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Impact of surface dielectric barrier discharge cold atmospheric plasma on quality and stability of fresh-cut iceberg lettuce

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ABSTRACT

This study evaluated the impact of cold atmospheric plasma (CAP) on fresh-cut iceberg lettuce (FIL) quality and stability. CAP treatments were performed using a Surface Dielectric Barrier Discharge, applying 6 kV at 23 kHz and exposition times varying from 15 to 60 min. FIL was analysed before and after treatments for polyphenoloxidase (PPO) and peroxidase (POD) activity, microbial contamination, colour, surface microstructure properties, chlorophylls content, polyphenols (TPC), ascorbic acid (AA), and antioxidant activity (AoA). TPC, AA and AoA were evaluated also after storage (4 °C for 5 days). CAP treatments provoked a reduction of PPO and POD activity with a processing time- and ozone-concentration-dependent effect. After treatments, a slight inactivation of mesophilic and psychrotrophic bacteria was observed, while yeast counts remained unaffected. With increasing treatment times, increasing cell damages and colour variations were detected, and a decrease of chlorophylls, TPC, AA, and AoA. After storage, an overall AoA increase was observed due to increased extractability or production by living cells of antioxidant compounds. These findings provide insights into the efficacy of CAP treatment in preserving fresh-cut iceberg lettuce quality. Further investigations are needed to optimize the process conditions and increase microbial inactivation while limiting qualitative damage to the product.

1. Introduction

Fresh-cut vegetables and fruits are a rich source of nutrients and health-enhancing compounds, but they often suffer from rapid deterioration due to mechanical and physical stresses during processing. These stresses, such as peeling, cutting, and slicing, trigger physiological responses, leading to cellular breakdown, cut surface browning, tissue softening, off-flavors, reduced nutritional value, and growth of spoilage and pathogenic microorganisms, diminishing the shelf-life of the product, ultimately (Kim et al., 2014; Laurita et al., 2021; Soliva-Fortuny & Martín-Belloso, 2003).

Iceberg lettuce, a largely consumed minimally processed vegetable known for its crispness, aroma, and high phytochemicals, is highly susceptible to quality decay and enzymatic browning, making it a subject of extensive research (Degl'Innocenti et al., 2005; Kang & Saltveit, 2002; Zhang et al., 2023). Numerous strategies have been investigated to mitigate browning in fresh-cut lettuce, including chemical interventions i.e. washing in acetic acid and ethanol solutions (Huang et al., 2020), aqueous chlorine dioxide and sodium hypochlorite (López-Gálvez et al., 2010), physical treatments such as heat shock (Rico et al., 2008; Saltveit, 2000), or their combinations (Mukhopadhyay et al., 2024; Roura et al., 2008), alongside emerging biological approaches such as allicin, phytoncide (Kim et al., 2014; Peng et al., 2014). However, as described by Meireles et al. (2016), some of these methods are generally constrained by their toxicity, high cost, and potentially impairing the products' organoleptic properties and nutrient content.

Cold plasma, among novel non-thermal technologies, emerged as promising for prolonging plant food shelf-life due to its capability to

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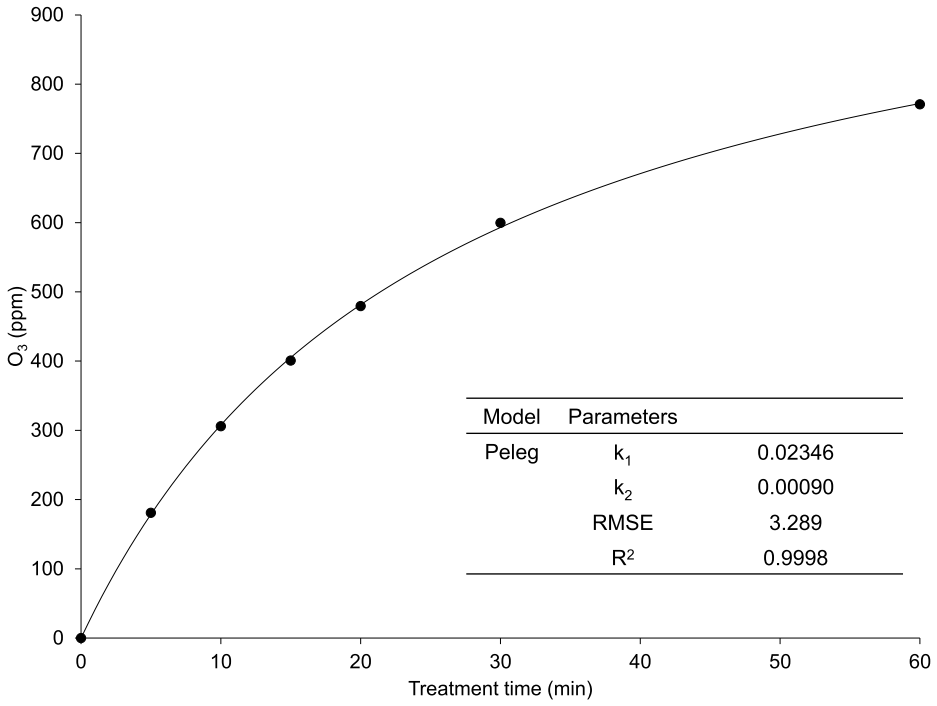

Fig. 1. O_3 concentration measured in the treatment chamber over time. The insert shows the parameters calculated fitting the data with the Peleg equation.

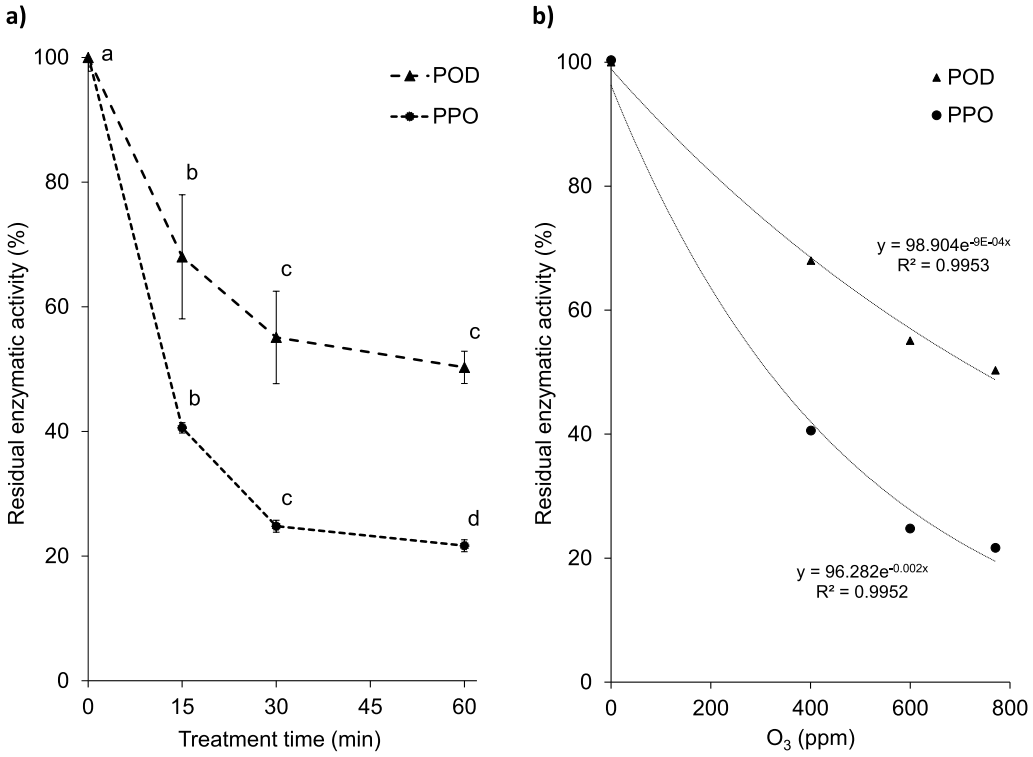

Fig. 2. Peroxidase (POD) and polyphenoloxidases (PPO) inactivation kinetic in iceberg lettuce as a function of the treatment time **(a)** and O_3 concentration **(b)**. Values are presented as means ± standard deviations (n = 3). Values with different superscripted letters are significantly different ($p < 0.05$).

inactivate microorganisms (Bangar et al., 2022) and enzymes (Mayookha et al., 2023) responsible for quality loss. However, the impact of cold plasma varies depending on the type of plasma and the operational conditions employed, making the prediction of the obtained results difficult. In particular, as recently reviewed by Özdemir et al. (2023) for fresh green leafy vegetables such as lettuce, numerous studies highlighted a significant variability or contradictory results regarding the effects of CAP on quality parameters and microbial inactivation and prevalently focused only on the evaluation of post-treatment effects without considering possible quality variations after storage. Moreover, studies on the use of surface dielectric barrier discharge (SDBD) as a plasma reactor to produce minimally processed lettuce are still limited

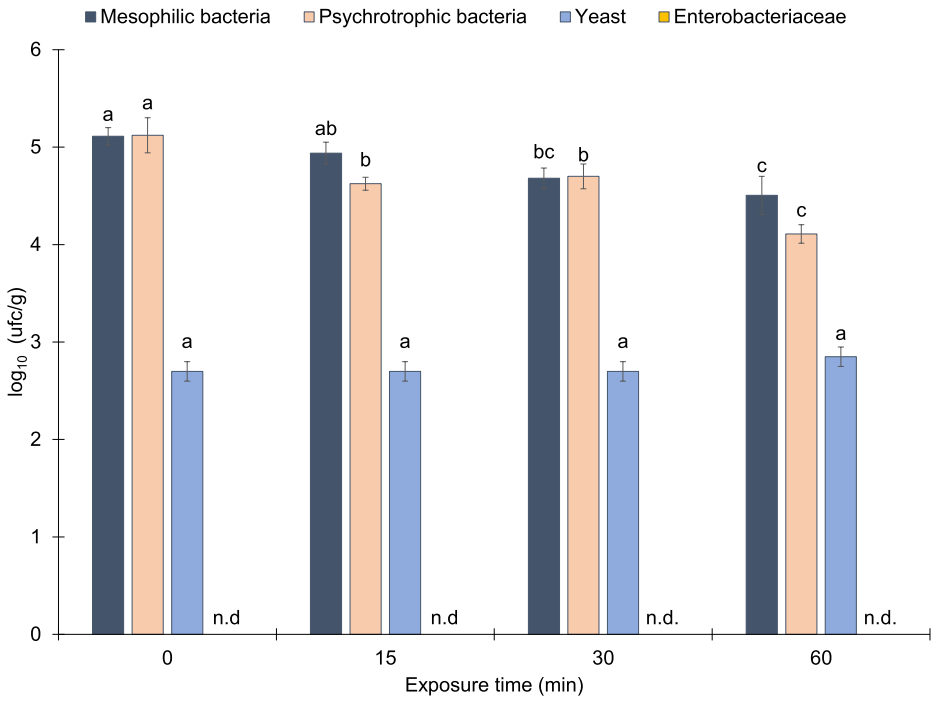

Fig. 3. Effects of CAP treatment time (min) on mesophilic bacteria, psychrotrophic bacteria, yeasts and Enterobacteriaceae on iceberg lettuce. Values with different superscripted letters are significantly different ($p < 0.05$).

(Mao et al., 2021; Pan et al., 2019; Özdemir et al., 2023). SDBD offers several advantages, including the use of low voltage, efficient generation of reactive species, high density of micro-discharges, and operation in ambient air without requiring a noble gas mixture (Abdelaziz et al., 2019; Pavlovich et al., 2014).

Thus, this study aimed to fill these knowledge gaps and highlight the effects of SDBD cold atmospheric plasma on iceberg lettuce. In particular, the investigations were focused on the inactivation of spoilage enzymes and microorganisms, colour, and surface microstructure. Additionally, to go deep inside the plant's physiological response induced by CAP, antioxidant compounds and in vitro antioxidant properties were assessed both after processing and refrigerated storage at 4 °C.

2. Materials

Heads of "iceberg" lettuce (*Lactuca sativa* L.) were purchased from a local producer between October and December 2021. All used reagents were purchased from Sigma–Aldrich (Steinheim, Germany).

2.1. Iceberg lettuce preparation

Before treatments, heads of iceberg lettuce were cleaned by removing the stem and outer leaves, cut into regular pieces of 4 × 4 cm, and washed with tap water. After removing the superficial water using a manual salad spinner, the lettuce pieces were immediately subjected to CAP treatment.

2.2. CAP treatments and storage conditions

All the treatments were performed by using the device "Plasma Assisted Sanification System" (PASS), developed by AlmaPlasma Srl (Bologna, Italy). The system comprises a surface dielectric barrier discharge (SDBD) plasma source, a treatment chamber, a cooling system, and a high-voltage generator (a more detailed description of the device was reported by Molina-Hernandez et al. (2022a). The device, operating in environmental air (relative humidity in the range of 20–40 %) was driven by a high-voltage generator (AlmaPULSE, AlmaPlasma Srl, Bologna, Italy). CAP treatments were carried out by applying a sinusoidal waveform with 6 kV of amplitude at 23 kHz to the high-voltage electrode, a 10% duty cycle and an average power of 42.54 ± 2.58 W. The temperature inside the treatment chamber was measured during the discharge time using a fiber optical temperature sensor (OpSense, OTG series). The iceberg lettuce samples were exposed to CAP treatment for 15, 30, and 60 min at a distance from the plasma source of 10 cm. Each treatment was replicated three times.

Fresh (untreated) and CAP-treated samples were portioned, individually packed into high O_2 barrier PA/PE (95 μm) bags (30 cm × 40 cm) and stored at 4 °C for 5 days.

2.3. Enzymatic activity

2.3.1. POD extraction and analysis

Peroxidase (POD) extraction and assay were carried out according to Martin-Diana et al. (2005) with slight modifications. An aliquote (20 g) of iceberg lettuce was added in a 1:2 (w:v) ratio with 0.5 M sodium phosphate buffer solution (pH 6.50) containing 50 g/L of polyvinylpyrrolidone (PVPP; Sigma, St. Louis, U.S.A.), and homogenised under ice for 3 min at 13500 rpm by a lab homogeniser (Ultra-Turrax yellow line DI25 basic, IKA-Werke, Staufen, Germany). After centrifugation (10 min, 3300×*g*, 4 °C), the extract was filtered and analysed. The spectrophotometric detection of peroxidase (POD) activity was conducted following Keesey (1987), with slight modifications. In 10-mm-path-length glass, cuvettes were added 25 μL of iceberg lettuce extract, 1450 μL of 2,2′-azinobis-(-3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (20 mmol/L), and 50 μ, of hydrogen peroxide at the concentration of 0.3% (w/w) as an oxidant. The POD activity was measured at 25 °C by monitoring the increase in absorbance at 405 nm using a spectrophotometer (Lambda Bio 20, PerkinElmer, Boston, MA, USA). The reaction rate was computed from the slope (ΔA/min) of the initial linear portion of the plot of absorbance vs time. The residual enzymatic activity was calculated according to the following equation:

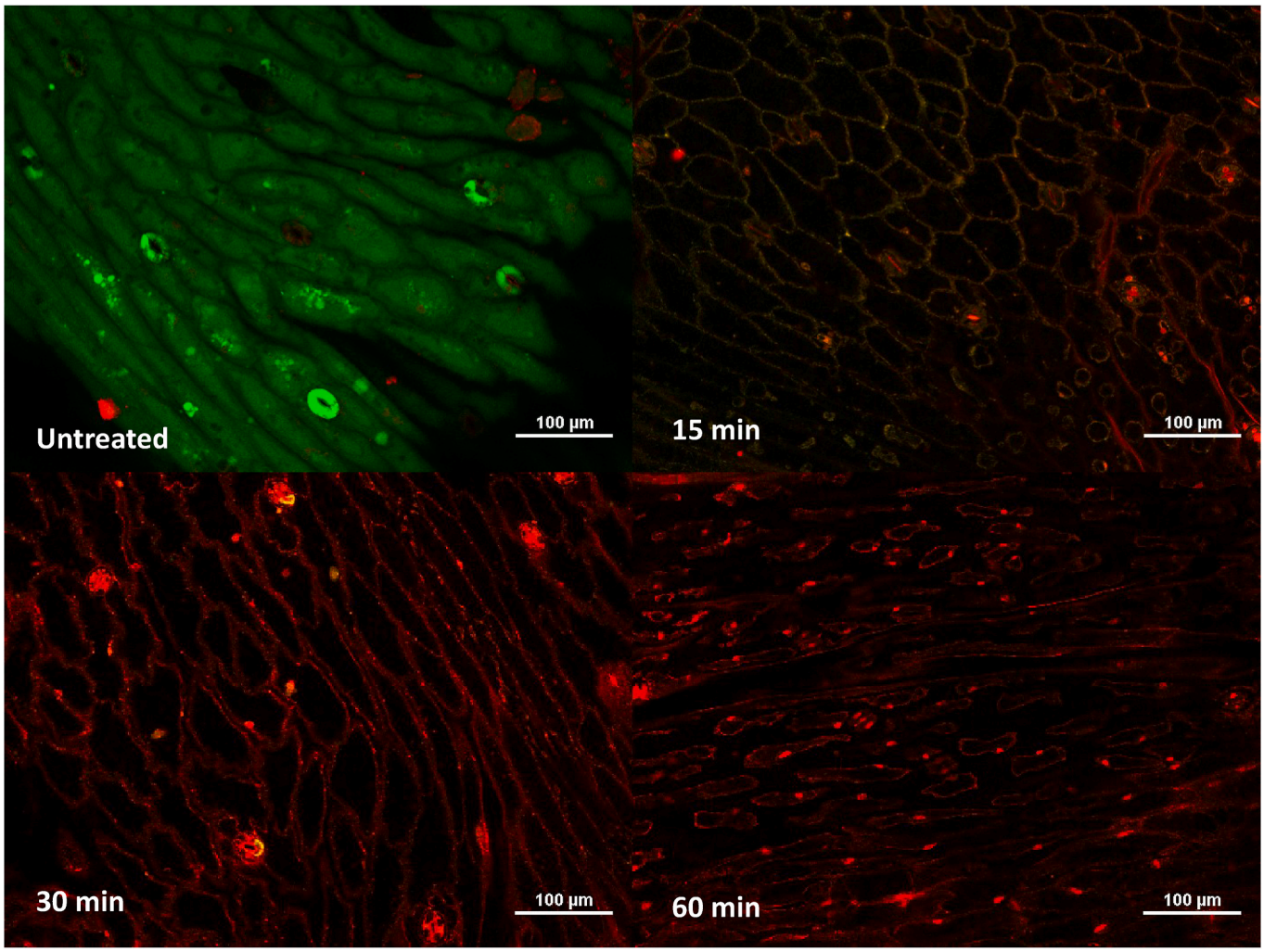

Fig. 4. Micrographs of lettuce leaves untreated and treated for different exposure times (15–60 min). The leaves were stained using green (carboxyfluorescein diacetate) and red (propidium iodide) as fluorescent dyes to evaluate live and dead cells, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$$R_A = A/A_0 \times 100 \quad (1)$$

where, R_A is the residual peroxidase activity expressed in percentage; A and A_0 show the sample absorption rate after cold plasma treatment and the absorption rate of the untreated sample, respectively. The analytical determinations were performed in triplicate.

2.3.2. PPO extraction and analysis

Polyphenoloxidase (PPO) extraction and assay were carried out according to Neri et al. (2019) with slight modifications. Briefly, an aliquot (20 g) of iceberg lettuce was added to 40 mL of McIlvaine buffer (pH 7.5) containing 0.5% di Triton X100, 25 mmol/L di ascorbic acid, and 12,5 g/L of polyvinylpyrrolidone (PVPP; Sigma, St. Louis, U.S.A.). The solution was homogenised for 3 min at 13500 rpm by a lab homogeniser (Ultra-Turrax yellow line DI25 basic, IKA-Werke, Staufen, Germany) under ice, centrifuged for 10 min at 3300×*g* at 4 °C, and paper filtered. The extract was used without further purification. The PPO assay substrate solution was obtained daily by preparing a 20 mmol/L 4-methylcatechol solution with McIlvaine buffer. PPO activity was tested by adding 50 µL of enzyme extract to 1450 µL of substrate solution in 10-mm-path-length glass cuvettes. PPO activity was measured at 25 °C by monitoring the absorbance at 420 nm using a spectrophotometer (Lambda Bio 20, PerkinElmer, Boston, MA, USA). The reaction rate was computed from the slope (ΔA/min) of the initial linear portion of the plot of absorbance vs time. The residual enzymatic activity was calculated according equation (1). The analytical determinations were performed in triplicate.

2.4. Microbiological analysis

To determine the effect of CAP in reducing the microbiota naturally associated with iceberg lettuce, treated and untreated samples were analysed after treatments. An aliquot of iceberg lettuce (10 g) was suspended in a sterile physiological solution (0.9% w/v) and homogenised in a Stomacher-400 Circulator (Seward, West Sussex, UK) for 5 min at room temperature.

Total mesophilic and psychrotrophic aerobic bacteria were determined on Plate Count Agar (PCA) at 30 °C for 48 h and 7 °C for 10 days, respectively. Enterobacteriaceae were enumerated on Violet Red Bile Glucose Agar (VRBGA) plates, at 37 °C after 48 h. Yeasts were determined on Potato Dextrose Agar (PDA), added of 150 ppm chloramphenicol, at 30 °C for 72 h. Results were expressed as log CFU/g. Microbial analyses were carried out in duplicate. All the culture media were puchesed from Liofilchem (Roseto degli Abruzzi, Italy).

2.5. Confocal Laser Scanning Microscopy (CLSM)

The microstructural response of iceberg lettuce leaves to CAP treatments was assessed through Confocal Laser Scanning Microscopy (CLSM) analysis utilizing a Nikon A1R confocal microscope. The system

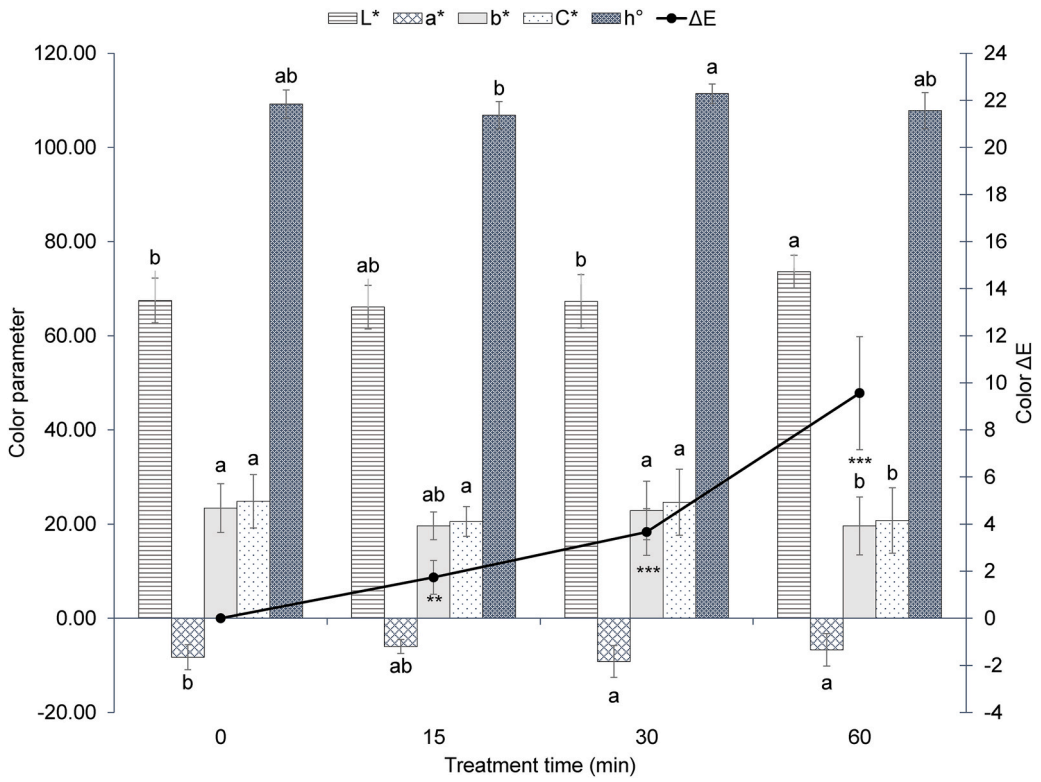

Fig. 5. Changes in iceberg lettuce colour (L*, a*, b*, C*, h°, ΔE) induced by CAP. Values with different letters differ significantly based on repeated measures ANOVA ($p < 0.05$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

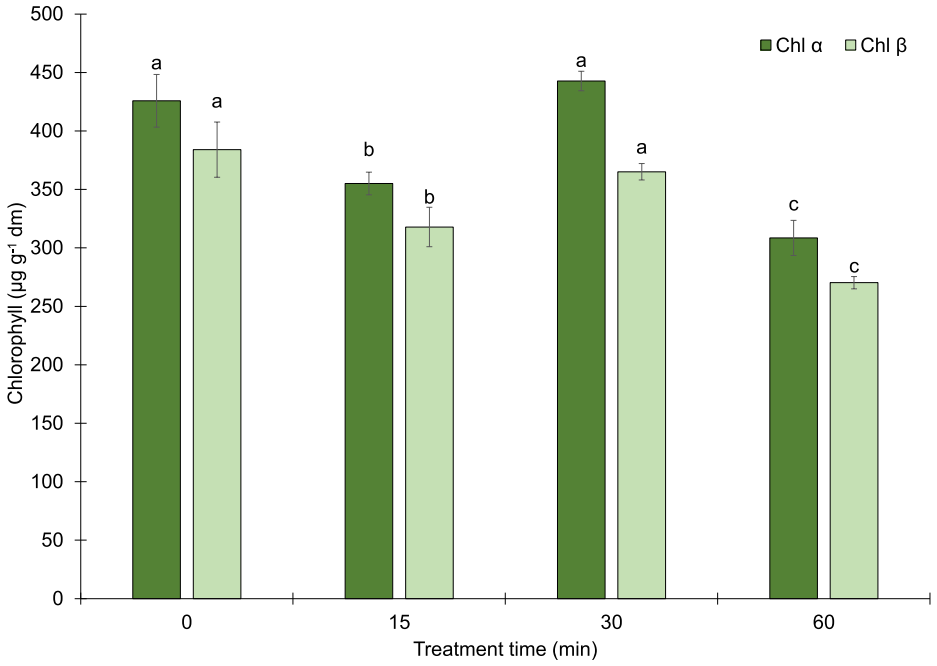

Fig. 6. Impact of CAP treatment on chlorophyll-α and chlorophyll-β content in iceberg lettuce. Values with different superscripted letters are significantly different ($p < 0.05$).

was controlled by Nikon NIS Elements software version 4.40 and equipped with a Plan Apo lambda 100× oil objective (Nikon Corporation, Tokyo, Japan). For the analysis, lettuce leaves were incised using a 6 mm biopsy punch. In the treated samples, leaf disks underwent CAP exposure for 15, 30, and 60 min, while a control sample was acquired without CAP treatment.

The CLSM analysis was carried out using carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) as fluorescent dyes (Molecular Probes, Eugene, OR, USA). In particular, leaf discs were initially treated with 50 μM cFDA and incubated at room temperature for 15 min in

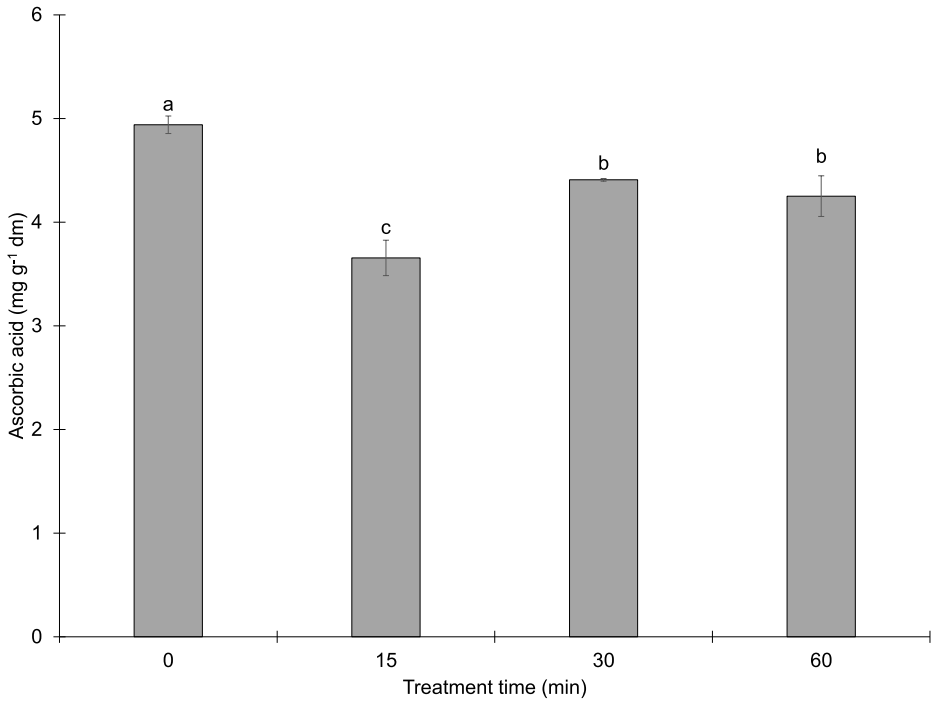

Fig. 7. Effect of CAP treatment on ascorbic acid content of iceberg lettuce. Values with different superscripted letters are significantly different ($p < 0.05$).

darkness. Subsequently, the samples underwent two washes with dH_2O_2 to remove the cFDA excess. Following this, leaf discs were incubated in 500 nmol/L PI (in dH_2O_2) for 5 min and rinsed with dH_2O_2. Finally, the leaves were observed with a Nikon A1R confocal imaging system (Nikon Corp., Tokyo, Japan).

2.6. Image analysis and colour determination

The colour parameters of the iceberg lettuce (L*: lightness, a*: magenta-greenness and b*: blueness-yellowness) were determined according to the method described by Yam and Papadakis (2004) using digital photography. Briefly, high-resolution pictures of 9 salad leaves were taken before and after CAP treatment with a digital camera (Nikon, Japan) after being placed into a wooden box internally black-painted to minimize external light and reflection. The box was equipped with a D_{65} white illumination. The camera was calibrated using a ColorChecker Passport target (X-Rite, USA). The pictures were saved in NEF format, corrected by applying the camera profile and analysed quantitatively using the Adobe Photoshop software through Histogram Windows. The Histogram Window displays the statistics of the L*, a* and b* colour parameters for a selected area in the salad image. The L*, a*, and b* values collected from the Photoshop Histogram Window were finally converted to CIE LAB using the following formulae:

$$L^* = Lightness/255 \times 100 \quad (2)$$

$$a^* = (240a/255) - 120 \quad (3)$$

$$b^* = (240b/255) - 120 \quad (4)$$

C* and h° were calculated as reported by Schanda (2007).

Total colour difference (ΔE) (before and after each CAP treatment) was calculated (Schanda, 2007) as follows:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (5)$$

2.7. Extraction and determination of ascorbic acid (AA) content

Ascorbic acid was determined by HPLC analysis. The extraction of AA was carried out in duplicate on freeze-dried (Labogene Scanvac Coolsafe freeze dryer, Allerød, Denmark) samples using the method reported by Neri et al. (2019) with some modifications. Briefly, an aliquot of 0.02 g of each sample was mixed with 1.5 mL of 0.2 Eq/L H_2SO_4, shaken at 300 rpm for 30 min in the darkness, and centrifuged at 10000×*g* for 10 min. The supernatants were filtered (0.45 μm Nylon filter) and injected into the chromatographic system.

Chromatographic analysis was performed by using a 1200 Agilent Series HPLC (Agilent Technologies, Milan, Italy), equipped with an Ion Exclusion column (Aminex HPX-87H, BIO-RAD, Hercules, CA) (300 mm, 7.8 mm) and a G1322A degasser, a G1311A quaternary pump, a G1316A Column Thermostat, a G1328B manual injection system, and a G13115D diode array detector. The system was controlled with Agilent ChemStation for Windows (Agilent Technologies). HPLC analysis was carried out using as mobile phase 0.009 Eq/L H_2SO_4. The other operating conditions were the following: flow rate of 0.4 mL min^{-1}, 20 min run, column temperature of 45 °C, and volume injection of 20 μL. Peaks were detected at 245 nm using a UV detector. Standard solutions with concentrations ranging from 100 to 500 ppm were used for AA identification and quantification.

2.8. Extraction and determination of total polyphenolic content (TPC) and antioxidant activity (AoA)

The extraction of phenolic compounds was performed using the procedure described by Boo et al. (2011) with slight modifications. Briefly, an aliquot of 0.25 g of each freeze-dried lettuce sample was added to 10 mL of extraction solvent composed of 85 mL of methanol, 15 mL of water and 0.5 mL of acetic acid, and homogenised for 1 min at 13500 rpm by a homogenizer (Ultra-Turrax yellow line DI25 basic, IKA-Werke, Staufen, Germany). After 5 min of sonication (100 Watt, 50 kHz) (LABSONIC LBS1, Falc Instruments, Treviglio, Italy), the samples were incubated in dark conditions for 20 min at room temperature. After centrifugation at 5000×*g* for 15 min, the supernatants were collected and stored at −40 °C until analyses.

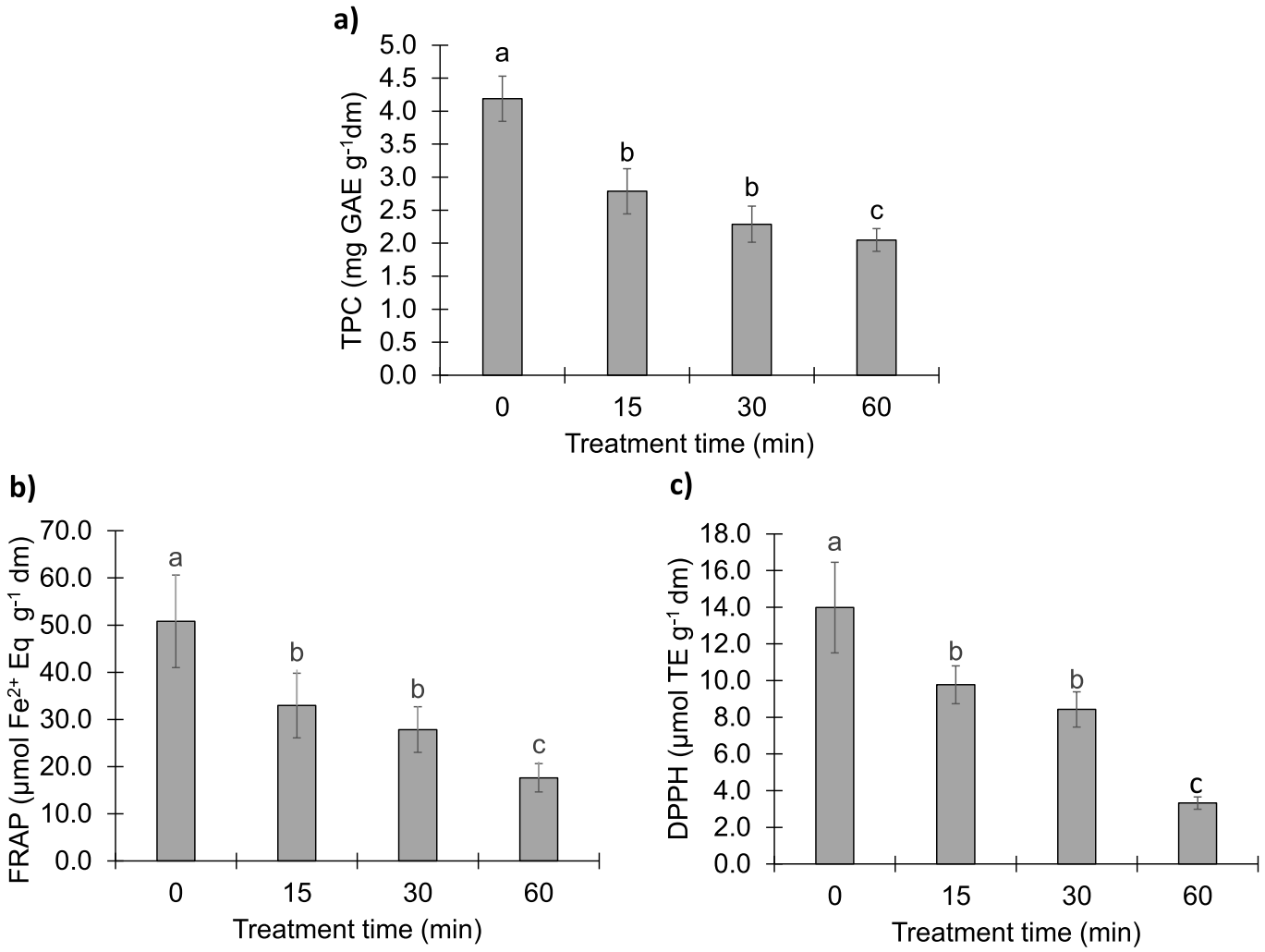

Fig. 8. Influence of CAP treatment on total phenolic content (TPC) **(a)**, ferric reducing antioxidant power (FRAP) **(b)**, and radical scavenging activity (DPPH) **(c)** of iceberg lettuce. Values with different superscripted letters are significantly different ($p < 0.05$).

Total polyphenol content (TPC) was determined by using the Folin-Ciocalteau reagent following the procedure described by Tatasciore et al. (2023). Gallic acid standard solutions were used to calibrate the method, and results were expressed as mg of gallic acid equivalent (GAE) per g of dry matter (dm) of iceberg lettuce. The results were the average of three analytical measurements.

For the antioxidant activity determination, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was performed according to Kaur et al. (2013). Briefly, 0.1 mL of each lettuce extract was added to 3.9 mL of 0.0634 mM solution of DPPH in methanol and shaken vigorously. Change in the absorbance of the obtained mixture was measured at 515 nm after 30 min. The data were expressed as percentage of inhibition of DPPH applying the following formula:

$$\%\ inhibition = \frac{A_0 - A}{A_0} \times 100 \quad (6)$$

where, A_0 is the beginning absorbance at 515 nm, and A is the final absorbance after extract addition. Trolox standard solutions were used to calibrate the method. The results are expressed as µmol of Trolox equivalent (TE) per g of iceberg lettuce dry matter. All analytical determinations were performed in triplicate.

Finally, FRAP reducing activity (Ferric Reducing Antioxidant Power) was determined according to the method described by Benzie et al. (1999) with some modifications. Each lettuce extract (0.2 mL) was added to 1.3 mL of FRAP reagent obtained by mixing acetate buffer (300 mmol/L, pH 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) solubilised in HCl 40 mmol/L and $FeCl_3$ 20 mmol/L in the ratio 10:1:1. The solutions were vortexed and incubated at 37 °C for 30 min. The absorbance was measured at 593 nm, and $FeSO_4{\cdot}7H_2O$ standard solutions were used to calibrate the method. Results are expressed as µmol $Fe2^{++}$Eq per g of iceberg lettuce dry matter. Analytical determinations were performed in triplicate.

2.9. Extraction and determination of chlorophyll α and chlorophyll β

Chlorophylls were extracted from freeze-dried lettuces following the method described by Lin et al. (2013) with slight modifications. Briefly, 0.05 g of each freeze-dried sample was added to 2 mL of 80% v/v acetone solution, mixed, and kept overnight at 4 °C. The samples were then centrifuged at 5000×*g* for 10 min, and the collected supernatants were used to determine absorbances at 663 and 645 nm by a Spectrophotometer UV–VIS (Jenway, Stone UK). The following formulae were used to calculate the concentrations (µg g^{-1} dm) of Chlorophylls α (Chl α) and Chlorophylls β (Chl β):

$$Chl\alpha = 12.72 \times OD_{663} - 2.59 \times OD_{645} \quad (7)$$

$$Chl\beta = 22.88 \times OD_{645} - 4.67 \times OD_{663} \quad (8)$$

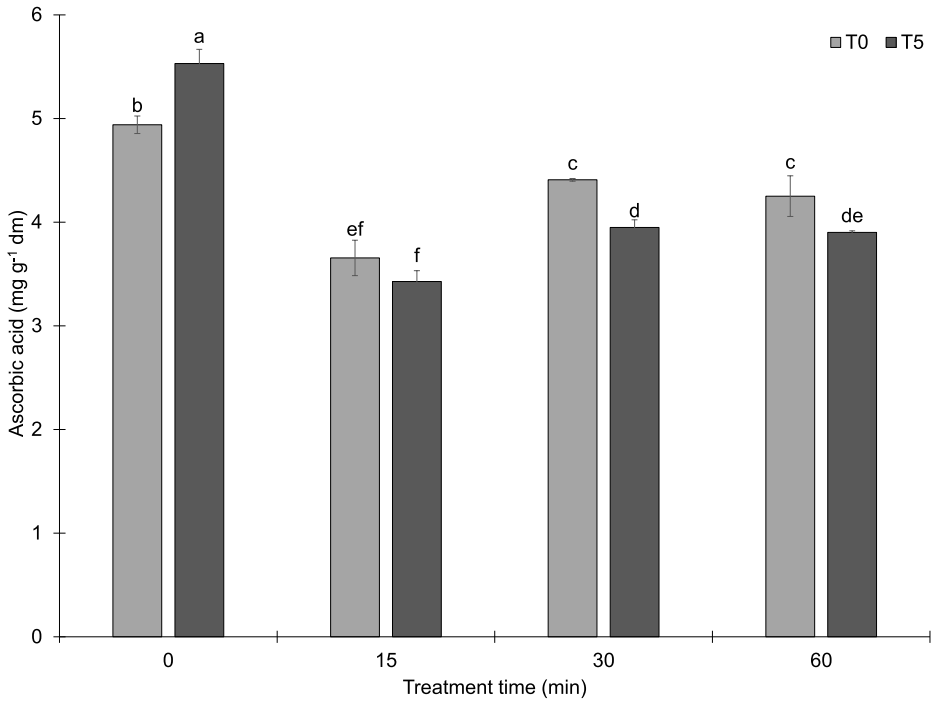

Fig. 9. Effect of storage (5 days at 4 °C) on ascorbic acid content of untreated and CAP-treated (15, 30, 60 min) iceberg lettuce. Values with superscripted letters are significantly different ($p < 0.05$).

2.10. Statistical analysis

Where not differently specified, data are reported as mean and standard deviation calculated on three independent replicates and analysed by one-way ANOVA. Tukey's multiple comparison tests computed significant differences between means at a significance level of $p \leq 0.05$. For colour analysis, repeated measures ANOVA, based on maximum likelihood estimation, was carried out. Data were processed using STATISTICA 12 for Windows (StatSoftTM, Tulsa, OK, USA) and XLSTAT (XLSTAT 2022; Addinsoft, New York, NY, USA) software.

3. Results and discussion

3.1. Concentration of reactive oxygen and nitrogen species produced by CAP

Plasma composition was evaluated by determining the ozone and nitrogen dioxide concentrations in the chamber at different process times (Fig. 1). As highlighted and discussed in a previous study (Laika et al., 2024), the low power condition promoted the production and accumulation over time of ozone while nitrogen dioxide concentration was below the detection limit (<5.5 ppm), indicating that the device was operating under the O_3 regime. Ozone concentration data over time were thus modelled using the Peleg equation, which showed a nearly perfect fit for the experimental data (Fig. 1 insert).

3.2. Effect of CAP treatment on iceberg lettuce

3.2.1. Effect on enzyme activity

The inactivation kinetics of polyphenoloxidase (PPO) and peroxidase (POD) due to CAP exposure are shown in Fig. 2 a. The activity of both enzymes decreased at the increase of the treatment time, reaching after 60 min, a residual activity of about 50% for the POD and 20% for the PPO. As the temperatures recorded within the chamber were below 30 °C, temperature was a negligible factor in the inactivation of the enzymes.

By plotting the POD and PPO residual activity *vs* ozone concentration in the chamber an exponential relationship was found ($R^2 > 0.99$) for both enzymes (Fig. 2 b). These results indicate that ozone and possibly other reactive species (e.g. •OH and H_2O_2) generated on the product surface due to the interaction with cellular and extracellular components were involved in the enzyme inactivation. Specifically, as showed in recent studies carried out on commercial peroxidase and PPO in model systems (Laika et al., 2022, 2023), the loss of catalytic activity is due to changes in protein tertiary structure and loss of secondary (alpha-helices) structures with alteration of the active sites (heme degradation for POD and probable loss of copper-binding site for PPO). The effectiveness of cold plasma in peroxidase and polyphenoloxidase inactivation in real and model systems was also highlighted by other authors (Mayookha et al., 2023); however, different inactivation percentages were found depending on the type of equipment, gas and process conditions employed, and the composition, microstructure, and porosity of fruits and vegetables (Waghmare, 2021).

3.2.2. Microbiological analysis

To assess the impact of cold atmospheric plasma (CAP) treatments on microbial contamination in iceberg lettuce, plate counts were conducted for mesophilic and psychrotrophic bacteria, yeasts, and Enterobacteriaceae, as depicted in Fig. 3. The initial microbial loads for untreated iceberg lettuce were measured at 5.11, 5.12, and 2.70 log CFU/g for mesophilic bacteria, psychrotrophic bacteria, and yeast, respectively. Enterobacteriaceae were not detected in any sample.

Overall, the results indicated a significant reduction of mesophilic and psychrotrophic bacteria, which was processing time-dependent, while no statistically significant reduction in yeast count was observed. Specifically, after the initial 15 min of CAP processing, no significant reduction in the microbial load was noted ($p > 0.05$) for mesophilic bacteria, whereas a reduction of 0.50 $\log_{10}$ was recorded for psychrotrophic bacteria. By increasing the exposure time, greater efficacy was observed, achieving reductions of approximately 0.61 and 1.01 $\log_{10}$ for mesophilic and psychrotrophic bacteria, respectively.

As highlighted in the review by Katsigiannis et al. (2022), several studies have demonstrated variations in the inactivation rates among different types of microorganisms. For instance, higher-order

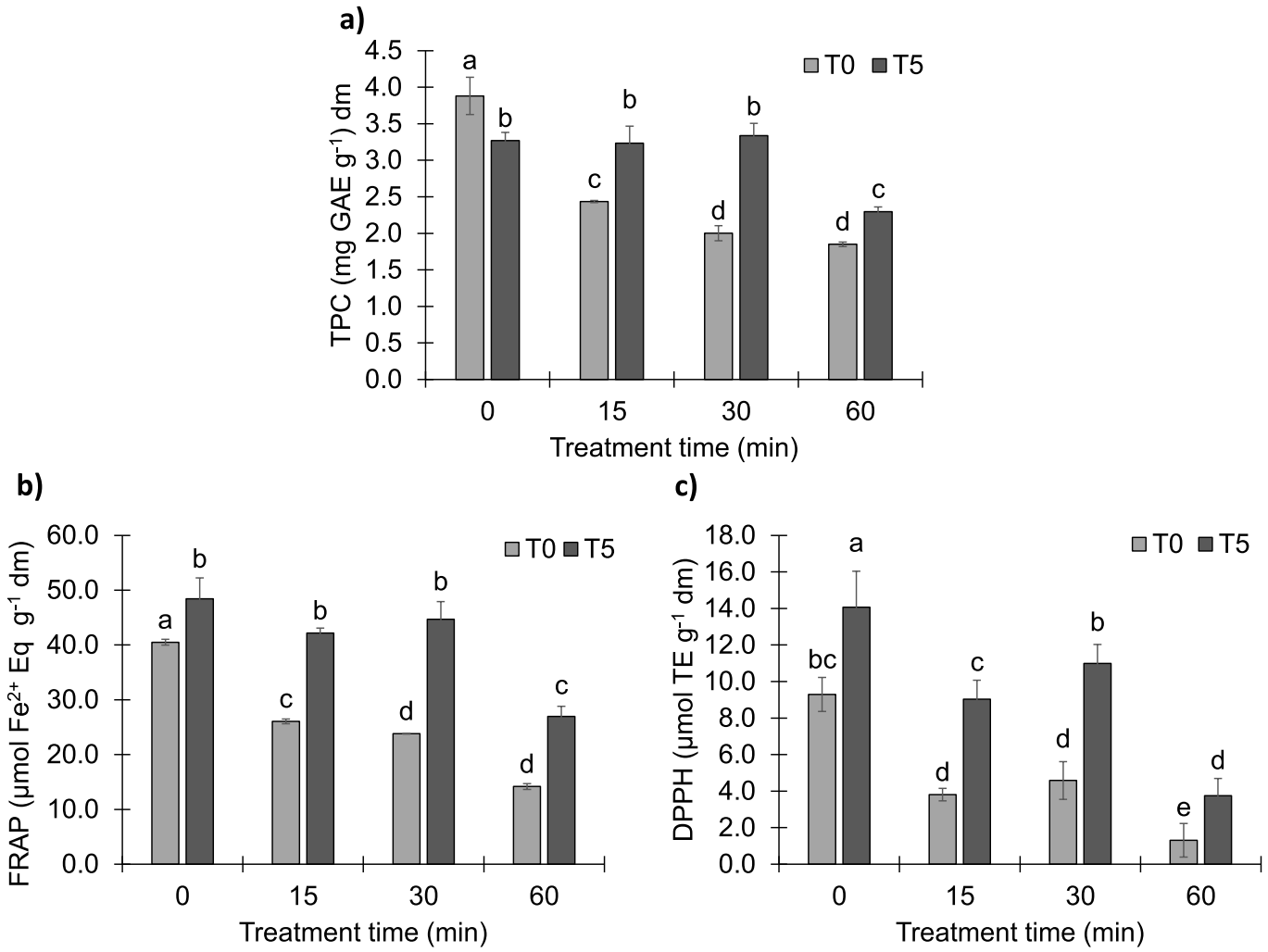

Fig. 10. Influence of storage (5 days at 4 °C) on total phenolic content (TPC) **(a)**, ferric reducing antioxidant power (FRAP) **(b)**, and radical scavenging activity (DPPH) **(c)** of untreated and CAP-treated (15, 30, 60 min) iceberg lettuce. Values with different superscripted letters are significantly different ($p < 0.05$).

microorganisms present greater resistance to inactivation compared to bacteria. The notable resistance of yeast can be attributed to its cell wall composition, which includes chitin, glucans (beta-glucan), and mannans. This composition provides a protective barrier against external damage and renders yeast cells less susceptible to damage induced by cold atmospheric plasma (CAP). In a comparative study utilizing plasma-activated water for 30 min, a reduction of 6 log CFU of bacteria was achieved, whereas only a 3 log CFU reduction of *S. cerevisiae* was observed (Kamgang-Youbi et al., 2009). Regarding bacterial susceptibility, gram-negative bacteria with thinner cell walls are more easily ruptured upon CAP treatment, leading to the leakage of intracellular components. In contrast, the main mechanism of CAP action against gram-positive bacteria involves the transport of reactive species inside the cell and subsequent reactions with intracellular components (Bourke et al., 2017).

Regarding the efficacy of CAP on microbial inactivation, analogous results were documented by Giannoglou et al. (2020), who assessed the effect of SDBD CAP device in reducing microbial loads in rocket leafy salad and found reductions in the total microbial load from 0.57 to 1.02 $\log_{10}$ after CAP exposure ranging from 5 to 20 min.

In contrast, other studies have reported a greater efficacy (Tappi et al., 2016; Ziuzina et al., 2015).

The variability in results among studies underscored by Mao et al. (2021) is attributed to numerous variables in plasma processing, complicating the comparison of cold plasma's efficacy in microbial inactivation with the food matrix and surface. In fact, cold plasma species could not reach the inside of coves generated by an irregular surface as well as the internal part of the product under its surface. It is well established that the decontamination impact decreases with increasing food product irregularity and porosity (Bhide et al., 2017; Xiong et al., 2011).

3.2.3. *Effect on microstructure*

To investigate the effect of CAP cellular microstructure and viability, CLSM analysis was combined with the use of Carboxyfluorescein Diacetate (cFDA) and Propidium iodide (PI) as fluorescent dyes. cFDA is a non-fluorescent, membrane-permeable dye that, upon penetration of the plasma membrane, is hydrolysed by intracellular esterases to produce a membrane-impermeable carboxyfluorescein, emitting green fluorescence, thereby serving as a positive assay for viable, metabolically active cells (Cui et al., 2015). Conversely, PI stains the nuclei of dead cells by passing through damaged cell membranes and intercalating with DNA, resulting in bright red fluorescence; as intact cell membranes exclude PI, it is an effective stain for identifying dead cells (Jones et al., 2016).

Micrographs of iceberg lettuce before and after CAP treatments are shown in Fig. 4. In the untreated sample, the prevailing fluorescence signal is green, indicating live cells. However, a distinct shift in fluorescence patterns is observed upon exposure to cold plasma. Treated leaves first exhibit a noticeable reduction in green fluorescence with increased red fluorescence (15 min). Subsequently, the green

fluorescence diminishes entirely with prolonged exposure (30 min), although the cellular boundaries remain distinguishable. Notably, the most pronounced effects are observed after 60 min of plasma treatment, where heightened red fluorescence and the complete absence of membrane integrity imply a comprehensive disruption of cellular integrity.

No analogous studies were identified in the literature; however, other investigations have indicated the impact of plasma on surface microstructures. Bermúdez-Aguirre et al. (2013) investigated the structure of lettuce following cold atmospheric plasma (CAP) treatment (10 min) via electron microscopy, revealing minimal changes with a comparable structure, including a roughened surface and stomata. In contrast, Grzegorzewski et al. (2011) found that while untreated lettuce leaves displayed thick platelets and small granular structures, plasma-treated (atmospheric pressure plasma jet, 20 s) leaves exhibited a roughened surface and the disappearance of granular structures with prolonged exposure time, ultimately resulting in visible desiccation and stress after 60 s of treatment.

3.2.4. Effect on colour

The effect of CAP treatments on iceberg lettuce colour is shown in Fig. 5. All colour parameters, L*, a*, b*, C*, and h° were slightly affected ($p < 0.05$) by CAP treatments. In particular, with the increase of the treatment time, b* and C* decreased considerably, highlighting the impact of CAP exposure in reducing the saturation and yellowness of the iceberg leaf. Furthermore, upon evaluation of the L* value of samples treated for 60 min, a significant increase was observed. Thus, following CAP treatments, the iceberg lettuce displayed a loss of colour, tending towards white. In addition, by calculating the ΔE parameter between iceberg lettuce before and after CAP treatments, a significant effect was observed ($p < 0.05$). Specifically, after 15 min of exposure, the ΔE showed a value between 1.0 and 3.3, indicating a colour variation perceptible only by experienced observers (Faghihi et al., 2021), while with increasing treatment duration, ΔE was higher than 3.3, which means the colour changes were easily distinguishable. These results are similar to those obtained by Giannoglou et al. (2020) on fresh-cut leafy rocket salad treated by SDBD. Additionally, Bermúdez-Aguirre et al. (2013) observed comparable values of ΔE and slight differences in L*, a*, and b* parameters on lettuce subjected to CAP under the following conditions: 3.95–12.83 kV, 60 Hz, Argon, 0.5–10 min. Conversely, Min et al. (2017) observed no significant alterations in the colour of Romaine lettuce processed with a dielectric barrier discharge (DBD) plasma source for 10 min.

3.2.5. Effect on Chlorophyll content

Fig. 6 illustrates the chlorophyll α and β content of iceberg lettuce subjected to various cold plasma treatments. The results indicate a significant ($p < 0.05$) reduction of both pigments in samples treated for 15 and 60 min (17% and 29%, respectively). The decrease in the amount of chlorophyll due to cold plasma was reported by several authors (Amorim et al., 2023; Makari et al., 2021) and could be associated with the Type II breakdown mechanism (Ramazzina et al., 2015) mediated by oxygen radicals. An unexpected result was observed in the sample treated for 30 min, where the chlorophylls α and β levels were nearly equal to the control sample. This effect could be due to the enhancement of the chlorophyll extractability from iceberg lettuce tissue as an effect of the cell damage induced by plasma-reactive species. A higher extractability of lycopene on sun-dried tomatoes after 15 min of CAP treatment was also observed by Molina-Hernandez et al. (2022b). The chlorophyll reduction highlighted after 60 min was possibly due to the accumulation of long-lifetime oxidising species such as ozone in the treatment chamber. These findings suggest that by previous optimisation of process parameters, cold plasma can enhance pigment extraction from plant tissues.

3.2.6. Effect on ascorbic acid (AA)

The effect of various CAP treatment times on AA concentration in iceberg lettuce is shown in Fig. 7. After 15 min of treatment, the AA concentration decreased ($p < 0.05$) by 27% compared to the untreated sample. However, at longer treatment times, i.e. 30 and 60 min, a reduction of the AA percentage loss was detected (10% and 9%, respectively). AA degradation over CAP treatments was also found by other authors (Zagarchi et al., 2024; Waghmare, 2021; Giannoglou et al., 2021) and could be attributed to the reaction with ozone and other oxidising plasma species and consequent deprotonation of the molecule forming ascorbate—ascorbate radical—ending in the formation of dehydroascorbate (Fernandes & Rodrigues, 2021). However, plasma technology inducing the hydrogenation of molecules can chemically transform dehydroascorbate into ascorbic acid, reversing the decay mechanism and increasing AA content. When the AA regeneration rate exceeds its destruction rate, its content increases (Castro et al., 2020). Finally, structural damages induced on cell structures could have enhanced the AA extraction, similar to what was observed for chlorophylls. All these phenomena could be responsible for the AA increase observed at exposure times longer than 15 min.

3.2.7. Total polyphenol content (TPC) and antioxidant activity (AoA)

The influence of CAP treatment time on TPC of iceberg lettuce is depicted in Fig. 8 a. TPC decreased with the increase of the CAP exposure time, reaching in 60 min the 50% of its initial value. The negative effect of CAP on phenolic compounds was also highlighted by other authors in various plant foods (Ali et al., 2021; Fernandes et al., 2019; Pankaj et al., 2018). The exact mechanisms responsible for this effect are still unknown, however, it can be hypothesised that plasma-inherent reactive oxygen species (ROS) have a crucial role in the TPC loss. As reported by Sruthi et al. (2022), the action of molecular ozone on phenolics' aromatic rings results in certain hydroxylated quinone compounds and, upon their rupture, in aliphatic compounds.

Antioxidant activity might be determined by using different methods evaluating metal reduction capacity, such as ferric reduction antioxidant power (FRAP), and organic radical-scavenging capacity, such as 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity. The combination of these assays provides a more reliable picture of the antioxidant activity of foodstuffs (Neri et al., 2020). The AoA of the differently CAP-treated lettuce measured by FRAP and DPPH is shown in Fig. 8b and c. For both assays, there was a decrease in the antioxidant activity just after 15 min of treatment (35% and 40% for FRAP and DPPH, respectively). No further losses were highlighted with the increase of the treatment time up to 30 min ($p < 0.05$). At 60 min treatment, AoA decreased to 65% for FRAP and 76% for DPPH. The loss of antioxidant activity due to the CAP treatment can be related to the losses of phenolic compounds, chlorophylls, and ascorbic acid highlighted on the lettuce samples (Figs. 6 and 7 a).

In the literature, variable results are reported regarding the effect of CP on antioxidant activity, and this highlights the influence of the food product composition and structure, plasma generation source, mode of exposure, and treatment parameters in modulating the effects of CP on food product antioxidant activity (Pankaj & Keener, 2017).

3.3. Storage effect

To evaluate the effect of refrigerated storage on the functional properties of iceberg lettuce treated by CAP, both untreated and CAP-treated samples were analysed after 5 days (t5) of storage at 4 °C for the ascorbic acid, total phenolic content, and AoA. After storage, the untreated sample showed an increase of ascorbic acid whilst, overall, those treated by CAP had a significant reduction (Fig. 9). The AA increase in the untreated sample could be due to its regeneration from dehydroascorbate mediated by dehydroascorbate reductase (Castro et al., 2020). Conversely, it is possible that in CAP-treated samples, this enzyme was inactivated by ROS; thus, the production of dehydroascorbate, due to oxidative reactions occurring during storage, was not counterbalanced.

Regarding the TPC, the untreated sample showed after storage a significant ($p < 0.05$) decrease, possibly due to POD and PPO activity (Fig. 2), while all the CAP-treated samples showed a significant ($p < 0.05$) increase in phenolic compounds (Fig. 10 a) despite the PPO and POD residual activity (Fig. 2). This result was also observed by Ji et al. (2020) on blueberries treated by CAP after 20 days of storage, and it could be due to a higher extractability as an effect of an increased cell disruption over storage or to the activation of enzymes responsible for the biosynthesis of phenolic compounds (Castro et al., 2020).

According to TPC, the AoA of CAP-treated samples significantly increased after storage (Fig. 10b and c). This result disagreed with previous studies (Giannoglou et al., 2020; Puligundla et al., 2018; Ramazzina et al., 2016) carried out on other plant matrices reporting that cold plasma has no effect on antioxidant activity after storage.

4. Conclusion

This study investigated the potential use of cold atmospheric plasma generated by Surface Dielectric Barrier Discharge (SDBD) on fresh-cut iceberg lettuce, highlighting the effect of different treatment times on various quality parameters after processing and refrigerated storage.

Results showed that SDBD cold atmospheric plasma can reduce the activity of polyphenoloxidase and peroxidase, with PPO being the most sensitive, and the extent of the inactivation is dependent on processing time. Microbiological analysis revealed a significant reduction in mesophilic and psychrotrophic bacteria following CAP exposure, while yeast counts remained unaffected, confirming the different susceptibility of microorganisms to CAP. On the other hand, microstructural analyses highlighted progressive plant cell damage and death with increasing treatment times. Moreover, CAP treatment negatively affected the antioxidant activity and the concentration of chlorophylls and ascorbic acid, potentially due to oxidation reactions induced by plasma reactive species. However, TPC and AoA increased after storage, suggesting a potential activation of enzymes responsible for phenolic compound biosynthesis during storage and/or a higher extractability of phenolic compounds.

Overall, the findings provide insights into the efficacy of CAP treatment in preserving the quality and enhancing the antioxidant properties of fresh-cut iceberg lettuce. Further research is needed to evaluate changes in microbial and quality attributes under different process conditions and after storage. This will provide a more comprehensive understanding of the potential of cold atmospheric plasma (CAP) as an innovative technology to produce minimally processed plant foods.

CRediT authorship contribution statement

Jessica Laika: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Simona Tatasciore:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Riccardo De Flaviis:** Writing – review & editing, Visualization. **Luca Valbonetti:** Visualization, Methodology, Investigation. **Junior Bernardo Molina-Hernandez:** Visualization, Methodology. **Romolo Laurita:** Investigation. **Antonella Ricci:** Visualization. **Clemencia Chaves Lopez:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition. **Lilia Neri:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

The authors do not have permission to share data.

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