Atmospheric cold plasma process for vegetable leaf decontamination: a feasibility study on radicchio (red chicory, *Cichorium intybus L.*)

Frederique Pasquali[a], Alexandros Ch. Stratakos[b], Anastasios Koidis[b] Annachiara Berardinelli[a*], Chiara Cevoli[a], Luigi Ragni[a], Rocco Mancusi[c], Gerardo Manfreda[a], Marcello Trevisani[c].

[a]Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Via Fanin 50, 40127 Bologna, Italy.

[b] Queen's University Belfast, Institute for Global Food Security, Belfast, Northern Ireland, UK.

[c]Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia (BO), Italy.

*Corresponding author:

Dr. Annachiara Berardinelli, Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Via Fanin 50, 40127 Bologna, Italy.

Tel: +39 0547338113, Fax: +39 0547382348

email: annachi.berardinelli@unibo.it

Abstract

Cold plasma is an emerging non-thermal processing technology that could be used for large scale leaf decontamination as an alternative to chlorine washing. In this study the effect of an atmospheric cold plasma apparatus (air DBD, 15 kV) on the safety, antioxidant activity and quality of radicchio (red chicory, *Cichorium intybus L.*) was investigated after 15 and 30 min of treatment (in afterglow at 70 mm from the discharge, at 22°C and 60 % of RH) and during storage. *Escherichia coli* O157:H7 inoculated on radicchio leaves was significantly reduced after 15 min cold plasma treatment (-1.35 log MPN/cm^2). However, a 30 min plasma treatment was necessary to achieve a significant reduction of *Listeria monocytogenes* counts (-2.2 log CFU/cm^2). Immediately after cold plasma treatment, no significant effects emerged in terms of antioxidant activity assessed by the ABTS and ORAC assay and external appearance of the radicchio leaves. Significant changes between treated and untreated radicchio leaves are quality defects based on the cold plasma treatment. Atmospheric cold plasma appears to be a promising processing technology for the decontamination of leafy vegetables although some criticalities, that emerged during storage, need to be considered in future studies.

Key words: cold plasma, decontamination, antioxidant activity, colour, *Listeria monocytogenes*, *Escherichia coli*

1. Introduction

In recent years vegetables are consumed more frequently due to their nutritional benefits. This has led to the development of a wide variety of minimally processed vegetable based products (Ramos et al., 2013). Commercially, fresh vegetables need to be decontaminated prior to packaging. Several chemical and physical technologies have been found to be efficient in reducing bacterial contamination in fresh vegetables (Parish et al., 2003). The majority of minimally processed fresh produce manufacturers use chlorine washing (50–200 mg/L). However due to the increasing safety

concerns regarding the formation of potentially carcinogenic chlorinated compounds in water, and the demonstrated limited efficiency of chlorine in reducing foodborne pathogens on fresh produce (Oliveira et al., 2012), alternative methods have been sought out by the food industry that can ensure safety and at the same time are environmentally friendly (Baur et al., 2004; Siroli et al., 2015).

Physical non-thermal technologies such as irradiation, ultraviolet light, pulsed light, high pressure processing, and ultrasound are considered more promising alternatives. Among these, cold plasma technology has drawn a lot of attention as a minimal processing technology (Olaimat & Holley, 2012; Srey et al., 2014; Ziuzina et al., 2014). Cold plasma is produced by excitation of gas molecules through the use of electrical discharges. According to the frequency used to excite the gas, cold atmospheric plasma sources can be classified in direct current (DC) and low frequency, radio frequency (RF) and microwave discharges. Corona discharge, characterized by a cathode, a wire or a needle, and an anode is an example of DC plasma source (Schütze et al., 1998) while the Atmospheric Gliding Arc and the Dielectric Barrier Discharge (DBD) devices characterized by two or more diverging metallic electrodes the first one, and by two electrodes (separation gap of few millimetres) and by one or more dielectric layers the second one, can operate at low frequencies (Moreau et al., 2008; Kogelschatz, 2003). The Atmospheric Pressure Plasma Jet (APPJ) works with low power RF sources in a capacitive coupled configuration (Hermann et al., 1999) while in a Microwave Plasma Jet device (MPJ) the jet is generated by the interaction between the microwave electrical field, the wave guide aperture and the gas nozzle (Pau et al., 2000). Generally, the treatment efficiency is correlated to several parameters such as the gas mixture, the power supply characteristics and the electrode geometry to name a few (Tendero et al., 2006).

The antimicrobial effect of cold plasma is the result of the action of charged particles and reactive species present in the plasma that can cause damages to the cell membrane, which can lead to further penetration of reactive species into the cell, DNA damage, and breaking of chemical bonds, (Fernández & Thompson, 2012). Even if the mechanism of interaction between the plasma species

71 and the microorganism has yet to be clarified plasma ions can catalyse processes such as oxidation
72 and peroxidation that take place inside the cell as well as in the external environment (Dobrynin et
73 al., 2009). Cold plasma efficiency also depends on biological parameters such as the type of
74 substrate and microorganism characteristics (type, load, physiological state) (Moreau et al., 2008;
75 Misra et al., 2011; Stratakos & Koidis, 2015). The decontamination efficiency of non-thermal gas
76 plasma treatments has been evaluated against gram-negative and gram-positive bacteria, spores,
77 yeasts, moulds and viruses (Montie et al., 2000). The first applications on agricultural products were
78 conducted targeting foodborne pathogens such as *Escherichia coli*, Salmonella spp., *Listeria*
79 *monocytogenes* as well as spoilage organisms inoculated on the surface of fruits and vegetables;
80 with results showing significant reductions depending on the treatment time and the technology
81 used to produce the gas plasma (Critzer et al., 2007; Perni et al., 2008).
82 The scientific community agrees that the main limitation to a potential industrial application is
83 related to the characteristics of the treated product. Qualitative properties could be modified
84 consequently to the reactive species action and a possible presence of residues of the oxidation
85 processes could be detected.
86 Alterations of the nutritional and quality/sensory characteristics could potentially take place
87 depending on the product characteristics and residues of the oxidation processes. Depending on
88 time and exposure conditions, pigments can be affected by the treatment; changes in colour
89 parameters of tomatoes and carrots as well as in the photosynthetic activity in cucumber and fresh
90 corn salad leaves have been shown (Baier et al., 2013; 2014; 2015). Reductions of antioxidant
91 compounds (e.g. vitamin C) were also observed on cold plasma treated cucumber (Wang et al.,
92 2012), on the surface of *Abate Fetel* pear in terms of ABTS antioxidant capacity (Berardinelli et al.,
93 2012), and on peel and pulp of *Fuji* apples as determined by the DPPH antioxidant assay (Gozzi et
94 al., 2013).
95 The present study explores the suitability of atmospheric cold plasma treatment generated by means
96 of a dielectric barrier discharge (DBD) device on the inactivation of *Escherichia coli* O157:H7 and

97 a mix of several *Listeria monocytogenes* strains experimentally inoculated on radicchio leaves (red
98 chicory, *Cichorium intybus L.*). Radicchio has been selected because it is part of many Ready-to-Eat
99 meals, normally chlorine washed and it has a delicate texture and a characteristic red colour which
100 is challenging to maintain after any processing. To complement this study, the effect of cold plasma
101 on radicchio was also assessed in terms of antioxidant activity, visual and sensory characteristics.
102 Differently from previous efforts, the effect of a maximum storage of 3 days at 4°C was also taken
103 into consideration. The temperature of 4°C was chosen as the lowest realistic temperature of
104 domestic refrigerators. The time of three days of storage was chosen upon preliminary experiments,
105 in which dehydration and browning of radicchio leaves were visibly unacceptable after 4 days of
106 storage in control radicchio leaves (stored at 4°C and 90% Relative Humidity).
107
108 **2. Materials and methods**
109
110 2.1 Gas plasma generator and vegetable treatments
111 Treatments were conducted at atmospheric conditions (at approximately 22 °C and 60 % of
112 Relative Humidity, RH) by placing radicchio leaves samples at about 70 mm beneath the plasma
113 emission generated between three independently supplied couples of parallel plates electrodes made
114 of common brass (Figure 1). Each circuit, generating the high voltage by switching transistors and
115 transformers, was DC powered by three independent power supplies at 19 V and about 3 A. The
116 voltage at the electrodes was of about 15 kV (peak to peak) with a dominant frequency of 12.5 kHz.
117 Other components were measured at 39.1, 64.6 and 91.0 kHz. One electrode of each couple was
118 covered by a 5 mm thick glass sheet according to a dielectric barrier discharge (DBD)
119 configuration. The gap spacing between the dielectric and the hot electrode was of 1.5 mm. The
120 discharge was directed on the vegetable surface by three fans (diameter of 77 mm) mounted over
121 the electrodes. Air speed was approximately 1.5 m/s (flow rate of about 7×10^{-3} m^3/s) at the
122 electrode and 0.5 m/s on the leaf. The electrodes and the treated samples were confined inside a

123 cabinet (about 3×10^{-2} m^3 of air volume) as described by Ragni et al. (2010). The chemical
124 characterisation of the emission in the 200-450 nm wavelength range (Fig. 2) was carried out by
125 means of an optic fibre probe (Avantes, FC-UV400-2) placed at about 20 mm from the discharge
126 and connected to a spectrometer (Avantes, AvaSpec-2048).
127 Radicchio also known as red chicory (*Cichorium intybus L.*) was purchased in bulk from local
128 wholesalers (Cesena, Italy) and was used unwashed. Treatment times of 15 and 30 min were chosen
129 after preliminary tests aimed at avoiding evident surface damages immediately after the treatment.
130 Control samples were conditioned at the same atmospheric (temperature and relative humidity) and
131 ventilation settings defined for the plasma tests.
132
133 2.2 Microbiological assessments
134 Radicchio samples were experimentally contaminated with a cocktail of five *Listeria*
135 *monocytogenes* strains (LR 102, 0227-359, VI 51028, 0113-131 and VI51010) or *Escherichia coli*
136 (O157:H7 VTx, Thermo Fisher NTCT12900). Cultures were grown at 37°C using brain heart
137 infusion (BHI, Thermo Fisher, Milan, Italy) and tryptic soy broth (TSB, Thermo Fisher) for *L.*
138 *monocytogenes* and *E. coli*, respectively.
139
140 *2.2.1 Listeria monocytogenes*
141 An aliquot of each of the five BHI *L. monocytogenes* overnight cultures was streaked on Agar
142 Listeria according to Ottaviani and Agosti (ALOA, Biolife, Milan, Italy) and incubated at 37°C for
143 24 h. Few colonies of each strain were re-suspended in physiological saline (NaCl 0.9%) and the
144 concentration adjusted to OD 0.08-0.1 at 625 nm. The five microbial suspensions were mixed with
145 a ratio of 1:1:1:1:1. The number of *L. monocytogenes* viable cells in the mixed suspension was
146 assessed by colony count on ALOA agar plates (Biolife) and ranged between 1.2 $x10^8$ and 1.6 x 10^8
147 CFU/ml. One hundred microliters of the microbial suspension were spotted on the surface of the
148 radicchio samples (4 x 4 cm). After inoculation, the leaves were stored under laminar flow in a

149 biohazard cabinet for 30 min in order to let the inoculum dry. After each treatment (0, 15 and 30
150 min, in afterglow at 70 mm from the discharge, at 22°C and 60 % of RH) and after 3 days of storage
151 at 4°C and 90% RH, each radicchio leaf was transferred into 160 mL of Buffer Peptone Water
152 (BPW; Thermo Fisher, Milan, Italy) and homogenised by a Stomacher® (Seward, UK) for 2 min at
153 normal speed. After one hour of storage at room temperature, serial ten-fold dilutions were
154 performed and plated onto Thin Agar Layer (TAL) plates for colony counting. The TAL method
155 involves overlaying 14 mL of nonselective medium (Tryptic Soy Agar, TSA, Thermo Fisher) onto a
156 prepoured, pathogen-specific, selective medium in order to allow the recovery of sub-lethally
157 injured cells. In the present study *L. monocytogenes* enumeration was performed on ALOA agar
158 plates (Biolife) overlayed with 14 mL of TSA. TAL plates and BPW were incubated for 24 h at
159 37°C. Upon observation of no colonies, the ISO 11290 was performed from the enriched BPW for
160 qualitative assessment of the presence/absence of *L. monocytogenes* in the sample.
161
162 *2.2.2 Escherichia coli*
163 The bactericidal effect of gas plasma on *E. coli* O157:H7 VTx- strain, Thermo Fisher NTCT12900,
164 was assessed in triplicate after 15 min of treatment using the most probable number counting
165 methods to enumerate the surviving bacteria on the surface of radicchio leave samples. This
166 experiment was set up to control the presence of interfering background flora that can also include
167 other *E. coli* strains. The use of selective supplements such as antibiotics, tellurite and bile salts
168 could inhibit bacterial cells exposed to the gas plasma treatment. An aliquot of a TSB overnight
169 culture of the *E. coli* O157:H7 VTx- strain, Thermo Fisher NTCT12900 was streaked on Sorbitol
170 MacConkey agar supplemented with cefixime (0.05 mg/L) and tellurite (2.5 mg/L) (CT-SMAC,
171 Thermo Fisher) and incubated at 37 °C for 24 h. Few colonies were re-suspended in physiological
172 saline (NaCl 0.9%). Three series of eight ten-fold dilutions of the microbial suspension with OD
173 0.08-0.1 at 625 nm containing approximately 10^8 CFU/mL were used for the inoculum of radicchio
174 leaf samples (1 cm x 1 cm). After treatment, all samples were transferred in tubes containing 10 mL

175 of BPW and homogenized for 1 min, then the tubes were incubated at 37 °C for 24 h. These
176 cultures were seeded on the surface of CT-SMAC (Thermo Fisher) and the agar plates were
177 incubated overnight at 37 °C. Sorbitol non-fermenting colonies were assessed with latex
178 agglutination test (*E. coli* O157 Latex Test Kit, Thermo Fisher). The BPW tubes containing viable
179 *E. coli* O157 were considered positive and on this basis, the most probable number (MPN) of *E.*
180 *coli* was assessed using MPN tables (USDA-FSIS, 2013).
181
182 2.3 Qualitative assessments
183 Qualitative assessments were conducted on samples of six radicchio leaves each before and
184 immediately after the treatments (15 and 30 min) and during storage at 4 °C and 90 % of Relative
185 Humidity. Antioxidant activity was evaluated immediately after the treatment; image and colour
186 analyses were conducted up to 1 day of storage (immediately after the treatment, 2h, 1d) while
187 sensory attributes were judged by assessors up to 3 days of storage (immediately after the treatment,
188 1d and 3d). Different storage times were selected according to the particular qualitative parameter
189 after preliminary tests aimed at finding storage conditions showing changes due to the exposure to
190 cold plasma.
191
192 *2.3.1. Antioxidant activity assays*
193 Treated and not treated radicchio samples were freeze-dried just after the treatments and then
194 analysed for ABTS radical-scavenging activity and oxygen radical absorbance capacity (ORAC).
195 The ABTS assay is based on the discolouration of the radical cation 3-ethyl-benzothiazoline-6-
196 sulfonic acid (ABTS•+; Sigma, UK.). The procedure was performed according to Miller et al.
197 (1993) and as improved by Re et al. (1999). The $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS
198 stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand overnight in
199 the dark at room temperature. The radical remained stable for 48 h when stored in the dark at room
200 temperature. A working solution of the $ABTS^{\bullet+}$ was prepared by diluting the radical stock solution

in 80% methanol to an absorbance of 0.70 ± 0.02 nm at 734 nm. Radicchio extract was obtained by vortexing 0.5 g freeze dried radicchio in 10 mL 80% methanol at 2500 rpm for 20 min and centrifuged for 10 min at 3800 rpm. Radicchio extract (20 µL) was added to 980 mL of $ABTS^{\bullet+}$ solution and incubated under dark at room temperature. Absorbance was measured at 734 nm after 10 min reaction. A calibration curve was constructed using Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid). All measurements were carried out three times, and in duplicate. The results are expressed as µmol Trolox equivalents per g of dried weight.

The ORAC assay was performed according to Huang et al. (2005) with some modifications. 2,2-Azobis (2-amidinopropane) dihydrochloride (AAPH; Sigma, UK.) was completely dissolved in 75 mM phosphate buffer (pH 7.4) to a final concentration of 369 mM. Fluorescein stock solution (4.19 µM) was made in 75 mM phosphate buffer (pH 7.4). A 0.586 µM fluorescein working solution was made fresh before analysis by further diluting the stock solution in 75 mM phosphate buffer. Trolox dissolved in 75 mM phosphate buffer (pH 7.4) was used to build the calibration curve. The procedure was as follows: 25 µL of radicchio extracts /blank/standard were added to a 96 well plate, subsequently 100 µl of fluorescein working solution was added to all wells. The plate was then heated to 37°C for 30 min. After the incubation, 75 µl of AAPH were added and the fluorescence of the samples was recorded for 100 min at 2 min intervals using a plate reader (Teca, Safire 2190, UK). Excitation wavelength was set at 485 nm and emission wavelength at 530 nm. ORAC values were calculated using the areas under the fluorescein decay curves (AUC), between the blank and the sample, using the following equation. Results were expressed as µM Trolox equivalents (TE) per g of dried weight.

$$AUC = 0.5 + \frac{f1}{f0} + \ldots \frac{fi}{f0} + \ldots + \frac{f99}{f0} + 0.5\left(\frac{f100}{f0}\right)$$

where: $f0$ = initial fluorescence reading at 0 min and fi = fluorescence reading at time i.

2.3.2 Digital Image analysis

226 A digital camera model D7000 (Nikon, Shinjuku, Japan) equipped with a 60 mm lens mod. AF-S
227 micro, Nikkor (Nikon, Shinjuku, Japan) was used to acquire digitalized images of radicchio leaves
228 (exposition time ½ sec; F-stop f/16) placed inside a black box under controlled lighting condition.
229 The digitalized images were analysed with Image Pro-Plus v. 6.2 (Media Cybernetics, USA). On
230 the basis of the chromatic characteristics, two different pixel ranges were defined corresponding to
231 "light red area" and "dark red area" for the samples evaluated until 2 h of storage at 4°C. For the
232 samples stored for 1 day, a different data analysis was conducted because the leaves were very
233 chromatically different from the other samples; two different pixel ranges were redefined
234 corresponding to "dark red area" and "brown area".
235 All pixels were then assessed in terms of percentage of each area on the total.
236
237 *2.3.3 Instrumental colour analysis*
238 Instrumental colour measurements were conducted by means of a Minolta ChromaMeter CR-400
239 reflectance colorimeter (Minolta, Milan, Italy). For each acquisition, an average value of three
240 measurements for each leaf taken at different spots was calculated. The CIELab system L^*, a^* and
241 b^* was considered (CIE, 1976) and the Chroma values were calculated as $C^*=\sqrt{a^{*2}+b^{*2}}$. The
242 acquisitions were performed on both white and red area of the radicchio leaves.
243
244 *2.3.4 Sensory test*
245 A hedonic test was conducted with 10 untrained assessors who scored the acceptability of 4
246 attributes (freshness, colour, odour and texture) using the following 1-5 point scale: 1)
247 unacceptable, very poor, strong defects; 2) poor, major defects; 3) fair, acceptable defects; 4) good,
248 acceptable defects; 5) typical attribute, very good without defects. In addition, 'overall
249 acceptability' was assessed using a 1-9 point scale ranged from 1 (dislike extremely) to 9 (like
250 extremely). Samples of 6 test settings were presented to the assessors (four cold plasma treated

samples for both treatment times and two respective controls) at 0, 1 and 3 days of storage. All test samples were appropriately randomised to avoid bias.

2.4 Data analysis

Significant differences ($p < 0.05$) between subgroups (control and treated samples as well as during storage) were determined by analysis of variance (ANOVA). Tukey test was used for post hoc comparisons. All analysis was conducted with SPSS 22.0 (IBM, Somers, New York).

3. Results and discussion

3.1 Emission characterisation

Irradiance values of the atmospheric dielectric barrier discharge emission (Fig. 2) show typical peaks of the second N_2 positive system (λ =290-440 nm, transition between $C^3\Pi_u$ and $B^3\Pi_g$ electronic states) and of the positive ion N_2^+ (λ= 391.4 nm transition between $B^2\Sigma_u^+$ and $X^2\Sigma_g^+$), as expected for air non-equilibrium discharges. The generation of NO (γ systems, transition between $A^2\Sigma^+$ and $X^2\Pi$) and OH radicals was also respectively detected at λ= 226-248 and λ= 305-309 nm. As previously described the presence of NO and OH radicals play an important role in microbial decontamination (Laroussi & Leipold, 2004).

3.2 Microbiological assessments

In several countries *Listeria monocytogenes* and *Escherichia coli* O157:H7 have been implicated in several food poisoning incidents resulting in serious illnesses and even deaths (Rangel et al. 2005; Olaimat & Holley, 2012). Cold plasma has been already described as a valuable decontamination technology on fruits and vegetables as cucumber, carrot and pear slices experimentally

276 contaminated by Salmonella (Wang et al., 2012). Reductions of *E. coli* O157:H7, *Salmonella* and *L.*
277 *monocytogenes* counts have also been reported for apples and lettuce (Misra et al., 2011).
278 In the present study the survival of a mix of *L. monocytogenes* strains as well as of *E. coli* O157:H7
279 NTCT12900 (Thermo Fisher) strain on radicchio leaves treated with atmospheric cold plasma was
280 evaluated immediately after treatment as well as after 3 days of storage at 4°C. Two treatment times
281 of 15 and 30 minutes were tested. The initial *L. monocytogenes* counts on radicchio leaves were not
282 significantly reduced by the 15 min cold plasma treatment ($p > 0.05$) (Table 1). However, a
283 significant reduction of approximately 2.20 log CFU/cm^2 of *L. monocytogenes* counts was observed
284 immediately after the 30 min cold plasma treatment. Storage results confirmed the decontamination
285 effect of this treatment . In particular, the *L. monocytogenes* log reduction was maintained
286 throughout the storage period with no occurrence of re-growth (Table 1).
287 Higher decontamination efficacy was described on strawberries and cherry tomatoes by means of a
288 DBD device (air, 70 kV_{RMS}) (Ziuzina et al., 2014). In particular a reduction of *L. monocytogenes*
289 counts by 4.2 log CFU/ sample and a complete eradication of the pathogen were observed
290 respectively after a 5 min treatment by cold plasma generated by a dielectric barrier discharge
291 system. The differing results compared to our study might be due to a matrix specific effect of cold
292 plasma as well as to a difference of the quantity of cold plasma reactive species produced. Cold
293 plasma reagents exclusively interact with the surface of the food product and cannot reach the
294 inside of coves generated by an irregular surface as well as the internal part of the product under its
295 surface. The higher the irregularity and porosity of the food product, the lower the decontamination
296 effect. Moreover the voltage was higher in the study of Ziuzina and colleagues in comparison to the
297 present one suggesting a different behaviour of the reactive species. Unfortunately, no informations
298 is available on the effect of such a high voltage on quality parameters of treated food products.
299 Regarding E. coli O157:H7 NTCT12900, a significant reduction in the number of surviving cells
300 was observed (-1.35 log MPN /cm^{2,} passing from 6.32 ($CI_{95\%}$ 5.35-4.64) to 4.97 ($CI_{95\%}$ 4.25-5.62)
301 log MPN /cm^2), after the 15 min treatment (Table 1). Similar results were found by Bermúdez-

302 Aguirre et al. (2013) who reported reductions in *E. coli* counts of 1.5 and 1.7 log CFU in lettuce and
303 tomato respectively, after a 10 min cold plasma treatment with tested voltages (3.95 kV up to 12.83
304 kV at 60 Hz) similar to the present study.
305 The results presented here illustrate the decontamination efficiency of the cold plasma on radicchio
306 experimentally inoculated with *L. monocytogenes* and *E. coli* O157:H7. The fact that a 30 min
307 treatment was needed to obtain a significant reduction in *L. monocytogenes* counts whereas a 15
308 min treatment was enough for *E. coli* could imply that Gram positive bacteria, such as *L.*
309 *monocytogenes*, are less susceptible to cold plasma treatment compared to Gram negative ones.
310 This is consistent with the study of Fröhling et al. (2012) who found, using membrane integrity
311 measurements, that different modes of plasma action exist against Gram-positive bacteria and
312 Gram-negative bacteria. Higher efficacies on the reduction of *E. coli* and *Salmonella* counts versus
313 *L. monocytogenes* counts were also described by Ziuzina et al., (2014).
314 For *E. coli*, the presence of sub-lethally injured cells (not culturable) due to the cold plasma
315 treatment should be excluded, since the long enrichment in a non-selective culture medium (i.e.
316 BPW for 24 h) can allow their recovery. Consequently, these cells could not recover or grow during
317 storage on radicchio leaves.
318 The treatment applied was able to significantly reduce but not eliminate the bacterial pathogens
319 inoculated on the surface of radicchio leaves. However, in this study a worst case scenario was
320 adopted (initial load of approx. 10^4-10^5 CFU/cm^2) whereas usually a load of maximum 10^2
321 CFU/cm^2 (Crépet et al., 2007) is present on the surface of leafy vegetables. Therefore, the treatment
322 could be effective in eliminating the pathogenic microorganisms although further experiments need
323 to be performed to confirm this.
324
325 3.3 Qualitative assessments
326
327 *3.3.1 Antioxidant activity*

328 The study of the interactions between plasma and food bioactive compounds is still at early stages.
329 Radicchio is rich in phenolic compounds, caffeic acid derivatives, chlorogenic acid, and some
330 flavonoids (Di Venere et al., 2005; Koukounaras & Siomos, 2010). The results from both ABTS
331 and ORAC antioxidant assays showed that, cold plasma at either treatment times (15 or 30 min) did
332 not cause any significant decrease in the antioxidant activity of polar fraction of the radicchio leaves
333 (Table 2). The polar profile of the radicchio extracts was not chromatographically analysed in this
334 study since no significant changes were observed. A different study by Ramazzina et al. (2015)
335 using the same DBD system described here showed that plasma treatments of 10 and 20 min had no
336 significant effect on the antioxidant activity and antioxidant content of fresh-cut kiwifruit. Different
337 mechanisms have been proposed to explain the changes in the content of individual antioxidant
338 compounds of the polar fraction (enhanced extractability due to penetration or favoured
339 biosynthesis due to UV-B radiation) and the matter is under investigation (Grzegorzewski et al.,
340 2010; Grzegorzewski et al., 2011). In essence, the presence of multiple reactive species in cold
341 plasma render the investigation of its effect on total antioxidant activity difficult as synergistic
342 actions and several different reaction pathways may take place. Although in this study cold plasma
343 treatment did not appear to negatively affect the antioxidant activity of the radicchio leaves, further
344 mechanistic studies need to be conducted in order to understand the interactions between plasma
345 and the antioxidant components.
346
347 *3.3.2 Image and colour analyses*
348 Results of digital image analysis, in terms of mean values of the calculated dark red area, are
349 reported in Table 3. For control samples, no significant differences emerged during 1 days of
350 storage at 4°C for both 15 (from 72.7 ± 5.5% before treatment to 76.5 ± 8.2 after 1 day) and 30 min
351 (from 73.0 ± 3.7% before treatment to 74.9 ± 6.1 after 1 day). For treated samples, significant
352 increases in terms of dark red area were observed after 1 day of storage (with respect to "before
353 treatment samples": about 20.8% and 35.5% for 15 and 30 min, respectively). Immediately and

354 after the first 2 h from the treatments, no significant changes on the radicchio leaves could be
355 observed. Results of the colour measurements, in terms of Chroma (C*), are summarised in the
356 Table 4. C* value was selected because it is considered the quantitative expression of colourfulness
357 perceived by consumers (Pathare et al., 2013). In relation to the white area, no significant
358 differences were observed for all samples. Concerning the red area, a significant decrease of the C*
359 values was observed during storage for the 15 min (from 29.1 ± 1.5 before treatment to 14.9 ± 0.7
360 after 1 day) and 30 min (from 25.8 ± 1.5 before treatment to 15.6 ± 2.1 after 1 day) treated samples.
361 For the control samples, this parameter showed a slight but not significant decrease ($p > 0.05$)
362 during storage at 4 °C for 1 day (from 28.3 ± 1.9 before 15 min treatment to 25.3 ± 1.6 after 1 day;
363 from 25.5 ± 4.5 before 30 min treatment to 21.7 ± 3.3 after 1 day).
364 The results obtained by digital image and instrumental colour analyses are in agreement with
365 previous studies carried out on lettuce leaves. Although different methods to generate the ionized
366 gas and different storage times were used, results suggested that the treatment can induce an
367 irreversible damage to the cellular structure of lettuce leaves (Grzegorzewski et al., 2011;
368 Bermúdez-Aguirre et al., 2013). Accordingly in the present study, a surface erosion of radicchio
369 leaves caused by oxidation of cell components can be hypothesized. This hypothesis is in line with
370 the visual observation of treated leaves after 1 day of storage (Tables 3 and 4).
371
372 *3.3.3 Sensory evaluation*
373 The mean scores of the organoleptic analysis are reported in Table 5. The results show that, after
374 one day of storage, the treated samples behaviour was significantly different ($p < 0.05$) from the
375 control samples ones. The mean scores for both control and plasma treated samples for freshness,
376 colour, odour, texture and overall acceptability decreased significantly during storage at 4°C for 3
377 days. Although, the scores were significantly reduced for control samples during storage illustrating
378 the very perishable nature of the radicchio leaves, cold plasma treated samples had an even lower
379 score. In terms of "Freshness", after 1 day of storage the treated samples were characterised by a

mean score of 2 ± 0 (15 min) and 1.1 ± 0.3 (30 min) while the control ones showed a mean score of 4 ± 0 for both treatment times. The results from the sensory evaluation are consistent with the decreased of C* values observed during storage. The results obtained in previous study conducted on fresh-cut apples with the same DBD prototype and operative conditions, suggested that the treatment could have a role in the alteration of the cellular respiratory pathway (Tappi et al., 2014). Negative effects on sensory attributes could be explained through the appearance of an anaerobic condition in the vegetable tissue. Further metabolic determinations could clarify this behaviour.

4. Conclusions

The present work presents the results of a critical study conducted on the efficiency of atmospheric cold plasma technology in the decontamination of radicchio leaves. Results indicate maximum significant reductions of 1.35 log MPN/cm^2 for *E. coli* (15 min of treatment) and approx. 2 log CFU/cm^2 for *L. monocytogenes* (30 min treatment). These reductions can be considered promising in terms of safety considering that this kind of product can be characterised by a maximum contamination load of 10^2 CFU/cm^2. The nutritional quality of the radicchio leaf, if conventionally expressed here as the antioxidant capacity of its polar fraction, remained relatively intact after the cold plasma treatments. Further evaluation of nutritional compounds need to be considered also in relation to the storage. In relation to the possible effects caused by the interaction of reactive species with the product, the treatments appeared to negatively affect the quality of the leaves during storage and this technology will only be promising for radicchio treatment if the quality defect can be excluded. Although immediately after the treatment and after 2 h of storage, no quality defects could be observed, a significant impact in terms of visual quality was observed after 1 day of storage with respect to the control. Since the cold plasma system described in this study operates in open air and does not require water, it could be easily incorporated in existing food production lines. Off-line gas plasma long time treating cells or a more enhanced equipment (different electrodes configurations) placed directly on the line conveyor belt for short treatments, could be

406 considered. Further optimisation needs to be undertaken to reduce or remove the negative effect on
407 quality evidenced during the storage. Moreover, besides taking into consideration evident damages,
408 the formation of undesirable substances in the food, due to the gas plasma reactions, should be
409 carefully investigated.
410
411 **Acknowledgements**
412 The research leading to these results has received funding from the European Union's Seventh
413 Framework Programme for research, technological development and demonstration under grant
414 agreement No. 289262. Theme KBBE.2011.2.1-01, research project STARTEC: "Decision Support
415 Tools to ensure safe, tasty and nutritious Advanced Ready-to-eat foods for healthy and vulnerable
416 Consumers".
417
418 **5. References**
419
420 Baier, M., Ehlbeck, J., Knorr, D., Herppich, W. B., & Schlüter, O. (2015). Impact of plasma
421 processed air (PPA) on quality parameters of fresh produce. *Postharvest Biology and Technology*,
422 *100*, 120-126.
423
424 Baier, M., Foerster, J., Schnabel, U., Knorr, D., Ehlbeck, J., Herppich, W. B., et al. (2013). Direct
425 non-thermal plasma treatment for the sanitation of fresh corn salad leaves: Evaluation of physical
426 and physiological effects and antimicrobial efficacy. *Postharvest Biology and Technology*, *84*, 81-
427 87.
428
429 Baier, M., Görgen, M., Ehlbeck, J., Knorr, D., Herppich, W. B., & Schlüter, O. (2014). Non-thermal
430 atmospheric pressure plasma: Screening for gentle process conditions and antibacterial efficiency
431 on perishable fresh produce. *Innovative Food Science & Emerging Technologies*, *22*, 147-157.

Baur, S., Klaiber, R., Hammes, W. P., & Carle, R. (2004). Sensory and microbiological quality of shredded, packaged iceberg lettuce as affected by pre-washing procedures with chlorinated and ozonated water. *Innovative Food Science and Emerging Technology*, *5*,45–55

Berardinelli, A., Vannini, L., Ragni, L., & Guerzoni, M. E. (2012). Impact of atmospheric plasma generated by a DBD device on quality-related attribute of "Abate Fetel" Pear Fruit. In Z. Machala, K. Hansel, & Y. Akishev (Eds.), *Plasma for Bio-Decontamination, Medicine and Food Security* (pp. 457-467), Nato Series, Springer.

Bermúdez-Aguirre, D., WemLinger, E., Pedrow, P., Barbosa-Cánovas, G., & Garcia-Perez, M. (2013). Effect of atmospheric pressure cold plasma (APCP) on the inactivation of Escherichia coli in fresh produce. *Food Control*, *34*, 149–157.

Crépet, A., Albert, I., Dervin, C., & Carlin, F., (2007). Estimation of microbial contamination of food from prevalence and concentration data: application to *Listeria monocytogenes* in fresh vegetables. *Applied and Environmental Microbiology*, *73*, 250–258.

Critzer, F. J., Kelly-Winterberg, K., South, S. L., & Golden, D. A. (2007). Atmospheric plasma inactivation of foodborne pathogens on fresh produce surfaces. *Journal of Food Protection*, *70*, 2290-2296.

Di Venere, D., Linsalata, V., Sergio, L., Cardinali, A., Pieralice, M., Vanadia, S., et al. (2005). Antioxidant phenolics in escarole and radicchio during storage of fresh-cut 'ready-to-use' product. ISHS Acta Horticulturae 682: V International Postharvest Symposium.

458 Dobryinin, D., Fridman, G., & Fridman, A. (2009). Physical and biological mechanisms of direct
459 plasma interaction with living tissue. *New Journal of Physics*, *11*, 115020.
460
461 Fernández, A., & Thompson, A. (2012). The inactivation of Salmonella by cold atmospheric plasma
462 treatment. *Food Research International*, *45*, 678–684.
463
464 Fröhling A., Baier M., Ehlbeck J., Knorr D., & Schlüter O. (2012). Atmospheric pressure plasma
465 treatment of *Listeria innocua* and *Escherichia coli* at polysaccharide surfaces: Inactivation kinetics
466 and flow cytometric characterization. *Innovative Food Science and Emerging Technologies*, *13*,
467 142–150.
468
469 Gozzi, G., Berardinelli, A., Ragni, L., & Vannini, L.(2013). Effect of cold atmospheric plasma on
470 natural Microflora and enzymatic activities of Fuji apples. In: Proceedings of the Spoiler 2013
471 International Symposium, Quimper, France, 1-2-3 July, 2013.
472
473 Grzegorzewski, F., Ehlbeck, J., Schlüter, O., Kroh, L. W., & Rohn, S. (2011). Treating lamb's
474 lettuce with a cold plasma–Influence of atmospheric pressure Ar plasma immanent species on the
475 phenolic profile of *Valerianella locusta*. *LWT-Food Science and Technology*, *44*, 2285-2289.
476
477 Grzegorzewski, F., Rohn, S., Kroh, L. W., Geyer, M., & Schlüter, O. (2010). Surface morphology
478 and chemical composition of lamb's lettuce (*Valerianella locusta*) after exposure to a low-pressure
479 oxygen plasma. *Food Chemistry*, 122, 1145–1152.
480
481 Hermann, H. W., Henins, I., Park, J., & Selwyn G. S. (1999). Decontamination of chemical and
482 biological warfare agents using an atmospheric pressure plasma jet. Physics of Plasmas, 6, 2285-
483 2289.

484 Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays.
485 *Journal of Agricultural and Food Chemistry*, *53*, 1841–1856.

486

487 Kogelschatz U. (2003). Dielectric-barrier discharges: their history, discharge physics, and industrial
488 applications. *Plasma Chemistry and Plasma Processing*, *43*, 1–46.

489

490 Koukounaras, A., & Siomos, A. S. (2010). Changes in antioxidant activity of radicchio during
491 storage. *Acta Horticulturae*, *877*, 1281-1286.

492

493 Laroussi, M., & Leipold, F. (2004). Evaluation of the roles of reactive species, heat, and UV
494 radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *International*
495 *Journal of Mass Spectroscopy*, *233*, 81-86.

496

497 Miller, N. J., Rice-Evans, C, Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method
498 for measuring antioxidant capacity and its application to monitoring the antioxidant status in
499 premature neonates. *Clinical Science (London)*, *84*, 407-412.

500

501 Misra, N., Tiwari, B., Raghavarao, K. S. M. S., & Cullen, P. (2011). Non-thermal plasma
502 inactivation of food borne pathogens. *Food Engineering Reviews*, *3*, 1-12.

503

504 Montie, T. C., Kelly-Wintenberg K., & Reece, J. R. (2000). An overview of research using the one
505 atmosphere uniform glow discharge plasma (OAUGDP) for sterilization of surfaces and materials.
506 *Plasma Science, IEEE Transactions*, *28*, 41-50.

507

508 Moreau, M., Orange, N., & Feuilloley, M. (2008). Non-thermal plasma technologies: new tools for
509 bio decontamination. *Biotechnology Advances*, *26*, 610-617.
510
511 Olaimat, A. N. & Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: a
512 review. *Food Microbiology*, *32*, 1–19.
513
514 Oliveira, M., Viñas, I., Anguera, M., & Abbeys, M. (2012). Fate of *Listeria monocytogenes* and
515 *Escherichia coli* O157: H7 in the presence of background natural microbiota on conventional and
516 organic lettuce. *Food Control*, *25*, 678–683.
517
518 Pathare, P. B., Opara, U. L., & Al-Said, F.A.J. (2013). Colour Measurement and Analysis in Fresh
519 and Processed Foods: A Review. *Food Bioprocess Technology*, *6*, 36–60.
520
521 Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garrett, E. H., Farber, J. N., et al. (2003).
522 Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Reviews*
523 *in Food Science and Food Safety*, *2*, 161–173.
524
525 Pau, C. F., Yan, J. D., & Wylie S. R. (2000). The influence of the gas flow rate and microwave
526 source power on the behaviour of a microwave generated argon plasma jet. Proceedings of the XIII
527 International Conference on Gas Discharges and their Applications, Glasgow, UK.
528
529 Perni, S., Liu D.W., Shama G., & Kong, M. (2008). Cold atmospheric plasma decontamination of
530 the pericarps of fruit. *Journal of Food Protection*, *71*, 302-308.
531

532 Ragni, L., Berardinelli, A., Vannini, L., Montanari, C., Sirri, F., et al. (2010). Non Thermal
533 atmospheric gas plasma device for surface decontamination of shell eggs. *Journal of Food*
534 *Engineering*, *100*, 125-132.
535
536 Ramazzina I., Berardinelli A., Rizzi F., Tappi S., Ragni L., Sacchetti G., & Rocculi P. (2015). Effect of cold
537 plasma treatment on physico-chemical parameters and antioxidant activity of minimally processed kiwifruit.
538 *Postharvest Biology and Technology*, *107*, 55–65.
539
540 Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva C.L.M. (2013). Fresh fruits and
541 vegetables—An overview on applied methodologies to improve its quality and safety. *Innovative*
542 *Food Science and Emerging Technologies, 20*, 1-15.
543
544 Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., & Swerdlow, D. L. (2005). Epidemiology
545 of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*,
546 *11*, 603–609.
547
548 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).
549 Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical*
550 *Biology and Medicine, 26*, 1231-1237.
551
552 Schütze, A., Jeong, J. Y., Babayan, S. E., Park, J., Selwyn, G. S., & Hicks R. F. (1998). The
553 atmospheric-pressure plasma jet: a review and comparison to other plasma sources. *IEEE*
554 *Transactions on Plasma Science*, *26*, 1685-1694.
555

556 Siroli, L., Patrignani, F., Serrazanetti, D., Tappi, S., Rocculi, P., Gardini, F., et al. (2015). Natural
557 antimicrobials to prolong the shelf-life of minimally processed lamb's lettuce. *Postharvest Biology*
558 *and Technology*, *103*, 35–44.
559
560 Srey, S., Park, S. Y., Jahid, I.K., & Ha, S.-D. (2014). Reduction effect of the selected chemical and
561 physical treatments to reduce *L. monocytogenes* biofilms formed on lettuce and cabbage. *Food*
562 *Research International*, *62*, 484–491.
563
564 Stratakos, A. C., & Koidis, A. (2015). Suitability, efficiency and microbiological safety of novel
565 physical technologies for the processing of ready to eat meals, meats and pumpable products.
566 *International Journal of Food Science & Technology*, *50*, 1283-1302.
567
568 Tappi, S., Berardinelli, A., Ragni, L., Dalla Rosa, M., Guarnieri, A., & Rocculi, P. (2014).
569 Atmospheric gas plasma treatment of fresh-cut apples. *Innovative Food Science & Emerging*
570 *Technologies*, *21*, 114-122.
571
572 Tendero, C., Tixier, C., Tristant, P., Desmaison, J., & Leprince P. (2006). Atmospheric pressure
573 plasmas: a review. *Spectrochimica Acta Part B*, *61*, 2-30.
574
575 USDA-FSIS. (2013). Most Probable Number Procedure and Tables. QD-F-Micro-0004.07.
576 Accessed online: http://www.fsis.usda.gov/wps/wcm/connect/8872ec11-d6a3-4fcf-86df-
577 4d87e57780f5/MLG-Appendix-2.pdf?MOD=AJPERES
578
579 Wang, R. X., Nian, W. F., Wu, H. Y., Feng, H. Q., Zhang, K., Zhang, J., et al. (2012) Atmospheric-
580 pressure cold plasma treatment of contaminated fresh fruit and vegetable slices: inactivation and

physiochemical properties evaluation. *The European Physical Journal D-Atomic, Molecular, Optical and Plasma Physics, 66*, 1-7.

Ziuzina, D., Patil, S., Cullen, P. J., Keener, K. M., & Bourke P. (2014). Atmospheric cold plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* inoculated on fresh produce. *Food Microbiology*, *42*, 109-116.

1 **Figure captions**

2 **Figure 1.** Atmospheric cold plasma treatments of radicchio leaves.

3 **Figure 2**. Irradiance values of the emission acquired at about 20 mm from the discharge (input voltage: 19

4 V). Values in brackets refer to vibrational transition (v'→ v'').

Figure 1

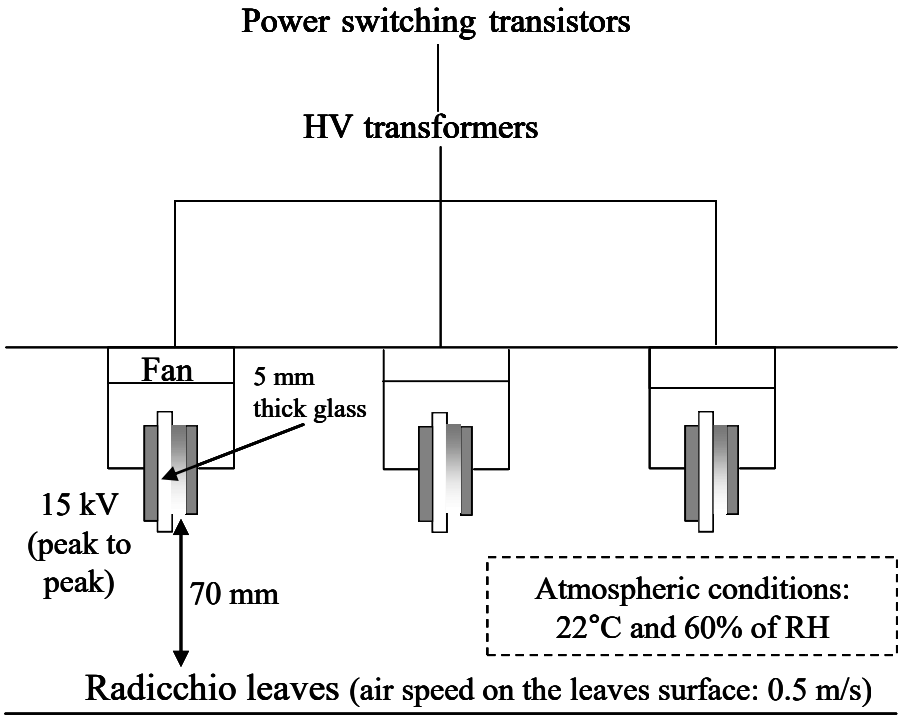

Fig.1

Figure 2

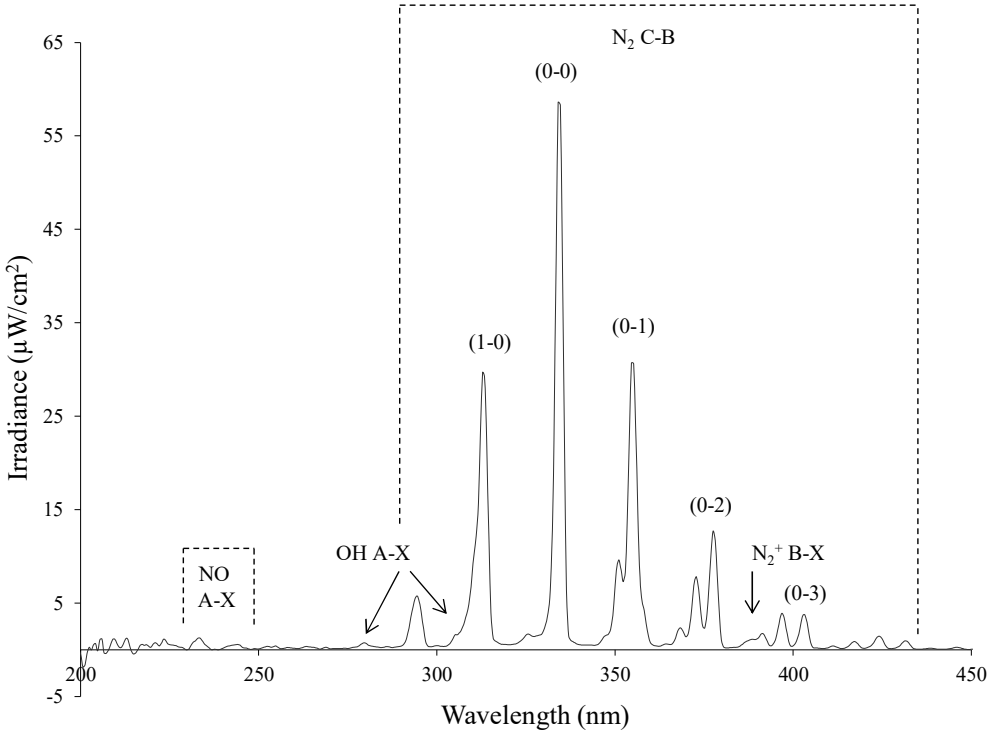

Fig.2

Table 1. *L. monocytogenes* and *E. coli* survival on the cold plasma treated radicchio leaves.

Listeria monocytogenes Log CFU/cm^2			
Treatment time (min)	Sample	After the treatment	After 3 days of storage
15	C	5.92 ± 0.16^a	5.85 ± 0.14^a
	T	5.59 ± 0.30^b	5.87 ± 0.16^a
30	C	4.17 ± 0.21^a	3.49 ± 0.66^a
	T	1.96 ± 0.16^b	1.21 ± 0.56^b
Escherichia coli Log MPN/cm^2			
Treatment time (min)	Sample	After the treatment	
15	C	6.32 ($CI_{95\%}$ 5.35-4.64)	
	T	4.97 ($CI_{95\%}$ 4.25-5.62)	

Note: C: control, T: treated (value ± standard deviation). The same lowercase letters denote no significant differences ($p < 0.05$).

Table 2. Effect of cold plasma on the antioxidant activity of radicchio leaves assessed by ABTS and ORAC values (μM TE/g dried weight) (n=6).

Treatment time (min)	Sample	ABTS	ORAC
	C	193 ± 22^{a}	98 ± 1^{a}
15	T	219 ± 8^{a}	117 ± 5^{a}
30	T	213 ± 18^{a}	97 ± 18^{a}

Note: C: control, T: treated (value ± standard deviation). The same lowercase letters denote no significant differences ($p < 0.05$).

Table 3. Mean values of the dark red area of untreated and cold plasma treated radicchio leaves.

Treatment time (min)	Sample	RED AREA (%)			
		Before treatment	After treatment	2 h	1 day
15	C	72.7 ± 5.5^{a}	72.4 ± 5.5^{a}	75.3 ± 3.1^{a}	76.5 ± 8.2^{a}
	T	79.7 ± 3.8^{a}	78.5 ± 4.9^{a}	79.6 ± 2.7^{a}	19.7 ± 9.2^{b}
30	C	73.0 ± 3.7^{a}	72.7 ± 3.7^{a}	75.2 ± 2.5^{a}	74.9 ± 6.1^{a}
	T	72.7 ± 6.2^{a}	78.1 ± 5.8^{a}	76.1 ± 7.3^{a}	16.6 ± 7.9^{b}

Note: C: control, T: treated (standard deviation in brackets). The same lowercase letters denote no significant differences during storage, within the same sample (Tukey HSD test, $p < 0.05$).

Table 4. Instrumental colour (C*) values of untreated and cold plasma treated radicchio leaves.

Treatment time (min)	Sample	Radicchio area	CHROMA (C*)			
			Before treatment	After treatment	2 h	1 day
15	C	White	5.5 ± 0.5^{a}	5.4 ± 0.6^{a}	5.8 ± 0.4^{a}	6.1 ± 0.6^{a}
		Red	28.3 ± 1.9^{a}	26.5 ± 2.4^{b}	25.8 ± 1.9^{b}	25.3 ± 1.6^{b}
	T	White	4.3 ± 0.6^{a}	4.3 ± 0.4^{a}	4.2 ± 0.6^{a}	4.2 ± 0.7^{a}
		Red	29.1 ± 1.5^{a}	20.6 ± 4.2^{b}	17.6 ± 2.7^{c}	14.9 ± 0.7^{d}
30	C	White	5.3 ± 0.9^{a}	5.5 ± 1.2^{a}	5 ± 0.7^{a}	5.2 ± 0.5^{a}
		Red	25.5 ± 4.5^{a}	23.4 ± 3.6^{ab}	22.4 ± 3.3^{b}	21.7 ± 3.3^{b}
	T	White	4.2 ± 0.7^{a}	4 ± 0.6^{a}	4.4 ± 0.4^{a}	4.9 ± 1.3^{a}
		Red	25.8 ± 1.5^{a}	19.5 ± 4.3^{b}	17.7 ± 2.4^{bc}	15.6 ± 2.1^{c}

Note: C: control, T: treated (standard deviation in brackets). The same lowercase letters, in the same row, denote no significant differences during storage, within the same sample (Tukey HSD test, $p < 0.05$).

Table 5

Table 5. Sensory analysis of untreated and cold plasma treated radicchio leaves stored for 3 days.

Treatment time (min)	Storage time (days)	Freshness		Colour		Odour		Texture		Overall acceptability	
		C	T	C	T	C	T	C	T	C	T
15	0	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	9 ± 0^{a}	9 ± 0^{a}
	1	4 ± 0^{b}	2 ± 0^{b}	3.6 ± 0.5^{b}	2.6 ± 0.2^{b}	3.8 ± 0.6^{b}	2 ± 0^{b}	3.8 ± 0.4^{b}	2.6 ± 0.2^{b}	8 ± 0^{b}	2 ± 0^{b}
	3	4 ± 0^{b}	2 ± 0^{b}	3.7 ± 0.6^{b}	2.1 ± 0.3^{c}	3.7 ± 0.5^{b}	2.1 ± 0.3^{b}	3.7 ± 0.5^{b}	2.1 ± 0.3^{c}	8 ± 0^{b}	2 ± 0^{b}
30	0	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	9 ± 0^{a}	9 ± 0^{a}
	1	4 ± 0^{b}	1.1 ± 0.3^{b}	3.6 ± 0.5^{b}	2.1 ± 0.3^{b}	3.8 ± 0.6^{b}	2.1 ± 0.3^{b}	3.8 ± 0.4^{b}	2.6 ± 0.2^{b}	8 ± 0^{b}	2 ± 0^{b}
	3	4 ± 0^{b}	1.1 ± 0.3^{b}	3.7 ± 0.6^{b}	1.1 ± 0.3^{c}	3.7 ± 0.5^{b}	1.1 ± 0.3^{c}	3.7 ± 0.5^{b}	2.1 ± 0.3^{c}	8 ± 0^{b}	1 ± 0^{b}

Note: C: control, T: treated (standard deviation in brackets). The same lowercase letters denote no significant differences during storage, within the same sample ($p < 0.05$).