Non-thermal plasma-bubbling system for food decontamination: Identification of reactive species and its bactericidal effect on iceberg lettuce

A.G. Komarudin[1,2], I. Sotome[1], T. Araki[1]

[1] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan*
[2] *School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia*

Abstract: This study investigated the potential of non-thermal atmospheric plasma integrated with a bubbling-assisted method as an alternative for fresh produce decontamination strategy. Using a multigas plasma jet with pure oxygen as the primary feeding gas, we evaluated the *in-vitro* bactericidal effect of the plasma-bubbling system, determined the primary reactive species by electron spin resonance (ESR) analysis, and applied the system to inactivate *E. coli* O157:H7 on iceberg lettuce.

Keywords: plasma-bubbling, bactericidal effect, singlet oxygen, hydroxyl radical

1. Introduction

Consumption of fruits and vegetables has been promoted and increased in response to recommendations of public health authorities and nutrition professionals. In contrast, the increased consumption positively correlates with an increased foodborne outbreak of microbial infection [1]. High microbial loads in fresh produce due to improper harvest and handling conditions might also lead to rapid deterioration of the product's quality and its highly perishable nature. Mostly being eaten raw and requiring no more cooking process, improving fresh produce's safety and shelf life has become a more challenging task both for the industry and consumers.

Employing non-thermal atmospheric plasma (NTAP) as a food safety intervention technology continues to be a key area of research. The plasma reactive species can diffuse through the microbial cell membrane, causing damage, surface erosion, and microbial cell death at last [2]. With the emergence of NTAP research in food and agriculture, ensuring uniform exposure of plasma species to food products with complex surface morphology (e.g., pores and rough surfaces) and finding the balance of keeping the food safe yet retaining its physical attributes are still a challenge to solve [3].

A plasma-bubbling system has been introduced to enhance the distribution of the reactive species in the liquid system to obtain a better uniformity of antimicrobial effect inside the plasma-treated sample [4-6]. However, there are still limited studies reporting NTAP treatment with a bubble-assisted approach, and the contribution of the generated reactive species still needs to be thoroughly investigated. Therefore, this study aimed to evaluate the *in-vitro* bactericidal effect of the plasma-bubbling system and to investigate the involved reactive species in the bactericidal activity using electron spin resonance (ESR) analysis. We also extended the results to treat inoculated iceberg lettuce and evaluate the potential of the plasma-bubbling system to prevent cross-contamination.

2. Materials and Methods

E. coli O157:H7 (CR-3, isolated from bovine feces) was cultivated in tryptone-soya broth at 37ºC for 24 h. The bacterial suspension was prepared at a final concentration of 8.0 $\log_{10}$ CFU/mL.

A non-thermal multi-gas plasma jet at atmospheric pressure was used as the plasma source, operated at 9 kV and 16 kHz, provided by an AC power supply (Plasma Concept Tokyo, Inc., Japan). The plasma jet was connected to a cylindrical gas injection tube (diameter x length = φ8 mm x 180 mm; filter size=φ15 mm×20 mm) to create the bubbling system. Pure oxygen with a flow rate of 8 L/min was used as the feeding gas. A schematic diagram of the plasma jet device is shown in **Fig.1**.

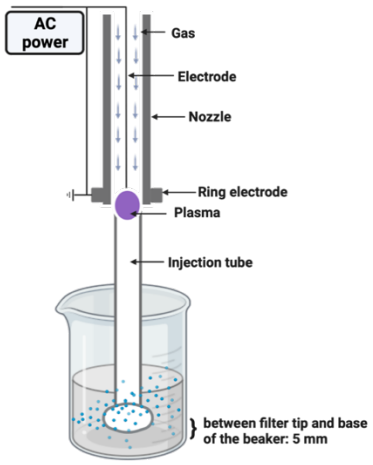

Fig. 1. Schematic diagram of experimental setup

The cylindrical tube connecting to the plasma jet was inserted into a beaker containing 50 mL of bacterial suspension, leaving a gap between the filter tip and the base approximately 5 mm. The suspension was treated by plasma for 0, 30, 60, 120, and 180 seconds. Subsequently, treated samples were serially diluted in phosphate-buffered saline and plated on Tryptic Soy agar plates, incubated at 37 ◦C for 24 h.

Prior to ESR analysis, the treated bacteria sample was prepared by centrifugation at 13000 rpm for 5 min at room temperature. The supernatant was subsequently used for ESR measurement. Two types of spin trapping reagents were used: 2-(5,5-dimethyl-2-oxo-2λ5-[1,3,2] dioxaphosphinan-2-yl)-2-methyl-3,4-dihydro-2H- pyrrole 1-oxide (CYPMPO) for detecting •OH radicals; and 2,2,6,6-tetramethyl-4-piperidone (TMPD) for detecting

$^1O_2$. Untreated and treated samples were analyzed by The Bruker EMX Plus with measurement conditions as follows: room temperature (23−25 ℃); magnetic field range, 3521 ± 100 G; data points, 1000; microwave power, 1 mW; time constant, 20.48 μs; conversion time, 20.00 ms; sweep time, 20.0 s; scan, 10 times; modulation intensity, 1.00 G.

Following the *in-vitro* test, the disinfection performance of the plasma-bubbling system on fresh produce was also evaluated. Approximately 100 μl of *E. coli* O157:H7 suspension were spotted on iceberg lettuce cuts (3*x*3 cm). First, the inoculated lettuce cuts were air-dried inside a clean bench for 90 min. Subsequently, 10 g of inoculated lettuce immersed in 200 mL of distilled water (inside a 500-mL beaker glass) were subjected to the plasma-bubbling system for 5 min. Treated lettuce and the wash water were analyzed to determine the bacteria inactivation and the remaining microbial load after treatment. Inoculated lettuce washed with distilled water only was used as the control. Plasma-treated samples were serially diluted in phosphate-buffered saline and plated on Sorbitol MacConkey agar plates. The number of surviving viable cells was determined by plate counts following the incubation of the plates at 37 ◦C for 24 h. The disinfection ability of plasma treatment was calculated using the following formula:

$$\text{Log}_{10}\text{ reduction} = \text{Log}_{10}(\text{CFU}_{\text{Untreated}}) - \text{Log}_{10}(\text{CFU}_{\text{Treated}}) \quad (1)$$

All experiments were carried out in triplicate. Results are demonstrated as the mean and standard deviation of these determinations. The results were subjected to a one-way analysis of variance (ANOVA), followed by a Tukey HSD test using RStudio version 4.0.2. Significant differences among the mean values were considered at a significance level of $p < 0.05$.

3. Results and Discussions

As shown in **Fig. 2**, treatment of *E. coli* with plasma-bubbling for 120 seconds led to a 5-log reduction of the viable cells from an initial population of 8.21 log CFU/mL. In addition, the viable counts were under the detection limit after 180 sec of treatment. Increasing treatment time generates more plasma-generated species, leading further to the decreasing population of bacteria. Plasma-bubbling generates more active species, including free electrons, monoatomic compounds, and charged particles. In addition, other physical mechanisms also might involve in inactivating the bacteria, including UV/VUV photons and ions through charge exchange reactions and photolysis [7] and change of osmotic pressure [8]. Results from previous research using a similar plasma jet reactor also agreed with this study, where the plasma treatment effectively inactivated the d *E. coli* population to 6 log reduction within 1 min [9]. Their higher antibacterial activity compared to antibacterial activity in this study could be due to the difference in the sample size, working gas, and experimental setup.

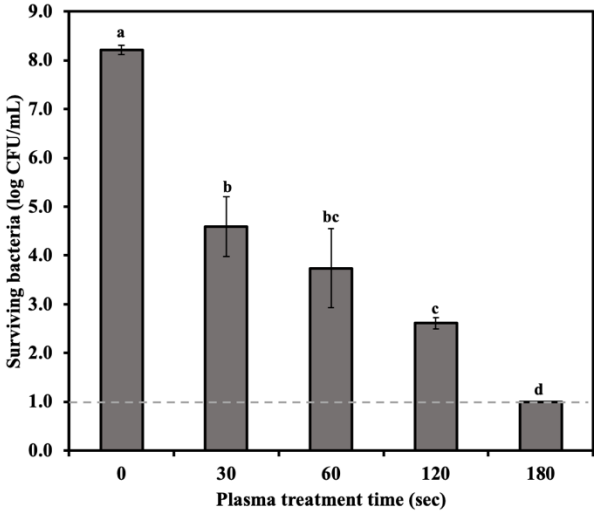

Fig. 2. *E. coli* population after different treatment times of plasma-bubbling. [a-d] Values with different letters were significantly different at $p < 0.05$ (n=3); the detection limit was 1.0 log CFU/mL

The spectra obtained from ESR are further used to identify the type of ROS by the shape and the numerical parameters, such as *g*-values and hyperfine coupling constants (*hfcc*) [10]. The *g*-value is calculated from the resonance magnetic field and the observed resonance frequency. *hfcc* values are characterized as the splitting of an ESR spectrum and determined by analyzing the splitting of the spectrum of the spin adduct. From the ESR spectra (data not shown), spin adduct CYPMPO-OH (g = 2.008) was able to be distinguished from the consecutive peaks in the spectrum with the following *hfcc*: isomer 1: A_H = 1.36 mT, A_N = 1.37 mT, A_P = 4.89 mT; isomer 2: A_H=1.23 mT, A_N=1.35 mT, A_P=4.70 mT. These *hfcc* values were similar to those •OH radical adduct spectrum in previous studies [11-12]. In addition, with similar protocols, $^1O_2$ was also identified using TMPD as the spin trap on both samples of two plasma treatment approaches (g = 2.004). According to the obtained ESR spectra, the *hfcc* for the $^1O_2$ were as follows: $A_H = A_N = A_P$ = 1.45 mT, which was comparable to the reported values [13]. Therefore, these findings suggested that •OH and $^1O_2$ were confirmed to exist in the supernatant of plasma-treated bacterial suspension in our study.

The signal intensity of the spin adducts is directly proportional to the concentration of the formed free radicals R•. **Fig. 3** depicts the increase of the signal intensity of CYPMPO-OH and TMPD-$^1O_2$ with the plasma treatment time. •OH began to appear on the spectrum after 30 sec of plasma treatment, while $^1O_2$ was subsequently identified after 60 sec. The intensity of both ROS adducts continued to elevate up to 1.09 ± 0.11 for CYPMPO-OH and 0.45 ± 0.09 for TMPD-$^1O_2$ at 180 sec. Due to their high reactivity, one might speculate that those reactive species attacked the bacteria in the suspension immediately after the plasma discharge. For this reason, these ROS could not

be identified by ESR before 60 sec and 120 sec of plasma treatment for •OH and $^1O_2$, respectively.

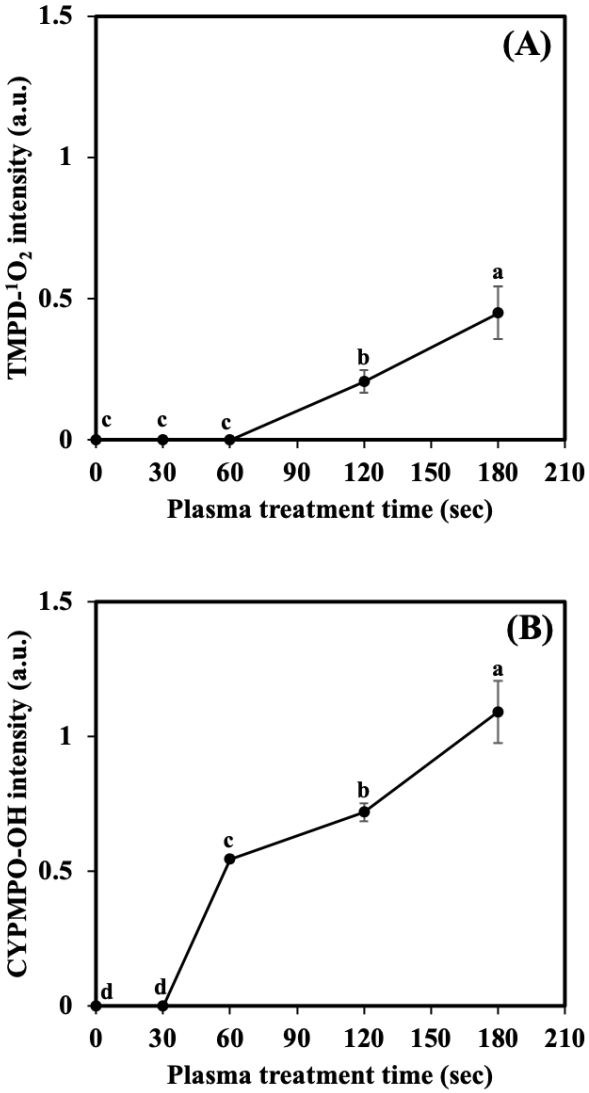

Fig. 3. Signal intensity of TMPD-$^1O_2$ spin adduct (A) and CYPMPO-OH spin adduct (B) after different treatment times of plasma-bubbling towards *E. coli.* [a-d] Values with different letters were significantly different at $p < 0.05$ (n=3)

•OH and $^1O_2$ are highly reactive with biological substances and have been reported to be important in microbial inactivation by non-thermal plasma [14-15]. As the most reactive among all RONS, •OH can react with proteins and lipids on the cell membrane, damaging the components of intracellular materials, such as DNA. While •OH is reported to react non-selectively with many chemical bonds, $^1O_2$ might be more crucial in eliminating bacteria in this plasma-bubbling system. Wu et al. [16] suggested that $^1O_2$ has a relatively long lifetime compared to •OH and is more easily diffused into the bacterial cell membrane.

Plasma-bubbling was assessed as a washing treatment for fresh produce, using ready-to-eat iceberg lettuce as a model. As a control, washing inoculated lettuce with untreated distilled water resulted in 1.2 ± 0.12 log CFU/g lettuce, whereas washing with plasma bubbling yielded 2.1 ± 0.07 log CFU/g lettuce, as shown in **Table 1**. These results are in accordance with other studies using plasma-water-based treatments (e.g., plasma-activated water) reporting that the disinfection performance is mainly attributed to the formation of reactive species [3]. Compared to the *in-vitro* results, the antibacterial activity of plasma bubbling against inoculated fresh produce is significantly lower. The less effective log reduction was due to iceberg lettuce's complex chemical and biological nature as food. Food products are rich in enzymes, proteins, sugars, lipids, and other macro and micronutrients, consisting of various ions and ionic compounds [17]. These components might provide a protective effect against the plasma reactive species targeting the bacteria, whereas previously, the bacteria were suspended in a less complex buffered environment.

Table 1. Viable cell counts of surviving bacteria after washing treatments

	Inoculated lettuce (log CFU/g)	Log reduction (Equation 1)	Wash water (log CFU/mL)
Before washing	6.02 ± 0.30	-	-
Distilled water	4.76 ± 0.40	1.26 ± 0.12	6.00 ± 0.33
Plasma-bubbling	3.91 ± 0.29	2.11 ± 0.07	n.d.

n.d.: below detection limit
The average value and standard deviation of the results evaluated are shown (n =3). The detection limit was 1.0 log CFU/mL.

Wash water samples were evaluated to measure the surviving bacterial populations remaining in the water. 6.00 ± 0.33 log CFU/mL viable bacteria were detected from the distilled water after washing. In contrast, plasma-bubbling reduced the bacterial load of the wash water to below the detection limit after treatment (< 1 log CFU/mL). This result demonstrates the potential for the application of the plasma-bubbling treatment to enhance the microbiological safety of the washing process of fresh produce while washing with distilled water has no disinfection effect on the detached bacteria from the lettuce.

From cost and sustainability perspectives, the generation of reactive species continuously during treatment shows the potential of the wash water to be reusable for subsequent washing treatment in a continuous system. However, the accumulated organic matter and debris might interfere with the bioactivity of the plasma treatment, and further research to anticipate such interference is required.

4. Conclusions

Treatment of the plasma bubbling system for 180 seconds could eliminate 8 log CFU/mL *E. coli* viable cells. Based on ESR analysis, two primary short-lived ROS are generated from the plasma system, which are •OH and $^1O_2$. The plasma treatment could reduce the bacterial load attached to iceberg lettuce cuts. In addition, the surviving bacteria load released from the treated lettuce was not detected from the washing water. Based on the current results, our developed non-thermal plasma bubbling system is prospective to be a food safety intervention for sanitizing fresh produce and preventing cross-contamination.

5. Acknowledgements

This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Number 21K05845. The authors thank Dr. Daisuke Nei and Dr. Hiromi Kameya (Institute of Food Research, NARO, Japan) for their technical inputs and help with the ESR measurements. AGK acknowledges the financial support from LPDP Indonesia Endowment Fund for Education (S-1254/LPDP.4/2022) for her doctoral study.

6. References

[1] S. O. Aiyedun, B. A. Onarinde, M. Swainson, R. A. Dizon, Foodborne outbreaks of microbial infection from fresh produce in Europe and North America: a systematic review of data from this millennium. *International Journal of Food Science and Technology*, *56*(5), https://doi.org/10.1111/ijfs.14884 (2021).

[2] R. Zhou, R. Zhou, P. Wang, Y. Xian, A. Mai-Prochnow, X. Lu, … K. Bazaka, Plasma-activated water: generation, origin of reactive species and biological applications, *Journal of Physics D: Applied Physics*, *53*(30), 303001. https://doi.org/10.1088/1361-6463/ab81cf (2020).

[3] Y. Q. Chen, J. H. Cheng, D. W. Sun, Chemical, physical and physiological quality attributes of fruit and vegetables induced by cold plasma treatment: Mechanisms and application advances, *Critical Reviews in Food Science and Nutrition*, *60*(16), 2676–2690 (2020).

[4] S. Aparajhitha, R. Mahendran, Effect of plasma bubbling on free radical production and its subsequent effect on the microbial and physicochemical properties of Coconut Neera, *Innovative Food Science & Emerging Technologies*, *58*, 1-9, (2019).

[5] R. Lokeswari, R. Mahendran, Effect of Plasma Bubbling on Textural and Engineering Properties of Ready-to-Eat Pearl Millet Flakes and Puffs, *IEEE Transactions on Plasma Science*, 1–7, (2022).

[6] Y. Suenaga, H. Kawano, T. Takamatsu, Y. Matsumura, N. Ito, A. Iwasawa, A. Okino, Ultrasonic-Combined Plasma Bubbling for Adherent Bacteria Disinfection on Medical Equipment, *Plasma Chemistry and Plasma Processing*, *42*, 572-586, (2022).

[7] M. Laroussi, Low temperature plasma-based sterilization: Overview and state-of-the-art, *Plasma Processes and Polymers*, *2*(5), 391–400, (2005).

[8] P. Lukes, J. Brisset, B. R. Locke, Biological Effects of Electrical Discharge Plasma in Water and in Gas–Liquid Environments. In *Plasma Chemistry and Catalysis in Gases and Liquids* (pp. 309–352), (2012).

[9] T. Takamatsu, K. Uehara, Y. Sasaki, M. Hidekazu, Y. Matsumura, A. Iwasa, … A. Okino, Microbial inactivation in the liquid phase induced by multigas plasma jet. *PLoS ONE*, *10*(7), 1–9 (2015).

[10] M. Kohno, Applications of electron spin resonance spectrometry for reactive oxygen species and reactive nitrogen species research, *Journal of Clinical Biochemistry and Nutrition*, Vol. 47, pp. 1–11, (2010).

[11] M. Kamibayashi, S. Oowada, H. Kameda, T. Okada, O. Inanami, S. Ohta, … Y. Kotake, Synthesis and characterization of a practically better DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO), *Free Radical Research*, *40*(11), 1166–1172, (2006).

[12] S. Oowada, N. Endo, H. Kameya, M. Shimmei, Y. Kotake, *Scavenging Capacity in Serum*. *51*(2), 117–121., (2012).

[13] O. I. Fromov, M. O. Kostenko, A. V. Petrunin, A. A. Popova, O. O. Parenago, N. V. Minaev, … M. Y. Melnikov, Solute diffusion into polymer swollen by supercritical co2 by high-pressure electron paramagnetic resonance spectroscopy and chromatography, *Polymers*, *13*(18), 1-16, (2021).

[14] T. Takamatsu, A. Kawate, K. Uehara, T. Oshita, H. Miyahara, D. Dobrynin, … A. Okino, Bacterial inactivation in liquids using multi-gas plasmas, *Plasma Medicine*, *2*(4), 237–248, (2012).

[15] H. Xu, R. Ma, Y. Zhu, M. Du, H. Zhang, Z. Jiao, A systematic study of the antimicrobial mechanisms of cold atmospheric-pressure plasma for water disinfection, *Science of the Total Environment*, *703*, 134965, (2020).

[16] H. Wu, P. Sun, H. Feng, H. Zhou, R. Wang, Y. Liang, … J. Fang, Reactive oxygen species in a non-thermal plasma microjet and water system: Generation, conversion, and contributions to bacteria inactivation -An analysis by electron spin resonance spectroscopy, *Plasma Processes and Polymers*, *9*(4), 417–424, (2012).

[17] N. N. Misra, O. Schlüter, P. J. Cullen, Plasma in Food and Agriculture, In *Cold Plasma in Food and Agriculture* (pp. 1–16), (2016).