Contrary to these results, Aday et al. (2014) and Alexandre et al. (2012), observed lesser changes in colour in treated 'Camarosa' strawberries than in control fruit throughout refrigerated storage, when applying lower ozone doses than those used in this work.

3.4. Anthocyanins and total phenolic contents

Total monomeric anthocyanin content (TAC) and total phenolic content (TPC) of assayed samples are presented in Table 4. TAC and TPC values in the raw fruit (day 0) (224.7 ± 68.5 mg/100 g DW and 1655.5 ± 234.2 mg GAE/100 g DW, respectively) were within the range of those reported in the literature for fully ripe strawberries cv. Albion (Ornelas-Paz et al., 2013). Significant interaction between ozone exposure time and storage time was found for TAC ($F_{4,81} = 6.1$, $p < 0.0002$) and TPC ($F_{4,81} = 3.5$, $p < 0.012$). Anthocyanin concentration significantly increased in control samples after 8 days of storage. Exposure to ozone for 5 min did not induce significant changes in anthocyanin accumulation as compared to the control, whereas the content of this pigment in samples treated for 15 min was significantly lower ($p < 0.05$) at day 8 of storage.

Control fruit exhibited a significant increase in TPC at day 8, which was in accordance with the variation observed in anthocyanin concentration. TPC values of samples treated for 5 min also increased during this storage period and did not differ significantly from control. In contrast, despite the decline observed in anthocyanin concentration in strawberries ozonized for 15 min, TPC also showed a slight but not significant increase after 8 days of storage. Correlation between TAC and TPC was not significant ($p > 0.5$), due to the different accumulation trend observed in strawberries treated for 15 min along storage. This lack of correlation could be associated with changes in the profile of other phenolic compounds present in strawberries, induced by the 15-min ozone treatment. In addition, it must be taken into consideration that the measurement of total phenolic compounds by the Folin-Ciocalteu reagent could be overestimated due to the reaction of other non-phenolic compounds, such as ascorbic acid, glucose, fructose, etc (Slinkard and Singleton, 1977).

Contrary to the findings in this study, Alexandre et al. (2012) observed a better retention of anthocyanin content in ozonized strawberries cv. Camarosa stored at 4 ± 1 °C when compared to untreated fruit. These discrepancies could be partly associated with differences in the ozone doses applied, and the different strawberry cultivars evaluated.

3.5. Antioxidant activity

3.5.1. In vitro assays

Results of ORAC and ABTS assays are presented in Table 4. Statistical analysis indicated no significant interaction between the treatment applied and storage time for ABTS ($F_{4,81} = 1.76$, $p > 0.1$) and ORAC ($F_{4,81} = 1.25$, $p > 0.1$). When analysing the main effects, both TEAC and ORAC values of samples significantly changed over time ($p < 0.05$ and $p < 0.0001$, respectively) but there was not a significant effect of treatment ($p > 0.05$). Mean TEAC values significantly diminished on day 4, mainly in ozonized fruit. In general, TEAC values tended to increase on day 8 but did not differ significantly from value on day 0. However, ORAC values of samples presented a different trend; both treated and control fruit showed a significant increase in ORAC values along storage.

Pearson correlation analysis revealed a weak but significant positive correlation between TPC and TEAC ($r = 0.59$, $p < 0.0001$), TPC and ORAC ($r = 0.45$, $p < 0.001$), and TAC and ORAC ($r = 0.39$, $p < 0.004$). Despite the fact that phenolic compounds, including anthocyanins, are plentiful in strawberries, a low correlation with antioxidant activity was also reported by other authors (Cordenunsi, Genovese, Oliveira do Nascimento, Hassimotto, dos Santos and Lajolo, 2005; Meyers et al., 2003). However, in some studies a strong positive correlation was found (Kalt et al., 1999). These contrasting results could be partly attributed to the fact that fruit phenolic may exhibit antagonistic as well as additive/synergistic activities when interacting with other phenols and phytochemicals. Therefore, total antioxidant activity could be affected by the relative concentration and the existence of particular phenolic compounds (Meyers et al., 2003). In addition, the low correlation between TAC and ORAC could also be associated with another of the strawberry's constituents such as vitamin C that contribute to the antioxidant activity. According to Kalt et al. (1999), this vitamin represented about 9–15 % of the ORAC value of berries.

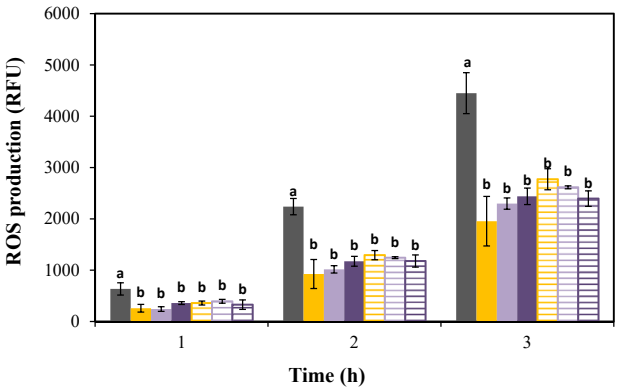

Figure 2. ROS accumulation during thermal stress at 37 °C in *C. elegans* previously exposed to different strawberry extracts. Results are expressed as means ± standard deviation of relative fluorescence units (RFU). At each given stress-time point, different lowercase letters indicate significant differences among treatments ($p < 0.05$). ■ Control worms, day 0; ■ control, day 0; ■ 5 min O_3, day 0; ■ 15 min O_3, day 0; ≡ Control worms, day 4; ≡ control, day 4; ≡ 5 min O_3, day 4; ≡ 15 min O_3, day 4.

3.5.2. In vivo assay

Results of ROS accumulation during thermal stress at 37 °C of living nematodes previously exposed or not to the strawberry extracts are shown in Figure 2. Thermal stress led to an increase in intracellular ROS accumulation in the nematode, as evidenced by the time-dependant rise in fluorescence intensity observed in both control worms and extract-exposed nematodes. At the different times of exposure to stress analysed (1, 2 and 3 h), statistical analysis indicated that all strawberry extracts significantly ($P < 0.0001$) reduced the amount of ROS generated in nematodes under conditions of thermal stress when compared to control worms. However, no significant differences were observed among nematodes exposed to ozonized and untreated fruit extracts at any exposure time. Regarding the effect of ozonisation treatments, these results are in agreement with the *in vitro* evaluation of antioxidant capacity previously described. Thus, these findings demonstrated that processing strawberries with aqueous ozone for 5 or 15 min did not provoke neither a beneficial nor detrimental effect in their antioxidant potential. Considering the storage effect, although significant modifications in ORAC and ABTS values were detected during refrigerated storage, changes in the anti-oxidative effect were not observed after 4 days in *in vivo* studies.

An improvement in the antioxidant status in berries and other fruit subjected to aqueous and gaseous ozone treatments has been reported by several authors (Minas et al., 2010; Onopiuk et al., 2017). This could be related, as mentioned above, to the elicitation of host defences which are triggered when plant organs are exposed to abiotic stressful conditions, such as ozone. However, a significant improvement in the antioxidant potential was not observed at the ozone doses assayed in this work, neither *in vitro* nor *in vivo* studies.

It is important to highlight that, as far as we know, there are no reports about the protective effect of strawberry extracts against oxidative stress in *C. elegans* or in the use of this nematode for the evaluation of the

effect of ozone processing on the antioxidant properties of foods, being this work a novel contribution on this subject. Nevertheless, the beneficial effect of phytochemicals against oxidative stress using this animal model has been demonstrated in different matrices. Song, Zheng, Li & Liu (2020a) found that raspberry extracts could ameliorate oxidative stress, improve antioxidant enzyme activity and enhance resistance to oxidative stress induced by paraquat exposure, leading to an increase in the mean lifespan of *C. elegans*. Similarly, Wilson et al. (2006) found that treatment with blueberry polyphenols or a proanthocyanidin-enriched fraction from blueberries produced both a moderate extensions of the mean maximum adult lifespan and improved thermo-tolerance of *C. elegans*. A recent study of Moraes et al. (2020) reported a protective effect against ROS generation in *C. elegans* after being fed with balckberry extracts. Moreover, the underlying action mechanisms of berry phytochemicals have been explored using this nematode, and it was demonstrated that these organisms could enhance oxidative stress resistance and promote lifespan by down-regulating the over expression of reactive oxygen species and by regulating the expression of certain antioxidant and anti-aging related genes (Song, Zheng, Li & Liu, 2020a, b).

3.6. Sensory analysis

Sensory analysis was carried out only with the 5-min ozonized samples, taking into account that longer exposure times did not significantly improve the inhibition of *B. cinerea*. The average scores given by the consumers were normalized by the blind control sample score, resulting in 0.02 and 0.05 for day 1 and 3, respectively. By incorporating the placebo effect, ozone treated samples were placed in the category of "slight changes", both after 1 and 3 days of storage. Statistical analysis determined that the judgments made by the consumer panel were homogeneous ($F_{55,\ 55} = 1.47$, $p > 0.07$at day 1; and $F_{55,\ 55} = 1.55$, $p > 0.05$ at day 3 of storage). In addition, score values obtained in ozonized and blind control fruit were not statistically significant ($F_{1,\ 55} = 0.01$, $p > 0.9$ and $F_{1,\ 55} = 0.11$, $p > 0.7$ at 1 and 3 day, respectively), indicating that the consumers did not discern significant differences in the sensory characteristics of the strawberries due to the ozone treatment.

4. Conclusions

Aqueous ozone treatment (maximum concentration 3.5 mg. L^{-1}) for 5 min delayed and reduced decay caused by *B. cinerea* along cold storage in strawberries cv. Albion. In agreement with previous studies on the response of native mycobiota at the same ozone doses, a longer treatment time (15 min) resulted less effective than 5-min exposure to ozone in decreasing *B. cinerea* growth lag phase. Overall, no significant differences were found in TSS, TA, TPC, TEAC and ORAC values, or in colour characteristics among untreated and ozone-washed fruit (at both doses); neither immediately after treatment nor during 8 days of storage. However, exposure to the largest dose (15 min) resulted in a small increase in pH and lower TAC values in comparison with control, and lower TPC content than 5-min exposed strawberries at the end of storage. *In vivo* studies using *C. elegans* as a model organism, in agreement with *in vitro* experiments, provided evidence that both ozone treatments would not affect the health benefits of strawberry extracts against pathologies caused by oxidative stress. Moreover, sensory analysis with frequent consumers of strawberries indicated no significant differences between untreated and 5 min-ozonized fruit. An aqueous ozone treatment for 5 min followed by cold storage, according to present results, could be an alternative strategy for extending the postharvest life of strawberries cv Albion, delaying *B. cinerea* infection without significant impact to colour properties, *in vitro* and *in vivo* antioxidant capacity, and total phenolics and anthocyanins contents. Future additional research is needed to assess the effectiveness of ozone washing treatment in other strawberry cultivars.

Declarations

Author contribution statement

Eunice V. Contigiani, María Florencia Kronberg, Gabriela Jaramillo Sánchez: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Paula L. Gómez: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Analía B. García-Loredo: Analyzed and interpreted the data.

Eliana Munarriz: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Stella M. Alzamora: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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