A Dissertation for the Degree of Master

Antimicrobial effects and mechanisms of plasma-activated acetic acid against *Salmonella* Typhimurium and its application for chicken meat

Salmonella Typhimurium에 대한 플라즈마 활성 초산의 항균 효능 및 메커니즘과 계육의 적용

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Abstract

Antimicrobial effects and mechanisms of plasma-activated acetic acid against *Salmonella* Typhimurium and its application for chicken meat

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We conducted research on plasma-activated acetic acid (PAAA) to improve the sterilization efficiency of each individual treatment of plasma-activated water (PAW) and acetic acid (AA) and apply it to poultry meats. PAAA was prepared by addition of different concentrations of AA into deionized water before plasma treatment. The aim of this study was to investigate growth-inhibitory activities of PAAA against *Salmonella* Typhimurium cells and biofilm and the underlying mechanisms, as well as identify the effect of PAAA on reduction of *S.* Typhimurium and quality traits of chicken meats.

Experiment I.

Growth-inhibitory activities of plasma-activated acetic acid against *Salmonella* Typhimurium cells and biofilm and the underlying mechanisms

This study aimed to examine the growth-inhibitory activities of plasma-activated acetic acid (PAAA) against *Salmonella* Typhimurium cells and biofilm and elucidate the underlying mechanism. PAAA was prepared by discharging plasma to 20 mL of 0.2% (v/v) acetic acid (AA) for 20 min (2.2 kHz and 8.4 kVpp) after the optimization test. The cells and biofilms on stainless steel were incubated with AA, and PAAA. The count of *S*. Typhimurium cells decreased by 5.71 log CFU/mL after 10 min of incubation with 0.2% PAAA compared with control (without any treatment). The *S*. Typhimurium count in the biofilms decreased by 4 log CFU/cm^2 upon treatment with 0.4% PAAA for 10 min when compared with the initial *S*. Typhimurium count in the biofilms (control) ($p < 0.05$). Confocal laser scanning microscopy (CLSM) revealed that 0.4% PAAA synergistically decreased the viability of *S*. Typhimurium in the biofilms. In in 0.2% PAAA, the concentrations of H_2O_2 and NO_3^- were directly proportional to the plasma discharge time, while NO_2^- was not detected. However, the pH values of both 0.2% PAAA and plasma-activated water (PAW) were inversely proportional to the plasma discharge time. Additionally, treatment with catalase, L-histidine, D-mannitol, and sodium azide inhibited the antibacterial activity of PAAA, which indicated that H_2O_2, $^1O_2$, ·OH, and NO_2^- are involved in the generation and decomposition of peroxynitrous acid (ONOOH). Therefore, ONOOH, which functions as an intermediate under acidic conditions, plays a key role in the bactericidal effects of PAAA. This study demonstrated that

PAAA has potential applications as a decontaminant in the food industry.

Experiment II.

Effect of plasma-activated acetic acid on reduction of *Salmonella* Typhimurium and quality traits of chicken meats

This study investigated the bactericidal effects of plasma-activated acetic acid (PAAA) on reduction of *Salmonella* Typhimurium and its impact on the physicochemical traits of chicken meats. Twenty mL of 0.8% (v/v) acetic acid (AA) was treated with plasma (2.2 kHz and 8.4 kVpp) for 30 min. The chicken skins, breasts, and drumsticks, inoculated with *S*. Typhimurium, were immersed in AA or PAAA and incubated for 10 min. The *S*. Typhimurium on the breasts and drumsticks were significantly susceptible to treatment with AA and PAAA, compared to the control (deionized water treatment), and the population of bacterial cells in PAAA-treated chicken breasts and drumsticks decreased by 0.98 and 1.19 log CFU/g, respectively, compared with AA. The values for pH and 2-thiobarbituric acid reactive substances (TBARS) of PAAA-treated samples decreased significantly compared to the control. The lightness (L^*) values of the chicken breasts after AA and PAAA treatments increased compared to the control, whereas the value for yellowness (b^*) decreased. The scanning electron microscopic images and the results for volatile compounds in chicken meat revealed similar patterns, with no significant differences between AA and PAAA treatments. Thus, the bactericidal effects and the potential industrial applications of PAAA were confirmed.

Keywords: Plasma-activated acetic acid; *Salmonella* Typhimurium; Antibacterial effect; Antibiofilm effect; Peroxynitrous acid; Chicken meats

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List of Abbreviations

·OH	:	Hydroxyl radical
$^1O_2$	:	Singlet oxygen
a^*	:	Redness
AA	:	Acetic acid
b^*	:	Yellowness
CFU	:	Colony-forming unit
CLSM	:	Confocal laser scanning microscopy
FDA	:	Food and Drug Administration
h	:	Hour
H_2DCFDA	:	2,7 –dichlorodihydrofluorescein diacetate
H_2O_2	:	Hydrogen peroxide
L^*	:	Lightness
min	:	Minutes
NO	:	Nitric oxide
NO_2	:	Nitrogen dioxide
NO_2^-	:	Nitrite
NO_3^-	:	Nitrate
$O_2\cdot^-$	:	Superoxide anion radical
O_3	:	Ozone
ONOOH	:	Peroxynitrous acid

PAAA	:	Plasma-activated acetic acid
PALA	:	Plasma-activated lactic acid
PAW	:	Plasma-activated water
PI	:	Propidium iodide
PTFE	:	polytetrafluoroethylene
RONS	:	Reactive oxygen and nitrogen species
ROS	:	Reactive oxygen species
s	:	Second
Saline	:	0.85% NaCl solutions
SEM	:	Scanning electron microscopy
TBARS	:	2-thiobarbituric acid reactive substances
XLD	:	Xylose lysine deoxycholate agar

Chapter I.

General introduction

1.1. Food safety

As the food industry is growing and food consumption is increasing, consumers are paying more attention to fresher, safer, higher quality foods (Ji et al., 2018). Thus, consumer's concerns of food-contamination also have increased. With regard to food safety, the Food and Drug Administration (FDA) reported that the incidence of food-borne pathogens have continued to increase over the years, causing the increases in outbreaks of food-borne illness throughout the world (FDA, 2008). *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* are general food-borne pathogens that cause severe illness in food industry (Reij et al., 2004). Above all, outbreaks of *Salmonella* infection are generally linked to chicken meat and eggs. (Wang et al., 2011). Common presenting symptoms of *Salmonella* in human include diarrhea, fever, chills, nausea and vomiting (Costerton et al., 1999). Hence, microbiological safety of the chicken meat has been a challenge for the poultry industry due to the contamination of *Salmonella*.

1.2. *Salmonella* Typhimurium

Salmonella Typhimurium, as the one of the most important food-borne

pathogens, is the most commonly isolated serotype of *Salmonella* spp. For this reason, it often causes the problems in animal-based foods, such as beef, pork and poultry meats (Ferrari et al., 2019). *S.* Typhimurium is able to produce biofilms which is defined as the assemblage of microbial cells (Merino et al., 2019). Thus, it is essential to develop a promising technology for decontamination of *S.* Typhimurium on chicken meats.

1.3. Antimicrobial agents

Antimicrobial agents have been developed and applied for many years in order to control spoilage and pathogenic microorganisms (Xu et al., 2016). Over the past few years, there were numerous efforts to inactivate microbial contaminants by utilizing various substances (Baek et al., 2020). The efficacy of chlorine-containing sanitizer was conducted to inactivate bacteria and the efficiency of slightly acidic electrolyzed water at different temperatures was also investigated for microbial inactivation on the surface of shell eggs (Cao et al., 2009; Chen et al., 2017). Actually, chlorine solution has widely been utilized to reduce the bacteria in chicken meat (Royintarat et al., 2020). In addition, various technologies including UV-C light, ultrasound with a chemical immersion and electrolyzed water, as a potential decontaminant of food-borne pathogenic bacteria, have already been studied for chicken products (Yang et al., 2017; Royintarat et al., 2020; Rahman et al., 2012). However, some technologies including the chlorine-based solutions still have some limitations with low efficiency, chemical residues and high cost of application

(Rahman et al., 2012).

1.4. Plasma technology

Recently, plasma has gained increasing attention as a novel technology for microbial inactivation among the non-thermal technology. In particular, plasma-activated water (PAW) could be produced by the treatment of non-thermal plasma for microbial decontamination against food-borne pathogens (Baek et al., 2020; Thirumdas et al., 2018). The antibacterial activity of the PAW is contributed by the presence of reactive chemical species, such as H_2O_2, ·OH, O_3, NO_2^- and NO_3^-, under acidic condition (Lu et al., 2017). The pH of PAW decreases with an increasing plasma discharge time due to the dissolution of nitrogen oxides, resulting that the H_2O_2, NO_2^-, NO_3^- is subsequently formed *via* various reactions (Zhou et al., 2018). In order to improve the antibacterial effect of PAW, various solvents were added into the water before plasma treatment. There were some studies for stronger bactericidal ability by adding 0.9% saline solution (Su et al., 2018), H_2O_2 solution (Wu et al., 2017) and phosphate buffer solution (Laurita et al., 2015). However, some solvents still have some problems because the efficiency and antibacterial activity of these methods are still low and limited, respectively (Oehmigen et al., 2011; Tian et al., 2015).

1.5. Plasma-activated acetic acid (PAAA)

Organic acids, as common preservative for decontamination of the chicken meats, were also used in combination treatments with other methods (Yuk et al., 2006; Lang et al., 2000; Park et al., 2013). Among the organic acids, several countries have studied and mainly used the lactic acid, citric acid and acetic acid for hygienic meat production (Kang et al., 2002; Seol et al., 2012). Based on this, some studies that the synergistic bactericidal effects of lactic acid with plasma treatment have already conducted by adding lactic acid into the water before plasma treatment (Qian et al., 2020; Qian et al., 2021). So far, no study on the synergistic bactericidal effects using plasma-activated acetic acid (PAAA) against chicken is reported. Here, we prepared PAAA by addition of different concentrations of acetic acid (AA) into deionized water before plasma treatment to enhance the sterilization efficiency of PAW and apply to chicken. The objective of this study was to investigate growth-inhibitory activities of PAAA against *S.* Typhimurium cells and biofilm and the underlying mechanisms, as well as investigated the effect of PAAA on reduction of *S.* Typhimurium and quality traits of chicken meats.

Chapter II.

Growth-inhibitory activities of plasma-activated acetic acid against *Salmonella* Typhimurium cells and biofilm and the underlying mechanisms

This manuscript consists of part of a paper submitted to Food Control as partial fulfillment of the Master's program of Taemin Kang.

2.1. Introduction

In the last two decades, consumer awareness of food safety has increased (Nerin et al., 2016). Food-borne diseases are typically caused by pathogenic bacteria, such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*, which must be controlled to avoid the outbreak of food-borne illnesses (Reij et al., 2004). In particular, the consumption of food contaminated with *S*. Typhimurium, a major food-borne pathogen that adversely affects human health, causes diarrhea, fever, chills, nausea, and vomiting. Additionally, *S*. Typhimurium can form biofilms in a hydrated extracellular polymeric matrix (Costerton et al., 1999). In the biofilm, the

microbial cells are attached to a substratum (Giaouris et al., 2014). The formation of biofilms on food surfaces and processing equipment results in cross-contamination and the spread of food-borne pathogens (Kim et al., 2012). Thus, the presence of *Salmonella* spp. biofilms can lead to food contamination.

In the food industry, disinfectants are used to control food-borne pathogens (Greene et al., 1993). Previous studies have reported the bactericidal and biofilm-inhibitory properties of agents, such as chlorine-containing chemicals, electrolyzed water, organic acids, and ozone generators (Kreske et al., 2006; Cao et al., 2009; Lee et al., 2010; Fan et al., 2020). PAW, an effective antibacterial agent, is prepared by treating water with non-thermal plasma discharge above or beneath the water surface (Kim et al., 2018; Ma et al., 2015; Porto et al., 2018). The advantages of PAW include easy preparation, safety, cost-effectiveness, and enhanced bactericidal properties (Qian et al., 2020). The antimicrobial properties of PAW can be attributed to the generation of reactive oxygen and nitrogen species (RONS) whose production is upregulated under acidic conditions (Jung et al., 2017; Kovačević et al., 2016; Xu et al., 2016). Additionally, the generation of peroxynitrite in PAW contributes to the antimicrobial properties of PAW (An et al., 2019; Lukes et al., 2014).

Several solvents are added to the water to enhance the antibacterial effect of PAW (Xu et al., 2016). Recently, Baek et al. (2021) enhanced the growth-inhibitory effect of PAW against *Staphylococcus aureus* through pretreatment with blue light. However, some solvents are associated with several disadvantages, such as low efficiency and limited antibacterial activity (Qian et al., 2020; Xu et al., 2016). In this study, PAAA was prepared by supplementing different concentrations of AA to deionized water to enhance the sterilization efficiency of PAW. Previous studies have

reported the antibacterial effect of plasma-activated lactic acid (PALA) (Qian et al., 2019; Qian et al., 2020). However, the growth-inhibitory effects of PAAA against *S.* Typhimurium viable cells and biofilm and the underlying mechanisms have not been reported previously. Therefore, this study aimed to examine the antibacterial and antibiofilm efficacies of PAAA and elucidate the underlying mechanisms to enable its industrial application.

2.2. Materials and methods

2.2.1. Bacterial strains and culture preparation

S. Typhimurium (ATCC 13311), which was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea), was cultured in nutrient broth (Difco, Becton Dickinson Co., Sparks, MD, USA) at 37°C for 48 h. The cultures were transferred to a 50-mL tube and centrifuged at 2,266 ×*g* and 2°C for 14 min (UNION 32R, Hanil Science Industrial, Co. Ltd, Gimpo, Korea). The pellets were washed twice with sterile 0.85% NaCl solution (saline) and the cells were resuspended in saline solution to a final concentration of approximately 10^8–10^9 colony-forming units (CFU)/mL.

2.2.2. Preparation of biofilm cells

Stainless steel coupons (20 × 20 × 10 mm^3) were used for the preparation of biofilms (An et al., 2019). The coupons were immersed in 70% ethanol for 10 min, rinsed with sterile deionized, and autoclaved at 121°C for 15 min as described previously (Ban et al., 2016). The sterile coupons were transferred to a Petri dish (60 × 15 mm) using sterile forceps. The cell suspension (4 mL; 10^8–10^9 CFU/mL) was transferred to the Petri dish containing the coupons. The samples were incubated at 4°C for 24 h to allow the cells to attach to the coupons. The coupons were aseptically removed from the Petri dish and gently agitated in 500 mL of sterile deionized water for 5 s (Kim et al., 2006). Next, the coupons were transferred to Petri dishes containing 4 mL of nutrient broth and incubated at room temperature (25°C) for 5

days without the replacement of broth.

2.2.3. Preparation and treatment of PAAA

An encapsulated atmospheric pressure dielectric barrier discharge plasma was used to prepare PAAA. The device was constructed using a rectangular plastic container (137 × 104 × 53 mm) containing copper electrodes with a polytetrafluoroethylene (PTFE) sheet attached to the inner walls (Figure 1). To prepare PAAA, 300 mL of deionized water was supplemented with 0.6, 1.2, 3.0, or 6.0 mL of 100% AA to obtain a final AA concentration of 0.2, 0.4, 1.0, or 2.0%, respectively. A glass dish containing AA was placed at the center of the container and exposed to plasma generated inside the container. Finally, 20 mL of AA was exposed to plasma discharge for 5, 10, 15, and 20 min. Various concentrations of PAAA (0.2%, 0.4%, 1.0%, and 2.0%) represent the AA concentrations in PAAA. Atmospheric air was used as a carrier gas and plasma discharge conditions were 2.2 kHz and 8.4 kVpp, which were the previously reported modified conditions (Yoo et al., 2021). The microbial and biofilm cells without any treatment were prepared as a control.

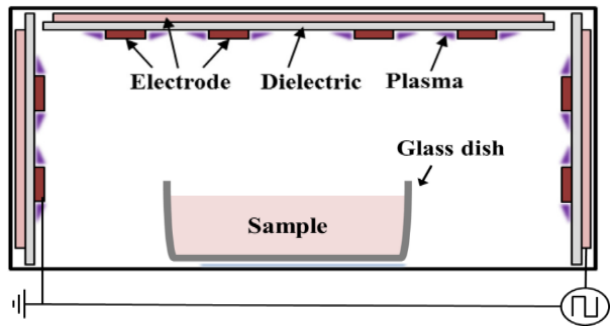

Figure 1. Schematic diagram of the experimental setup for the preparation of dielectric barrier discharge plasma.

2.2.4. Growth-inhibitory activity of PAAA against S. Typhimurium

AA or PAAA (9.9 mL) was added to a sterile 50-mL conical tube containing 0.1 mL bacterial cells (approximately 8–9 log CFU/mL). The suspension was mixed thoroughly by vortexing for 5 s and incubated at room temperature (25°C) for different time (Baek et al., 2020; Xiang et al., 2018). The cell suspension (100 μL) was collected at the indicated time points, serially diluted 10-fold, plated onto nutrient agar plates, and incubated at 37°C for 24 h. The bacterial colonies were counted, and the results were presented as log CFU/mL.

2.2.5. Chemical characterization of PAAA

The concentrations of hydrogen peroxide (H_2O_2), nitrate anions (NO_3^-), and nitrite anions (NO_2^-) in 0.2% PAAA prepared after exposure to plasma discharge for various time and PAW prepared after exposure to plasma discharge for 20 min were measured. The H_2O_2 concentrations were measured using the titanium oxysulfate assay with a TiOSO4 reagent. This assay is based on the reaction of H_2O_2 with titanium (IV) ions under acidic conditions. Additionally, sodium azide was added to prevent the decomposition of H_2O_2 under acidic conditions. The concentrations of nitrites and nitrates were measured using an ion chromatography system (ICS-3000, Dionex Co. Ltd., Sunnyvale, CA, USA). The pH values were measured using a pH meter (Seven 2Go, Mettler-Toledo International Inc., Schwerzenbach, Switzerland). The ONOOH in 0.2% PAAA was measured using fluorescence spectroscopy with the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Kooy et

al., 1997). The fluorescence intensity of the dye was measured using a fluorescence spectrometer (SpectraMax M2e; Molecular Devices, San Jose, CA, USA) with peak excitation and emission wavelengths of 495 and 521 nm, respectively (Tarabová et al., 2019).

2.2.6. Treatment with reactive oxygen and nitrogen species (RONS) scavengers

The RONS scavengers tiron ($O_2{\cdot}^-$ scavenger), catalase (H_2O_2 scavenger), L-histidine ($^1O_2$ scavenger), D-mannitol (·OH scavenger), and sodium azide (NO_2^- scavenger) were used in this study. Tiron (20 mM), catalase (1000 U/mL), L-histidine (150 mM), D-mannitol (150 mM), and sodium azide (10 mM) were added before or after plasma discharge based on the final concentration of the solution containing 0.2% AA or 0.2% PAAA obtained after exposure to plasma discharge for 20 min. Next, the solution was incubated with bacterial cells for 10 min after vortexing for 5 s as described above (Aboubakr et al., 2016; Guo et al., 2018).

2.2.7. Measurement of cell membrane integrity

The viability of biofilm cells was determined using the BacLight™ Live/Dead bacterial viability kit (L-7012; Molecular Probes, Eugene, OR, USA). The dye mixture (300 μL) containing propidium iodide (PI; red fluorescence), SYTO9 (green fluorescence), and sterile deionized was dripped on the surface of the coupons with biofilm. The red fluorescence of PI indicates bacterial membrane damage, whereas

the green fluorescence of SYTO9 indicates an intact bacterial membrane (Baek et al., 2020). The coupons were incubated for 20 min at room temperature (25°C) in the dark. Each coupon was dropped onto a glass slide (Paul Marienfeld GmbH & Co. KG, Laud-konigshofen, Germany) and covered with a cover glass (Marienfled Superior). The stained biofilm was examined under a CLSM (Leica TCS SP8X, Wetzlar, Germany) using appropriate filters with excitation/emission wavelengths of 483/490–540 nm for SYTO9 and 535/590–680 nm for PI.

2.2.8. Statistical analysis

All experiments were independently performed in triplicates. The data were analyzed using one-way analysis of variance, followed by Tukey's multiple range test and Student's *t*-test. The differences were considered significant at $p < 0.05$. All statistical analyses were performed using the SAS software program (version 9.4, SAS Institute Inc., Cary, NC, USA).

2.3. Results and discussion

2.3.1. Growth-inhibitory activity of PAAA against S. Typhimurium

The growth-inhibitory activities of PAAA against *S.* Typhimurium under different treatment conditions are shown in Figure 2. As shown in Figure 2A, the plasma discharge time significantly affected the *S.* Typhimurium inactivation efficiency of PAAA ($p < 0.05$). The initial number of bacterial cells was 5.71 log CFU/mL. The viability of bacterial cells was inversely proportional to the plasma discharge time. Additionally, the incubation time significantly affected the *S.* Typhimurium inactivation efficiency of PAAA ($p < 0.05$).

The viability of bacterial cells was inversely proportional to the incubation time (Figure 2B). The bacterial cells were not detected after incubation with 0.2% PAAA (obtained after a plasma discharge time of 20 min) for 10 min. These results were consistent with those of Xiang et al. (2018) who demonstrated that the incubation time and plasma discharge time affected the antibacterial effect of PAW.

Figure 2C shows the inactivation patterns of various concentrations of AA (0.2%−2%) after incubated for 10 min. The viability was not significantly different between control and 0.2% AA treatment groups. However, the bacterial count decreased by 1.40 and 5.84 log CFU/mL in the 1% and 2% AA treatment groups, respectively, when compared with the initial bacterial count ($p < 0.05$).

Figure 2D shows the antibacterial activity of PAW and 0.1%−0.2% PAAA (obtained after plasma discharge for 20 min) after 10 min of incubation. The viable population of bacterial cells decreased by 1.43, 1.45, 2.97, and 5.84 log CFU/mL in

the PAW, 0.1% PAAA, 0.15% PAAA, and 0.2% PAAA treatment groups, respectively ($p < 0.05$). AA at a concentration of 0.2% did not exhibit antibacterial activity (Figure 2C). However, the percentage viability of bacterial cells in the 0.2% PAAA treatment group was 0%. These findings are consistent with those of Qian et al. (2020) who reported the viable count of *Salmonella* Enteritidis decreased with the increase in both treatment time and lactic acid concentration. Similarly, Qian et al. (2019) reported that PALA (0.2%) exhibited the highest antibacterial activity. The antibacterial activity of PAAA with lower concentrations of AA significantly decreased. In this study, 0.2% PAAA exhibited the most potent growth-inhibitory activity against *S*. Typhimurium.

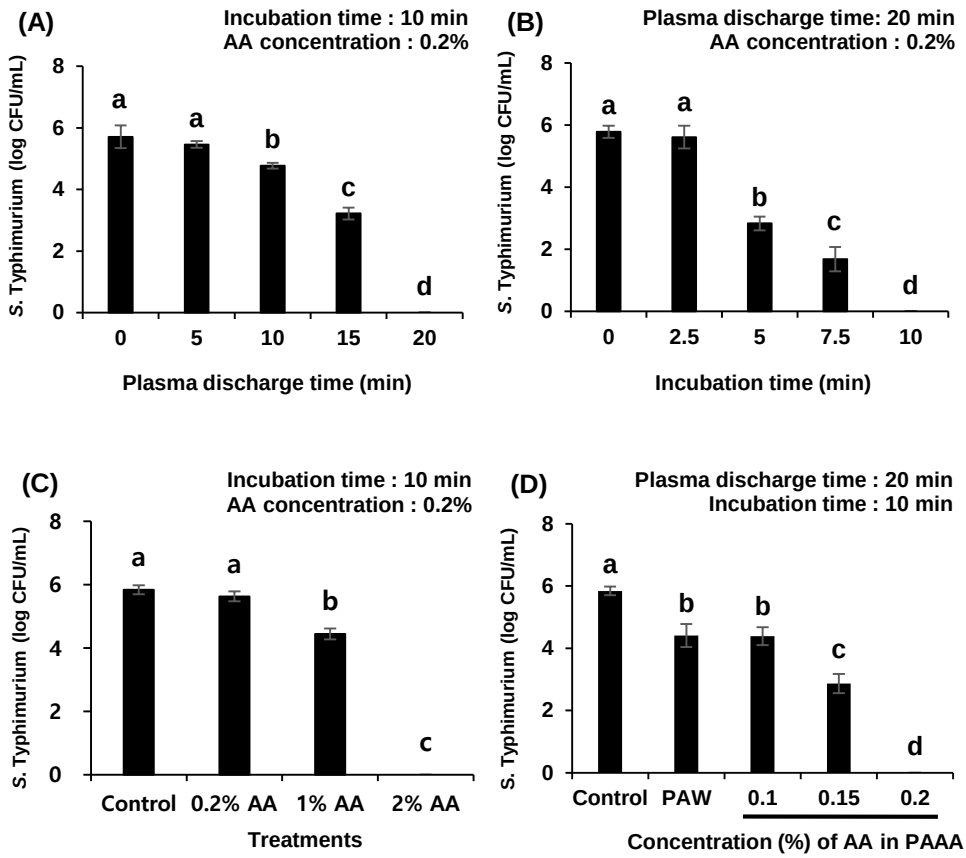

Figure 2. Effect of acetic acid (AA) and plasma-activated acetic acid (PAAA) on the viability of bacterial cells according to (A) plasma discharge time, (B) incubation time, (C) concentrations of AA and (D) concentrations of AA in PAAA. Control group, without ant treatment; AA, AA treatment; PAW, plasma-activated water treatment; PAAA, PAAA treatment. Error bars represent standard deviation. $^{a\text{-}d}$Different letters indicate a significant difference ($p < 0.05$) among the treatments.

2.3.2. Antibiofilm activity of PAAA

To determine the potential applications of PAAA as a disinfectant in the industry, the growth-inhibitory activity of PAAA against *S*. Typhimurium biofilms formed on stainless steel surfaces was examined. Figure 3A shows the viability of biofilm cells after treatment with various concentrations of AA (0.2%–0.4%) or deionized water for 10 min. The initial number of cells in the biofilm (control) was 8.15 log CFU/cm^2. The number of cells in the biofilm of the deionized water, 0.2% AA, and 0.4% AA treatment groups was slightly lower than that in the biofilm of the control ($p < 0.05$). The antibiofilm effects of PAW, 0.2% PAAA, and 0.4% PAAA are shown in Figure 3B. The number of cells in the biofilm of the 0.2% PAAA treatment group decreased by 1.64 log CFU/cm^2 when compared with that in the biofilm of the control. The 0.2% PAAA and PAW treatment groups exhibited a similar number of cells in the biofilm (Figure 3B). However, the number of cells in the biofilm significantly decreased by 4 log CFU/cm^2 upon treatment with 0.4% PAAA ($p < 0.05$). This suggests that PAAA induced cell detachment (Khan et al., 2016). In this study, treatment with 0.4% PAAA optimally decreased the cell count in the *S*. Typhimurium biofilm.

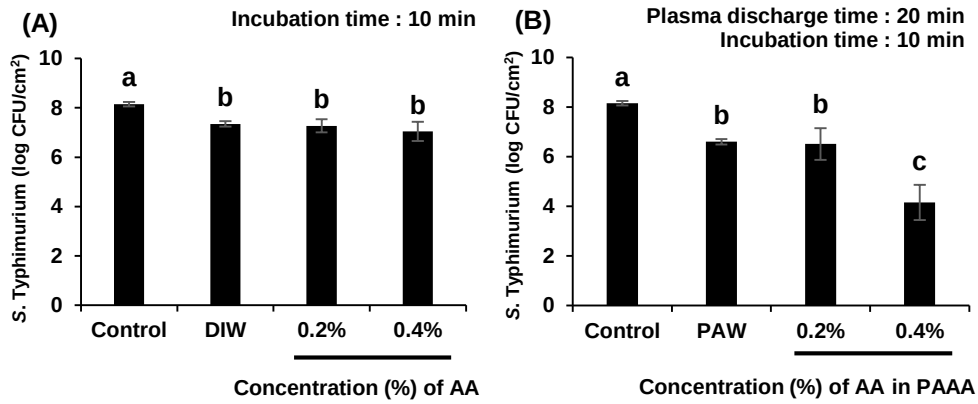

Figure 3. Effect of acetic acid (AA) and plasma-activated acetic acid (PAAA) on the viability of *Salmonella* Typhimurium cells in the biofilm according to (A) different concentrations of AA and (B) different concentrations of AA in PAAA. Control group, without ant treatment; DIW, deionized water treatment; PAW, plasma-activated water treatment; AA, acetic acid treatment; PAAA, plasma-activated acetic acid treatment. Error bars represent standard deviation. $^{a\text{-}e}$Different letters indicate a significant difference ($p < 0.05$) among the treatments.

2.3.3. Confocal laser scanning microscopy (CLSM)

The membrane integrity of cells in the *S*. Typhimurium biofilm was examined using CLSM after staining with SYTO9 and PI. The result of control and the effects of 0.4% AA treatment for 10 min on the membrane integrity are shown in Figures. 4A and 4B, respectively. Green fluorescence was detected in both groups, which indicated the presence of live cells. However, only some red fluorescence signals were detected (Figure 4B). Figure 4C presents the effect of 0.4% PAAA treatment for 10 min on the cells in the biofilm. The red fluorescence intensity in the 0.4% PAAA treatment group was higher than that in the control and 0.4% AA treatment groups. The 0.4% AA treatment group mostly exhibited green fluorescence signals with some red fluorescence signals (Figure 4B). However, 0.4% PAAA damaged most of the cells. An et al. (2019) examined the growth-inhibitory activity of sodium hypochlorite solution and plasma combination against the microbial biofilm based on membrane integrity using CLSM. The uptake of PI after plasma treatment indicates the formation of pores in the bacterial membrane, which increases the bacterial membrane permeability to PI (Dolezalova et al., 2015). Thus, the *S*. Typhimurium biofilm inactivation efficiency of 0.4% PAAA was higher than that of 0.4% AA.

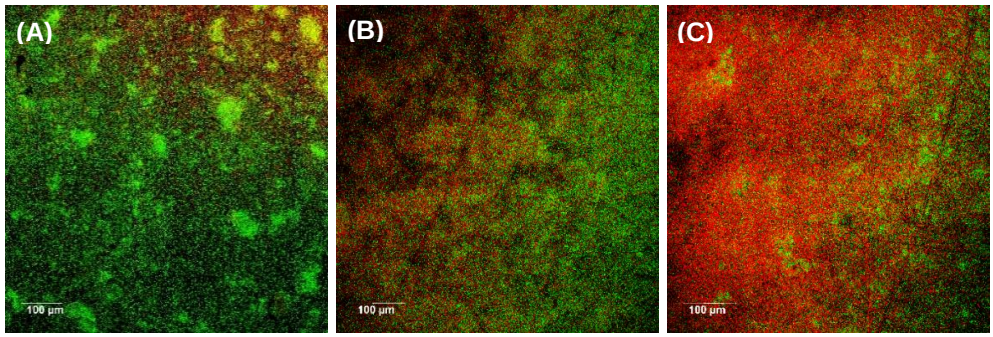

Figure 4. Evaluation of membrane integrity of cells in the *S.* Typhimurium biofilm using confocal laser scanning microscopy. (A) Control group cells; (B) 0.4% acetic acid (for 10 min)-treated cells; (C) cells treated with 0.4% plasma-activated acetic acid (PAAA; obtained after plasma discharge for 20 min) for 10 min. Control group, without ant treatment; AA, 0.4% acetic acid treatment; PAAA, 0.4% PAAA treatment. The fluorescence of red, damage of bacterial membrane; The fluorescence of green, intactness of bacterial membrane

2.3.4. Chemical characterization of PAAA

To further investigate the antimicrobial mechanisms of PAAA, the concentrations of H_2O_2, NO_2^-, and NO_3^- were measured. Reactive oxygen species (ROS) with a long half-life, such as H_2O_2, NO_2^-, and NO_3^- may be the major antimicrobial agents and mediate a series of complex chemical reactions in PAW (Bruggeman et al., 2016; Samukawa et al., 2012). This study considered H_2O_2 as an indicator of ROS formation as it is stable with a long half-life in aqueous conditions (Baek et al., 2020). Table 1 presents the concentrations of H_2O_2, NO_2^-, and NO_3^- in 0.2% PAAA produced after exposure to plasma discharge for various time and PAW prepared after plasma discharge for 20 min. The concentrations of H_2O_2 and NO_3^- increased significantly in 0.2% PAAA with the increase in plasma discharge time ($p < 0.05$) but NO_2^- was not detected. During plasma discharge, H_2O_2 is produced from ·OH radicals through reaction (1) (Lukes et al., 2014).

$$\bullet OH + \bullet OH \rightarrow H_2O_2 \quad (1)$$

Under acidic conditions, NO_2^- is converted to a relatively stable NO_3^- through reaction (2) and undergoes decomposition into nitric oxide (NO·) and nitrogen dioxide (NO_2·), which are acidified nitrites generated through reactions (3), (4), and (5) (Babaeva et al., 2012; Oehmigen et al., 2011; Park et al., 2015). As shown in Table 2, the pH value of 0.2% PAAA was less than pKa = 3.29 ($HNO_2 \leftrightarrow NO_2^- + H^+$) even before plasma discharge (Ryu et al., 2009). Additionally, NO_2^- contributes

to the formation of ONOOH, which is highly cytotoxic under acidic conditions (Lukes et al., 2014). This explains the reason for the non-detection of NO_2^- and the increased concentrations of NO_3^- and H_2O_2 with the increase in plasma discharge time.

$$3NO_2^- + 3H^+ + H_2O \rightarrow 2NO + NO_3^- + H_3O \quad (2)$$

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (3)$$

$$HNO_2 + H^+ \rightarrow NO^+ + H_2O \quad (4)$$

$$2HNO_2 \rightarrow NO\cdot + NO_2\cdot + H_2O \quad (5)$$

The acidity of 0.2% PAAA obtained after plasma discharge for 20 min was more than that of PAW (Table 2). Thus, the concentrations of H_2O_2 and NO_3^- in 0.2% PAAA were lower than those in PAW exposed for the same plasma discharge time (20 min) although 0.2% PAAA exhibited potent antibacterial activity. Oehmigen et al. (2010) suggested that the activity of reactive species is potentiated under acidic conditions, which leads to enhanced antibacterial activity. During plasma discharge, H_2O_2 is utilized for the NO_2^--dependent generation of ONOOH under acidic conditions through reaction (6) (Laurita et al., 2015; Lukes et al., 2014). The concentration of H_2O_2 in 0.2% PAAA obtained after plasma discharge for 20 min was lower than that of PAW. Thus, the results presented in Table 2 support the results of antibacterial activity presented in Figure 2D. The enhanced concentration of NO_3^- may partially contribute to low pH values of PAW (Jung et al., 2017), which is consistent with the results of this study. However, Qian et al. (2019) reported that

NO_3^- did not play a key role in the bactericidal effect of PALA. The pH values of 0.2% PAAA and PAW were inversely proportional to the plasma discharge time ($p < 0.05$), which is due to the dissolution of nitrogen oxides through reactions (7) and (8) (Table 2) (Zhou et al., 2018). Several studies have demonstrated that the pH value alone may not play a key role in the antibacterial activity of PAW. However, the pH value affects the activity of reactive species in plasma-activated liquid (Oehmigen et al., 2010; Qian et al., 2019).

$$NO_2^- + H_2O_2 + H^+ \rightarrow O{=}NOOH + H_2O \quad (6)$$

$$NO_2 + NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (7)$$

$$NO + NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (8)$$

Table 1. Chemical composition of 0.2% plasma-activated acetic acid (PAAA) according to plasma discharge time and plasma-activated water (PAW)

Plasma discharge time (min)	H_2O_2 (μM)	NO_3^- (mg/L)	NO_2^- (mg/L)
0	12.44 ± 2.78^{d}	ND	ND
5	33.56 ± 5.48^{d}	2.35 ± 0.54^{d}	ND
10	83.00 ± 7.62^{c}	7.99 ± 1.79^{cd}	ND
15	98.22 ± 14.61^{c}	18.60 ± 6.84^{bc}	ND
20	126.78 ± 13.18^{b}	27.70 ± 4.19^{ab}	ND
PAW*	195.00 ± 5.24^{a}	35.02 ± 4.19^{a}	ND

PAW*, PAW obtained after plasma discharge for 20 min.

ND, not detected (the detection limit for analysis of nitrites and nitrates was 1 mg/L).

The results are expressed as the mean ± standard deviation (n=3).

$^{a-d}$Different letters within the same column differ significantly ($P < 0.05$).

Table 2. The pH value of 0.2% plasma-activated acetic acid (PAAA) and plasma-activated water (PAW) according to plasma discharge time

Plasma discharge time (min)	pH value	
	0.2% PAAA	PAW
0	3.08 ± 0.01[a]	5.68 ± 0.02[a]
5	3.07 ± 0.01[a]	3.80 ± 0.02[b]
10	3.03 ± 0.01[a]	3.48 ± 0.03[c]
15	2.97 ± 0.02[b]	3.27 ± 0.03[d]
20	2.92 ± 0.03[c]	3.06 ± 0.04[e]

The results are expressed as mean ± standard deviation (n=3).

[a-e]Different letters within the same column differ significantly ($P < 0.05$).

2.3.5. Effect of RONS scavengers on the antibacterial effect of PAAA

RONS scavengers were used to identify the major reactive species involved in the antibacterial activity of 0.2% PAAA. As shown in Figure 5A, the antibacterial activity of 0.2% PAAA was inhibited upon treatment with RONS scavengers, except tiron ($O_2{\cdot}^-$scavenger). This indicated that H_2O_2, $^1O_2$, ·OH, and NO_2^- mediated the antibacterial activity of 0.2% PAAA during plasma discharge for 20 min. Catalase and sodium azide scavenge H_2O_2 and NO_2^-needed for the formation of ONOOH through reaction (6), which impairs ONOOH formation and consequently inhibits the activity of 0.2% PAAA during plasma discharge. Thus, the role of ONOOH in the antibacterial activity of 0.2% PAAA is also supported by the data presented in Figure 5A. Tarabová et. al. (2019) demonstrated the formation and activity of ONOOH using the fluorescent dye H_2DCFDA and RONS scavengers. ONOOH is a powerful oxidant that can diffuse through cell membranes, damage cells, and promote cell death through apoptosis and necrosis (Denicola et al., 1998; Huie et al., 1993). However, ONOOH damages the bacteria through its decomposition products, such as ·OH and $\cdot NO_2$ free radicals under acidic conditions due to their short half-life (Oehmigen et al., 2011). The free radicals mentioned above are generated by the decomposition of ONOOH through reactions (9) and (10) during plasma discharge (Lukes et al., 2014; Radi et al., 2000).

$$ONOOH \leftrightarrow \cdot OH + \cdot NO_2 \quad (9)$$

$$ONOOH \rightarrow HNO_3 \rightarrow NO_3^- + H^+ \quad (10)$$

Lukes et al. (2014) suggested that ONOOH can react directly or indirectly through the activity of ·OH and $\cdot NO_2$ radicals, which are formed by the H^+-catalyzed decomposition of ONOOH through reaction (9) at pH < 6.8. Additionally, ONOOH interacts with H_2O_2 to generate O_2NOOH, which can be dissociated into $^1O_2$, which plays a key role in the bactericidal properties of PAW through reactions (11)–(13) (Ikawa et al., 2016; Ma et al., 2020).

$$ONOOH + H_2O_2 \rightarrow O_2NOOH + H_2O \quad (11)$$

$$O_2NOOH \rightarrow O_2NOO^- \quad (12)$$

$$O_2NOO^- \rightarrow {}^1O_2 + NO_2^- \quad (13)$$

Thus, the dissociated species may be continuously generated and accumulated in 0.2% PAAA and consequently mediate the growth-inhibitory effect against *S.* Typhimurium. This was consistent with the results of the scavenger analysis presented in Figure 5B, which exhibited a similar pattern to that shown in Figure 5A. As shown in Figure 5B, the activity of ONOOH continued for a short period even after the cessation of plasma discharge. Piskarev et al. (2014) reported that the half-life of ONOOH is approximately 1.3 s under acidic conditions because ONOOH is continuously produced in PAW due to the presence of H_2O_2 and NO_2^-. As shown in Figure 5A, a series of activities that occurred during the plasma discharge also occurred for a short time immediately after the end of plasma discharge. Thus, the generation and decomposition of ONOOH are also considered to mediate the bactericidal effect of 0.2% PAAA immediately after plasma discharge.

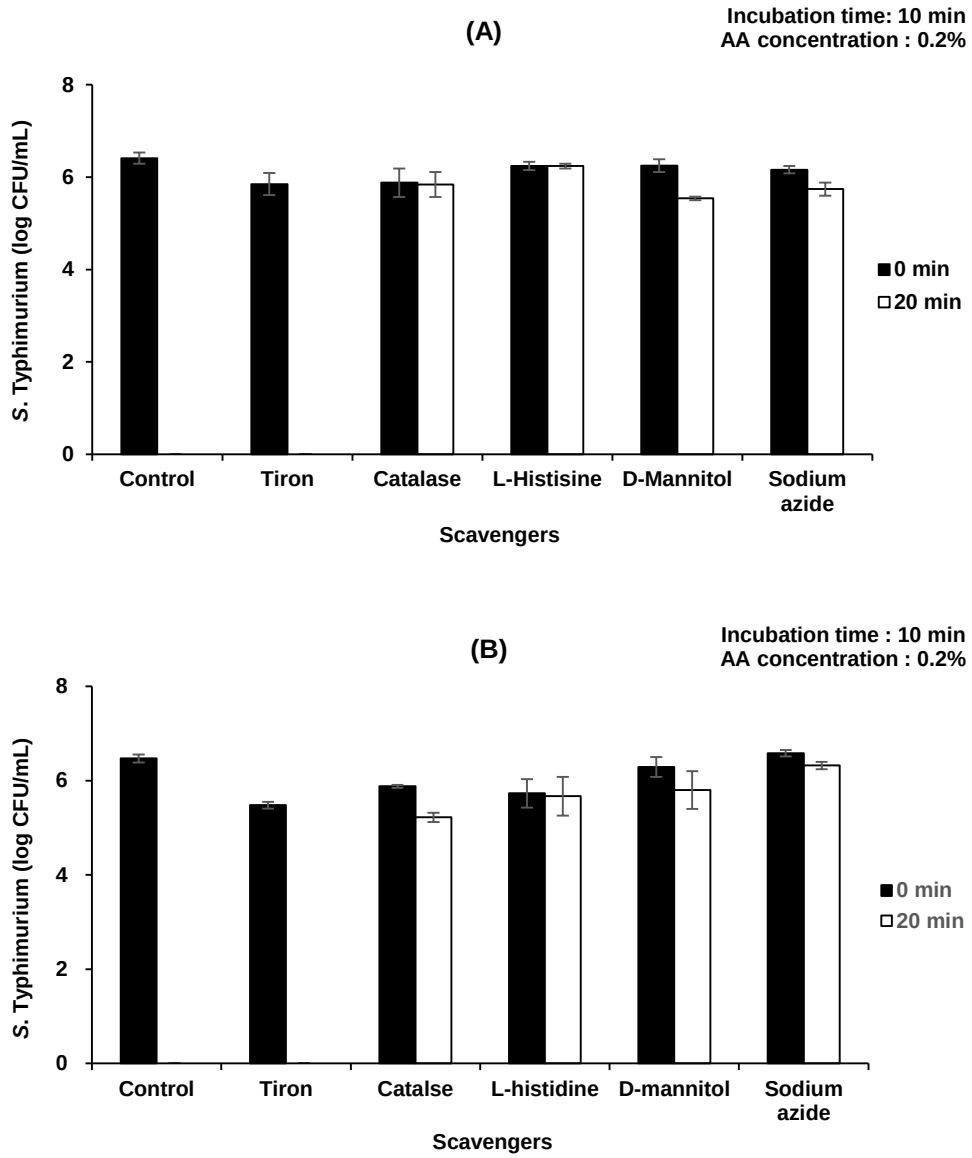

Figure 5. Effect of reactive oxygen and nitrogen species scavengers added before (A) or after (B) plasma discharge for 20 min on the viability of bacterial cells. Control group, without ant treatment. Error bars represent standard deviation.

2.3.6. Detection of peroxynitrous acid (ONOOH) in PAAA

As ONOOH is strongly dependent on the pH, it is important to understand the activity of ONOOH under acidic conditions (Tarabová et al., 2019). The generation of ONOOH in 0.2% PAAA according to different plasma discharge time and during post-plasma discharge time was examined. As shown in Figure 6A, ONOOH production did not significantly vary with the plasma discharge time. This is because the generation and decomposition of ONOOH occur simultaneously under acidic conditions. The change in the fluorescence signal of ONOOH in 0.2% PAAA (obtained after plasma discharge for 20 min) during the post-plasma discharge time was also investigated. As shown in Figure 6B, the fluorescence signal significantly decreased at 5 min post-discharge ($p < 0.05$). The changes in ONOOH levels post-discharge exhibited a similar trend as those before plasma discharge (Figure 6A). This was because of the decomposition of ONOOH under acidic conditions (Figure 6B). Consequently, the antibacterial effect of ONOOH decreased at 5 min post-discharge (Figure 6C).

Zhou et al. (2018) reported that the concentration of ONOOH in PAW varies according to the storage time and pH, which indicated that alkaline conditions play an active role in prolonging the bactericidal activity of PAW. Therefore, the results presented in Figure 6C suggest that the activities of various radicals produced in ONOOH could be limited during the post-plasma discharge time. This explains the decreased antibacterial effect of 0.2% PAAA at 5 min post-discharge. However, the viable bacterial population slightly increased at 5 min post-discharge (Figure 6C). Hence, other reactive species may also be involved in the antibacterial effect of 0.2%

PAAA. Further studies are needed to identify additional reactive species that contribute to the bactericidal effect of PAAA.

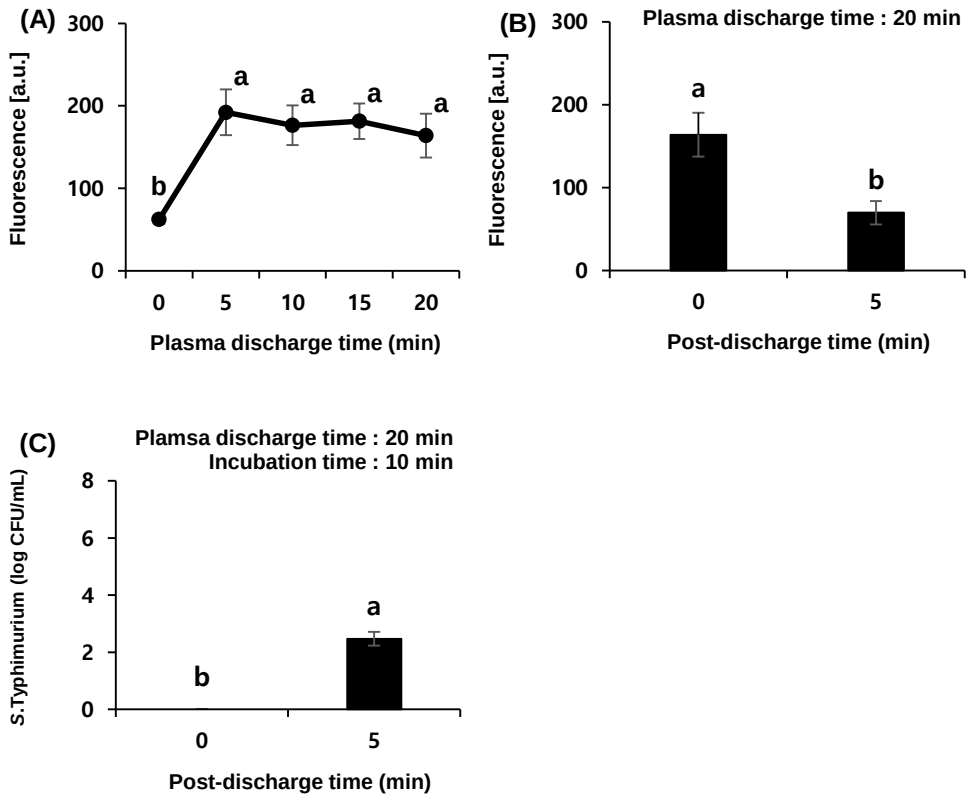

Figure 6. (A) Detection of peroxynitrous acid (ONOOH) in 0.2% plasma-activated acetic acid (PAAA) during plasma discharge using the fluorescence dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA); (B) Detection of ONOOH in 0.2% PAAA post-plasma discharge; (C) The viable bacterial cell count post-plasma discharge. Error bars represent standard deviation. $^{a\text{-}b}$Different letters indicate a significant difference ($p < 0.05$) among the treatments.

2.4. Conclusion

This study aimed to combine AA with plasma treatment to enhance its bactericidal effects. This treatment combination was hypothesized to exert a synergistic bactericidal effect when compared with the individual treatment. The growth-inhibitory activities of PAAA against *S*. Typhimurium cells and biofilms were examined under optimal conditions. The findings of this study indicated that PAAA effectively inactivated the bacteria. Additionally, the role of ONOOH in the bactericidal effect of PAAA was analyzed using scavengers and a fluorescence dye. The analysis revealed that ONOOH functioned as an intermediate agent and decomposed under acidic conditions. ONOOH can lead to the production of free radical species, such as ·OH, $\cdot NO_2$, and $^1O_2$, which play a key role in the bactericidal effect. Thus, PAAA is a potential novel decontamination agent that can reduce the pathogenic bacterial count. However, further studies are needed to examine the activity of other bactericidal agents, such as peroxyacetic acid in combination with PAAA. The increased understanding of the fundamental chemical processes enables further optimization of the efficacy and selectivity of this technology. Additionally, future studies must focus on the effect of PAAA treatment on the quality of food for applications in the food industry.

Chapter III.

Effect of plasma-activated acetic acid on inactivation of *Salmonella* Typhimurium and quality traits on chicken meats

This manuscript consists of part of a paper submitted to LWT – Food Science and Technology as partial fulfillment of the Master's program of Taemin Kang.

3.1. Introduction

With the increasing consumption of meat and meat product, the number of food-borne pathogens outbreaks related to meat has significantly increased (Zhao et al., 2001). Meat and meat products are highly susceptible to contamination by food-borne pathogens such as *Salmonella* spp., *Campylobacter* spp., Shiga toxin-producing strains of *Escherichia coli*, and *Listeria monocytogenes*, during their production, processing, and transportation (Kang et al., 2019; Nerin et al., 2016; Omer et al., 2018). Particularly, chicken meat is a highly perishable product because of its characteristics that can cause rapid and intensive spoilage (Noriega et al., 2011). Most of the pathogen contamination in chicken meat can occur in slaughter houses through spread of microorganisms between carcasses (Kim et al., 2019). A previous

study reported that *Salmonella* spp. account for majority of the food-borne pathogens identified in poultry and poultry products (Dominguez et al., 2002). Therefore, many chicken meat industries face problems in the effective inactivation of *Salmonella* spp. as well as in ensuring that the quality of chicken meat is maintained. However, the inactivation of pathogens and deterioration of the quality of chicken meat remains a significant challenge (Dirks et al., 2012). Numerous efforts have been made to inactivate microbial contaminants in chicken meat using thermal treatments, use of bacteriocins or lactic acid bacteria, and washing with agents such as chlorine and trisodium phosphate (Mani-López et al., 2012). However, these traditional methods have some limitations in inactivating pathogens and adversely affect the nutritional value or sensory quality of chicken meat (Berrang et al., 2007; Kim et al., 2002; Whyte et al., 2001).

In many countries, organic acids such as acetic, citric, and lactic acid are also used for the decontamination of chicken meat. According to the Food and Drug Administration (FDA), organic acids are recognized as safe food additives that can be used as antimicrobial agents in slaughterhouses (FDA, 1982). The antimicrobial property of organic acids is based on their ability to lower the pH, thereby causing instability in the bacterial cell membranes (Luck et al., 1998). With the increasing consumer demand that industries reduce their use of chemical additives, many food industries want to control the amount of organic acids used and simultaneously increase their antimicrobial effect in food products (Sagong et al., 2011).

Recently, non-thermal technologies that can ensure the safety of foods with minimum impact on quality have been developed as alternatives to traditional methods (Heo et al., 2021). Cold plasma has a minimal impact on the quality of meat

and meat products, is relatively inexpensive, and easy to install compared to other non-thermal technologies (Lee et al., 2016). In the present study, we treated plasma with acetic acid (AA) and the resulting plasma-activated acetic acid (PAAA) was expected to improve the bactericidal efficiency of AA. Some studies on lactic acid treated with plasma have studied the synergistic bactericidal effects (Qian et al., 2020; Qian et al., 2021). However, no studies have reported the antibacterial effects of PAAA and the impact on the physicochemical traits of chicken meat. Therefore, the objective of this study was to investigate the antibacterial effects of PAAA and its impact on the quality characteristics of chicken meat.

3.2. Materials and methods

3.2.1. Antibacterial activity of AA and PAAA treatments on chicken meats

3.2.1.1. Bacterial strains and culture preparation

Salmonella Typhimurium (ATCC 13311) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). *S.* Typhimurium was cultivated at 37°C in nutrient broth (Difco, Becton Dickinson Co., Sparks, MD, USA) for 48 h to obtain mid-log phase cells. The strain was then washed twice with 0.85% NaCl solution (saline), followed by centrifugation at 2,266 × *g* for 14 min at 2°C (UNION 32R, Hanil Science Industrial, Co. Ltd, Gimpo, Korea). Finally, the viable cell density of the re-suspended culture was adjusted to approximately 10^8–10^9 colony forming units (CFU)/mL using the optical density at a wavelength of 600 nm.

3.2.1.2. Sample preparation, sterilization and inoculation

Raw chicken skin, breasts, and drumsticks were purchased up to one week in advance from a local market in Seoul (Nonghyup Co., Seoul, Korea) and frozen at -20°C. Before the experiment, the samples were thawed overnight (24 h) at 4°C. The skin was punched into 2 cm^2 round pieces, and the breasts and drumsticks were cut into equal-sized pieces (3.00 ± 0.05 g) using a sterile knife. To study the antibacterial effect, the surface of each sample was exposed to ultraviolet light for 30 min to eliminate background microflora. Hundred microliters of the cell suspension were spot inoculated on the surface of the samples; the samples were then

placed for 1 h on a clean bench at room temperature (25°C) to allow bacterial adsorption to the surface of the samples.

3.2.1.3. Preparation of PAAA

As presented in Figure 1, an encapsulated atmospheric dielectric barrier discharge plasma, using a rectangular plastic container (137 × 104 × 53 mm) containing copper electrodes and a polytetrafluoroethylene (PTEE) sheet, was prepared for the generation of PAAA. Atmospheric air was used as the operating gas and plasma was generated at 2.2 kHz and 8.4 kVpp following the modified conditions from a previous study (Yoo et al., 2021). The AA immersed in a glass dish was placed in the center of the container, and then exposed to plasma generated inside the container. PAAA was obtained by exposing the plasma discharge to 20 mL of AA (v/v) for 30 min. The concentrations of AA used in this study were 0.2%, 0.6%, and 0.8% (v/v) and those of PAAA were 0.2%, 0.6%, and 0.8% (v/v), representing the corresponding AA concentration in PAAA. The bacterial cells in the samples treated with deionized water were used as a control.

3.2.1.4. Microbial analysis

To investigate the bactericidal effect of bacteria, inoculated samples were immersed in 50 mL conical tubes containing AA and PAAA for each treatment and incubated at 25°C for 10 min. Immediately after treatment, each sample was placed in a new conical tube containing 27 mL of saline solution for the detachment of

bacteria on the surface by vortexing at high speed for 2 min. The supernatants from the tube were serially diluted in saline, and each diluted sample (0.1 mL) was spread on xylose lysine deoxycholate agar (XLD; Difco). The agar plates were incubated at 37°C for 48 h, and the microbial counts were determined. The results of the skin were expressed as log CFU/cm^2, and the breasts and drumsticks were expressed as log CFU/g.

3.2.2. pH measurement

The pH values of the breasts and drumsticks were measured according to a previously described method (Heo et al., 2021). After each treatment, 1 g of the homogenized sample was added to each tube containing 9 mL distilled water and mixed thoroughly for 30 s using a homogenizer (T25 Basic, Ika Co., Staufen, Germany). After homogenization, the solution was centrifuged (Hanil Science Industrial Co., Ltd.) at 2,265 × *g* for 10 min at 4°C and the pH value of the resulting supernatant was measured using a pH meter (Seven 2Go, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

3.2.3. Lipid oxidation measurement

The lipid oxidation of the breasts and drumsticks was analyzed according to a previously reported method, which calculates the level of 2-thiobarbituric acid reactive substances (TBARS) (Lee et al., 2016). Three grams of each sample and 9 mL of distilled water were mixed and homogenized with 50 μL of butylated

hydroxytoluene (7.2% in ethanol) using a homogenizer at 9,600 rpm for 30 s. The homogenate (1 mL) of was then added to a 15 mL centrifuge tube containing 2 mL of thiobarbituric acid (20 mmol/L)/trichloroacetic acid (15%) solution, and the tubes were heated in a water bath at 90°C for 30 min, followed by cooling in water. The test tubes were centrifuged at 2,265 × *g* for 10 min, and the absorbance of the supernatant was measured at 532 nm using a spectrophotometer (DU 530; Beckman Instruments Inc., Brea, CA, USA). The TBARS value was presented as mg of malondialdehyde per kg sample, using a standard curve (Yim et al., 2020).

3.2.4. Color measurement

The color of the breasts and drumsticks was measured using a colorimeter (CM-5, Konica Minolta Co., Ltd., Osaka, Japan) to obtain the CIE lightness (L^*), redness (a^*), yellowness (b^*), hue angle (tan-1(b^*/a^*)), and chroma (($a^{*2} + b^{*2})^{1/2}$). The instrument was calibrated with a white and black standard tile before the analysis (Yoo et al, 2020). Measurements were taken randomly on the surface of the breasts and drumsticks at six different locations per sample with an 8 mm diameter measurement area. The color values were monitored by a computerized system using spectra Magic software (Konica Minolta Sensing, Inc.).

3.2.5. Scanning electron microscopy (SEM) analysis

The SEM images of the breasts and drumsticks were obtained following the method reported in our previous study (Shin et al., 2020). After each treatment, the samples were cut into 0.5 cm diameter and 0.2–0.3 cm thick pieces and were fixed

with Carnoy fluid (60% ethyl alcohol, 30% chloroform, 10% glacial acetic acid; v/v) at 4°C for 24 h. The samples were dehydrated using an increasing concentration of ethyl alcohol (70% for 12 h, 95% for 2 h, and 100% for 2 h). Each dehydrated sample was immersed in hexamethyldisilazane twice for 10 min and dried overnight (24 h) in a fume hood at 25°C. The dried samples were mounted on aluminum stubs using double-sided carbon tape and coated with a layer of platinum in a vacuum evaporator (EM AC E600, Leica Microsystem, North Ryde, NSW, Australia). Micrographs of the samples were visualized using a Zeiss Sigma field emission scanning electron microscope (AURIGA, Carl Zeiss Microscopy, Thornwood, NY).

3.2.6. Electronic nose analysis

The electronic nose (Heracles II, Alpha MOS, Toulouse, France) in each treatment was analyzed to determine the effect of PAAA on the odor of the treated chicken meat. The samples were ground using a meat grinder (MG510, Kenwood, Hampshire, UK), and each sample (5.00 ± 0.05 g) was taken in a 20 mL vial and cooked for 30 min at 80°C to obtain the volatile compounds (Li et al, 2020). The volatiles were then injected into an electronic nose equipped with dual columns of MXT-5 and MXT-1701 (10 m × 180 m × 0.4 m; length × diameter × thickness) (Restek, Bellefonte, PA, USA). Each peak was integrated and identified using the retention time and relevance index, indicating the percentage of matching probability, based on the comparison of Kovats retention index of the detected compound and the Kovats retention indices of known compounds from the AroChemBase library (Lee et al, 2019).

3.2.7. Statistical analysis

All experimental procedures were conducted independently in triplicates. The data were assessed by Tukey's multiple-range test using the SAS program (version 9.4, SAS Institute Inc., Cary, NC, USA) at a significance level of $p < 0.05$. Statistical analysis was performed using the Student's *t*-test and one-way analysis of variance. The standard deviation of the mean values is reported in the figures and tables. Scores plot based on principal component analysis (PCA) was generated using MetaboAnalyst 4.0, in accordance with the method mentioned by Kim et al. (2020).

3.3. Results and discussion

3.3.1. Antibacterial effect of PAAA on chicken meats

The population of bacterial cells in both AA and PAAA treatments significantly decreased with increasing concentrations of AA ($p < 0.05$, Figure 7(A)). The initial population of *S*. Typhimurium on the skin was 6.91 log CFU/cm^2. The population of *S*. Typhimurium decreased by 0.17, 1.05, and 2.33 log CFU/cm^2 after incubation for 10 min with 0.2%, 0.6%, and 0.8% PAAA, respectively. However, after treatment with AA, the population of *S*. Typhimurium decreased by 0.07, 0.93, and 1.23 log CFU/cm^2 under the same concentrations of AA (0.2%, 0.6%, and 0.8%), respectively.

The bactericidal efficiency of 0.6% PAAA ($p < 0.05$) and 0.8% PAAA ($p < 0.01$) were significantly higher than those of the control and 0.2% PAAA treatment groups. These data suggest that *S*. Typhimurium on chicken skin has a more significant susceptibility to PAAA treatment compared with that of AA, and it is significantly affected by the increasing concentration of AA. This provides a potential application in the slaughtering process. The reactive species in chemical reactions generated by plasma discharge induce lethal effects on bacteria due to the role of these short-lived species (Zhou et al., 2015). For solutions that were treated with plasma, the antibacterial effect of plasma-activated water (PAW) is attributed to the acidic pH and ONOOH generated by H_2O_2 and NO_2^- (Oehmigen et al., 2011; Naïtali et al., 2010). Qian et al. (2019) also found that the number of *S*. enteritidis inoculated on beef significantly decreased with increasing concentration of lactic acid in plasma-activated lactic acid, which can accelerate the generation of NO_2^-. Further, NO_2^-

induces the generation of ONOOH with H_2O_2, which is consistent with our experimental results, indicating that the antibacterial activity of PAAA is more potent with increasing concentrations of AA. As shown in Figure 7(A), when the concentration of AA was 0.8%, the bactericidal efficiency and the level of significance for differences between PAAA and AA treatment were the highest ($p < 0.01$). Therefore, 0.8% AA concentration was regarded as the optimal treatment condition and was used to investigate the antibacterial activity of the chicken breasts and drumsticks.

Figure 7(B) shows the antibacterial effect of 0.8% AA and PAAA on the breasts and drumsticks. Each sample was incubated for 10 min with 0.8% AA and PAAA obtained by plasma exposure for 30 min. After treatment with AA, the population of *S*. Typhimurium on the breasts and drumsticks significantly reduced by 1.35 and 1.56 log CFU/g, respectively, compared with the control ($p < 0.05$). whereas, after treatment with 0.8% PAAA, the population of *S*. Typhimurium on the breasts and drumsticks significantly reduced by 2.33 and 2.75 log CFU/g, respectively ($p < 0.05$). These findings indicate that the susceptibility of the bacteria in chicken meat treated with PAAA was significantly higher than that treated with AA, which is consistent with the results of chicken skin presented in Figure 7(A). According to a previous study, the efficacy of plasma was greatly reduced due to the surface topography on chicken skins, which may act as a physical barrier, resulting in the bacteria being protected from the reactive species generated by plasma, as compared to chicken muscle (Noriega et al., 2011). However, Rossow et al. (2018) identified that there were no significant differences in the bactericidal effects between the skin and breast samples by investigating the efficacy of parameters in plasma treatment. Consistent

with this study, no significant differences in bactericidal effects were observed between the skin and breast samples in our study. Therefore, these results suggest that *S*. Typhimurium can be effectively inactivated in chicken meat by treatment with PAAA.

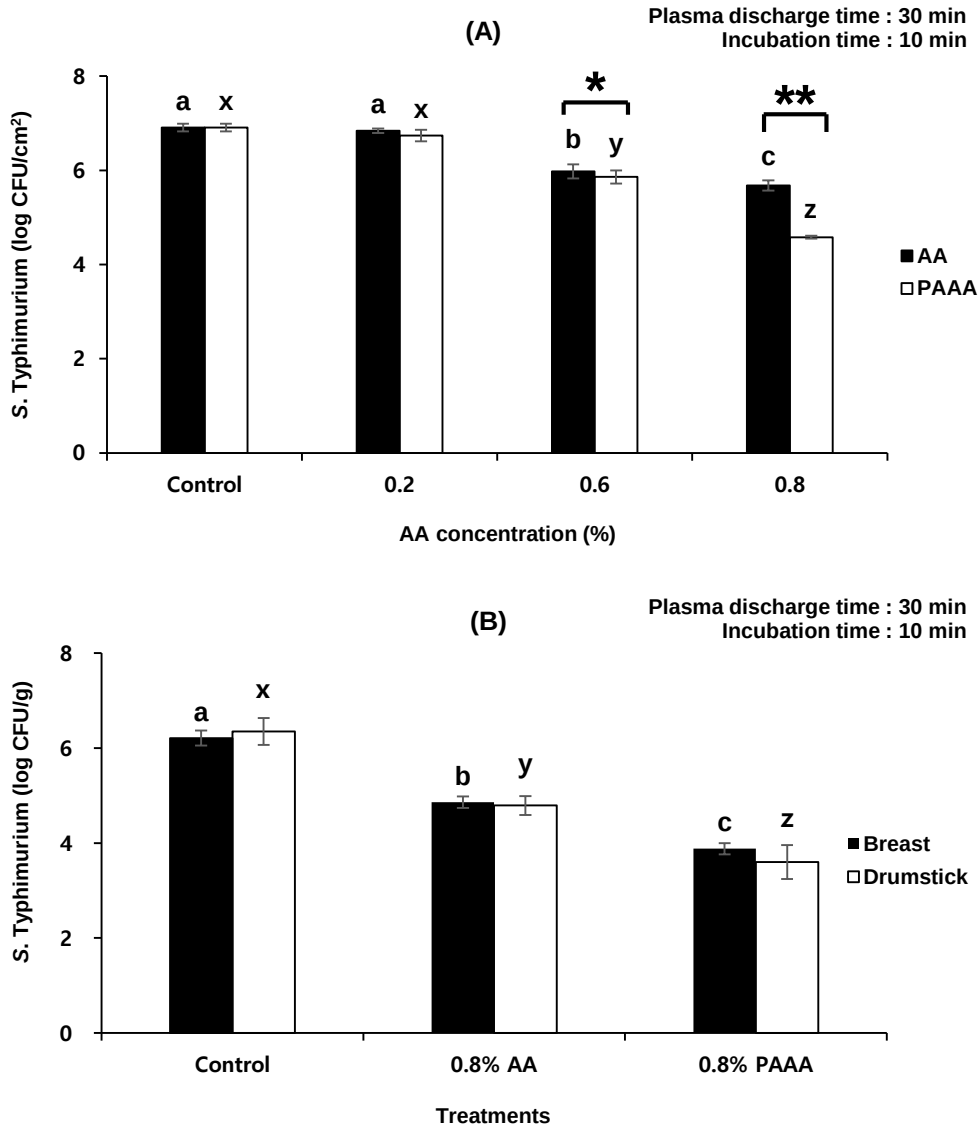

Figure 7. Effect of acetic acid (AA) and plasma-activated AA (PAAA) on population of *S.* Typhimurium according to (A) the concentration of AA in chicken skins and (B) the antibacterial effects of 0.8% AA and PAAA on chicken breasts and drumsticks. Control group, deionized water treatment; AA, AA treatment; PAAA, PAAA treatment. Error bars represent standard deviation. $^{a\text{-}c}$Different letters indicate a significant difference ($p < 0.05$) among the treatments. $^{x\text{-}z}$Different letters indicate a significant difference ($p <$

0.05) among the treatments. Student's *t*-test; *, $p < 0.05$ and **, $p < 0.01$ with respect to the untreated control.

3.3.2. pH

Table 3 presents the pH values of the breasts and drumsticks treated with AA or PAAA. The breasts and drumsticks showed significant differences between the control and treatment groups (0.8% AA and PAAA); the treatment group showed a decrease in pH values as compared to the control. However, no significant differences were identified between the 0.8% AA and PAAA groups ($p > 0.05$). The decrease in the pH of chicken meat was related to the combined action of AA and PAAA. Aktaş et al. (2003) identified that the type and concentration of acids used significantly affected the pH values of marinated meat due to the dissociation of hydrogen ions (H^+). The plasma-induced decrease in pH values in the samples was caused by the generation of acidogenic molecules from the plasma, resulting in their accumulation on the surface of the samples (Fröhling et al., 2012). In addition, the interaction of the reactive species generated by plasma, including O, O_3, and NO_X, with the moisture content of the samples caused a decrease in the pH values in the samples after plasma treatment *via* equations (14) to (16) (Liu et al., 2010). However, there were little additional effects of plasma treatment in the present study.

$$N_2 + O_2 \rightarrow 2NO \quad (14)$$

$$2NO + O_2 \rightarrow 2NO \quad (15)$$

$$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3 \quad (16)$$

3.3.3. TBARS

The TBARS values of the treatments (0.8% AA and PAAA) in breasts and

drumsticks were lower than those of the control (Table 3). Significant differences in TBARS values of the breasts and drumsticks were observed between the control and treatment groups. Several studies have shown a decrease in the TBARS value of meat treated with organic acids, such as acetic, lactic, and citric acid, compared with the control. Kang et al. (2002) reported that TBARS values of pork treated with acetic acid were lower than those of the control during storage, and this finding was supported by the inhibition of the generation of malondialdehyde, which is induced by products such as carbonyl complexes, alcohols, ketones, and aldehydes. According to previous research, the TBARS values of jerky treated with dielectric barrier discharge plasma decreased as the plasma treatment time increased, which was caused by the antioxidant effect of nitrite (Yong et al., 2019). The TBARS values of pork treated with plasma showed a decrease or no significant differences as the plasma treatment time increased, indicating that the decrease in TBARS values might be due to the action of plasma treatment. In the present study, however, there were no significant differences in TBARS values observed between the 0.8% AA and PAAA treatments ($p > 0.05$), suggesting that the action of plasma has little effect on 0.8% PAAA treatment.

Table 3. pH and lipid oxidation of breasts and drumsticks treated with acetic acid (AA) and plasma-activated acetic acid (PAAA)

Treatments	pH	TBARS value (mg malondialdehyde /kg)
Breast		
Control	5.88 ± 0.01^{a}	0.27 ± 0.02^{a}
0.8% AA	5.28 ± 0.07^{b}	0.10 ± 0.01^{b}
0.8% PAAA	5.36 ± 0.18^{b}	0.10 ± 0.01^{b}
Drumstick		
Control	6.77 ± 0.03^{a}	0.31 ± 0.03^{a}
0.8% AA	5.90 ± 0.02^{b}	0.17 ± 0.02^{b}
0.8% PAAA	5.95 ± 0.08^{b}	0.19 ± 0.04^{b}

Control group, deionized water treatment; 0.8% AA, acetic acid (0.80%, v/v) treatment; 0.8% PAAA, plasma-activated acetic acid (0.8%, v/v) treatment.

All values represent the Mean ± Standard Deviation.

[a, b]Different letters within column differ significantly ($p < 0.05$).

3.3.4. Color

The color of meat is the most common indicator of quality, which has an important influence on consumer preferences (Kang et al., 2019; Wadhwani et al., 2012). The L^* values of the breasts and drumsticks treated with 0.8% AA and PAAA increased, whereas the a^* and b^* values decreased compared to those of the control (Table 4). However, there were no significant differences in color values (L^*, a^*, and b^*) between the control and the treatments (0.8% AA and PAAA) in drumsticks. These results indicate that both samples had similar patterns in L^*, a^*, and b^* values, except for the significant differences. The color difference in meat is attributed to the amount of heme pigments and fibers type of predominant muscle (Berri et al., 2001). Lengerken et al. (2002) reported that breast meat contains more than 90% white fibers, whereas the proportion of red fibers and irregular surface color in leg meat is higher than in other meat types. This could be the reason why there were no significant differences among the treatments in drumsticks compared to the breasts. Stivarius et al. (2002) identified that ground beef treated with acetic acid was lighter ($p < 0.05$; L^*) and less red (a^*) and yellow (b^*) ($p < 0.05$) in color compared to the control. In addition, the ground beef from the treatment with acetic acid showed higher hue angle values than the control. Similarly, in the present study, the hue angles in the breasts and drumsticks were higher than those of the control. These results were related to the concentration of oxymyoglobin in meat, and could be attributed to the discolored meat, which caused lower redness values and oxymyoglobin content after treatment with acetic acid (Stivarius et al., 2002; Bell et al., 1986).

According to a previous study, the L^* and b^* values of chicken breasts treated with dielectric barrier discharge plasma also increased as the treatment time increased, whereas the a^* value decreased significantly (Lee et al., 2016). Another study also identified significant increases in the L^* values and significant decreases in the a^* and b^* values were observed in breasts treated with PAW (Kang et al., 2019). Fröhling et al. (2012) demonstrated that the hydrogen peroxide generated in plasma induced a green color in plasma-treated meats *via* reaction with myoglobin. Consistent with the previous discussion, the color of breasts and drumsticks treated with 0.8% PAAA was additionally affected by the action of plasma, although there were no significant differences between the 0.8% AA and PAAA treatments ($p > 0.05$).

Table 4. Surface color values of breasts and drumsticks treated with acetic acid (AA) and plasma-activated acetic acid (PAAA)

Treatments	L^{*a}	a^{*a}	b^{*a}	Chroma	Hue angle
Breast					
Control	51.33 ± 2.00[b]	3.15 ± 0.14[a]	13.65 ± 0.20[a]	14.01 ± 0.22[a]	77.09 ± 0.46
0.8% AA	55.93 ± 1.43[a]	2.08 ± 0.43[ab]	10.40± 0.20[b]	10.65 ± 0.27[b]	78.65 ± 2.17
0.8% PAAA	58.56 ± 0.32[a]	1.50 ± 0.80[b]	9.90 ± 0.06[b]	10.04 ± 0.10[b]	82.55 ± 5.77
Drumstick					
Control	52.02 ± 1.60	8.97 ± 1.53	15.05 ± 0.76	15.99 ± 0.84	61.17 ± 5.35
0.8% AA	51.65 ± 6.04	7.88 ± 0.37	14.68 ± 2.63	16.71 ± 2.13	61.33 ± 5.56
0.8% PAAA	53.13 ± 2.90	7.45 ± 2.38	13.82 ± 0.72	15.75 ± 2.03	63.16 ± 6.38

Control group, deionized water treatment; 0.8% AA, acetic acid (0.80%, v/v) treatment; 0.8% PAAA, plasma-activated acetic acid (0.8%, v/v) treatment.

All values represent the Mean ± Standard Deviation.

[a, b]Different letters within column differ significantly ($p < 0.05$).

[a] L^*: Lightness; a^*: redness; b^*: yellowness.

3.3.5. SEM

The skin was removed from the surfaces of breasts and drumsticks for each sample; these showed a smooth and regular shape on the SEM images (Figure 8). As shown in Figure 8, there were no remarkable morphological alterations observed after treatment with AA or PAAA. However, compared to the control in each sample, the images of AA or PAAA treatment clearly revealed the presence of pores. These could be correlated to the porous structure of chicken meat, which is caused by the weakening of structures under acidic conditions (Alagöz et al., 2020) identified that the combined treatment of PAW and ultrasound was more porous with the surface of chicken meat compared with individual treatment, resulting in bacteria being susceptible to PAW treatment. However, Lin et al. (2019) reported that the SEM images of eggs revealed less damage to the cuticles on eggs treated with PAW than on the commercially washed eggs. In the present study, no additional morphological changes were observed on the surface of PAAA-treated chicken breasts and drumsticks compared with the AA treatment. Thus, the morphological changes on the surface of chicken meat may not be attributed to the action of plasma in the present study.

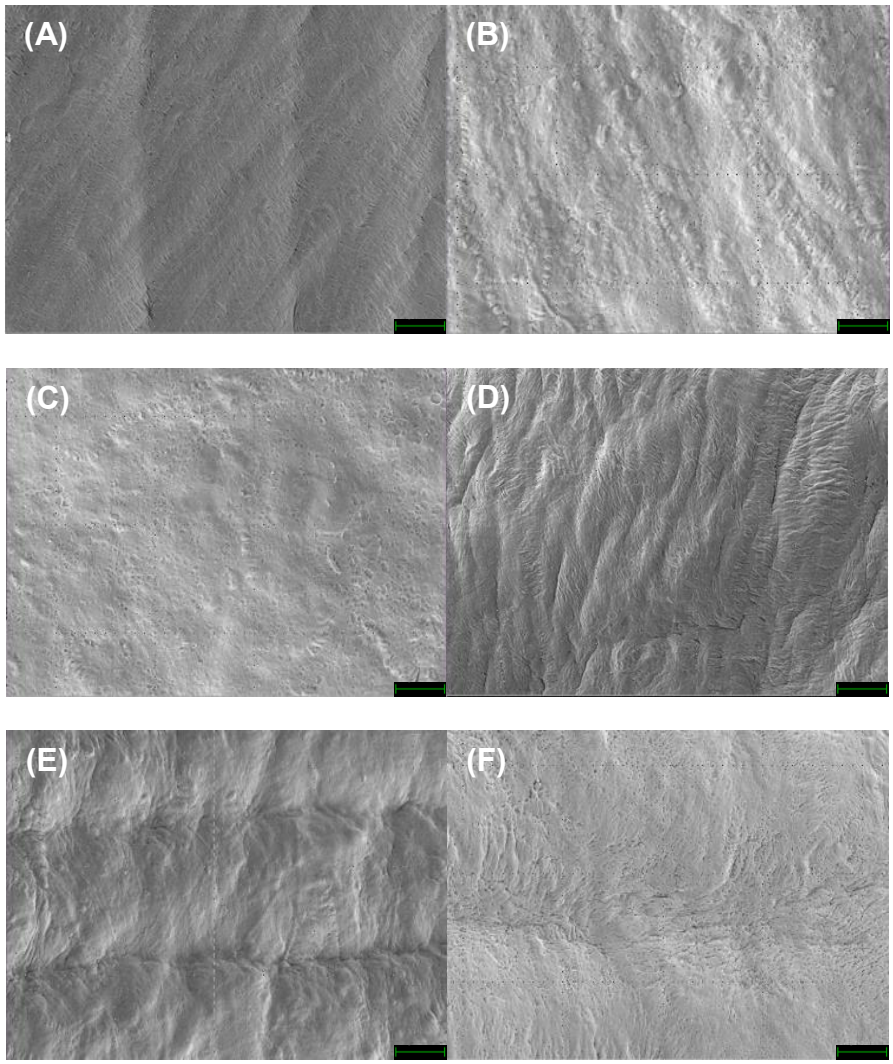

Figure 8. Evaluation of morphological images of chicken meats using scanning electron microscope (SEM). (A) Control group (breasts); (B) 0.8% AA treatment (breasts); (C) 0.8% PAAA treatment (breasts); (D) Control group (drumsticks); (E) 0.8% AA treatment (drumsticks); (F) 0.8% PAAA treatment (drumsticks). Control group, deionized water treatment; AA, 0.8% AA treatment; PAAA, 0.8% PAAA treatment. Bar, 10 μm.

3.3.6. Electronic nose

Electronic nose is a rapid analysis tool to detect and distinguish between various types of gaseous samples (Chen et al., 2021). It is possible to obtain comprehensive information on volatile compounds in samples (Lee et al., 2021). The PCA results obtained for the volatile compounds of chicken meat and are presented in Figure 9. The two principal components (PC), PC1 and PC2, of chicken breasts and drumsticks explained 81% and 79.2% of the overall variance, respectively. PCA is a simple method used for modelling and visualization of multidimensional data (Wiśniewska et al., 2016). PC1 was the most important variable for the different treatments (control, 0.8% AA, and 0.8% PAAA). As for chicken breasts presented in Figure 9(A), PC1 separated control from 0.8% AA and 0.8% PAAA and accounted for 65.1% of the total variation, which indicated that there was a significant difference in odors between the control and the treatment groups. However, the difference between 0.8% AA and PAAA was not identified in the PC1 data area, indicating that they were not significantly different in odors. These results were similar to those of the drumsticks presented in Figure 9(B). The overlapping area between 0.8% AA and PAAA in the results of drumsticks indicates that there were similarities of odor between 0.8% AA and PAAA treatments. Thus, the significant differences in odors between the control and treatment groups were mainly related to the action of AA, indicating that the changes in odor caused by the plasma treatment were negligible.

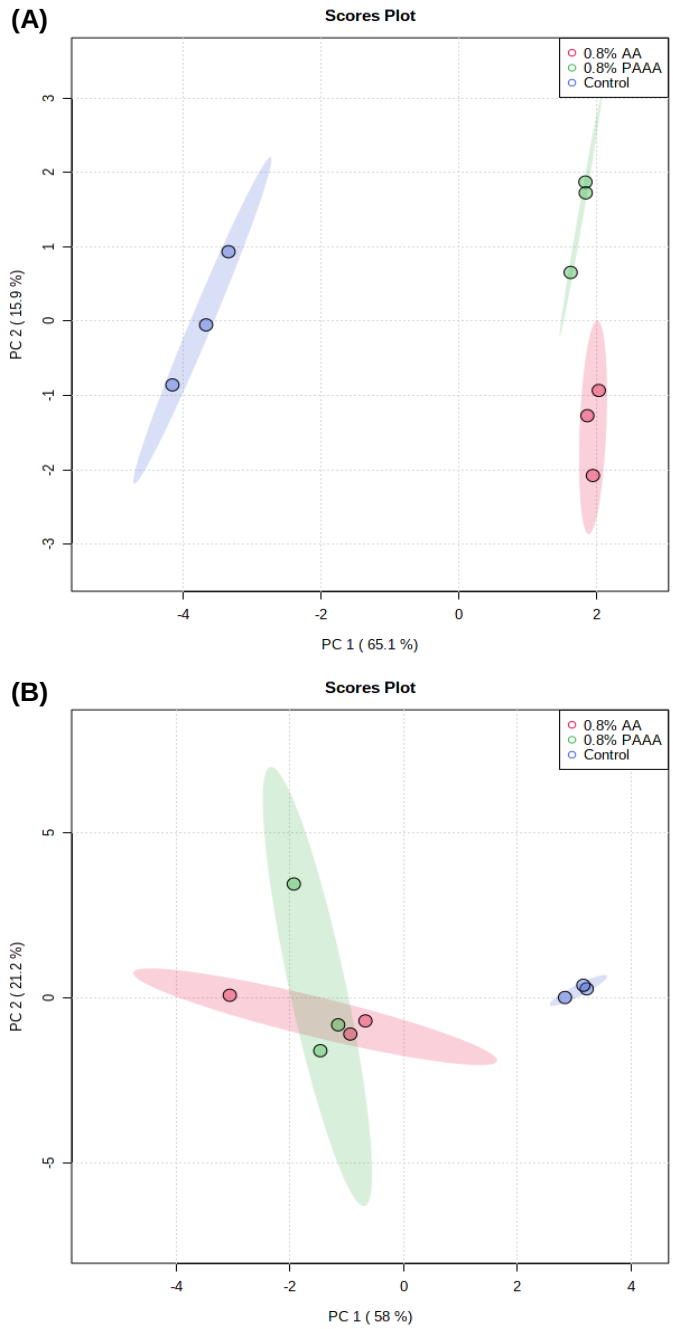

Figure 9. Principal component analysis (PCA) score plots for all samples in different type of treatments. (A) Chicken breasts; (B) Drumsticks. Control group, deionized water treatment; 0.8% AA, 0.8% AA treatment; 0.8% PAAA, 0.8% PAAA treatment.

3.4. Conclusion

This study identified the antibacterial activity of PAAA and its quality traits in chicken meat. PAAA showed a higher bactericidal efficiency than AA in breasts, drumsticks, and skin, and the bactericidal activity was proportional to the concentration of AA. Based on the 0.8% concentration of AA, which had a high bactericidal activity, there were no significant differences in the quality traits, including pH, surface color, and TBARS values between AA and PAAA treatments. Similar results were obtained for the morphological images and volatile compound areas analyzed by SEM and electronic nose analysis, respectively. Thus, PAAA is a potential decontaminating agent that can effectively inactivate food-borne pathogens, including *Salmonella*, and could be used as an alternative to AA to obtain hygienic chicken meat. However, further studies are needed to evaluate the sensory properties of chicken meat after treatment with PAAA and identify the optimal conditions for its application in the food industry. In addition, future research will focus on investigating the bactericidal activity of PAAA and its impact on the quality traits of various other food products and widen its applications in the food industry.

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Summary in Korean

Salmonella Typhimurium에 대한 플라즈마 활성 초산의 항균효능 및 메커니즘과 계육의 적용

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본 연구에서는 플라즈마 활성 초산(Plasma-activated acetic acid, PAAA)을 이용하여 *Salmonella* Typhimurium과 바이오필름(Biofilms)에 대한 살균력과 살균 기작을 확인하고, 실제 식품산업 현장의 적용 가능성을 확인하기 위하여 처리 후 계육의 살균 및 이화학적 특성을 조사하였다. 실험은 다음과 같이 두 개의 실험으로 진행되었다. 첫번째 실험의 경우, *Salmonella* Typhimurium과 더불어 이를 이용해 형성시킨 바이오필름에 대한 플라즈마 활성 초산의 살균력을 확인하고 스캐빈저(Scavengers) 및 형광 발색 시약을 통해 살균 메커니즘을 확인하였다. 두번째 실험의 경우, 플라즈마 활성 초산을 처리한 계육(닭 스킨, 닭 가슴살 및 닭 북채)의 살균 및 이화학적(pH, 색도, 지질 산패도 및 전자현미경) 특성 분석을 진행하여 식품 산업에서 적용 가능성 여부를 확인하였다.

첫번째 실험 결과, 일반 현탁액 형태의 미생물에서 플라즈마 활성 초산의 최적 살균 조건을 확인하였는데, 플라즈마 방전 시간 20분, 미생물과의

배양시간 10분, 초산의 농도 0.2% (v/v)일 때 가장 최적 조건임을 확인하였다. 또한, 바이오필름에도 효과적인 살균 능력을 보였으며, 0.4% (v/v) 농도의 초산을 20분 방전시킨 플라즈마 활성 초산의 처리 10분 뒤에는 대조군(Control) 대비 4 log CFU/cm^2의 감균효과를 나타내었다. 이에 대한 결과는 공초점레이저주사현미경(CLSM)을 통해서도 확인할 수 있었다. 살균 메커니즘을 확인하기 위한 스캐빈저 검증 결과, 플라즈마 방전 전후로 수산화라디칼(·OH), 과산화수소(H_2O_2), 일중항산소($^1O_2$) 및 아질산염 이온(NO_2^-)들이 살균에 유의적으로 기여하는 것을 확인하였으며 이는 과산화질산염(ONOOH)의 중간 작용에 기인한 것으로 판단되었다. 즉, 과산화 수소와 아질산염 이온에 의해 과산화 질산염의 형성을 유도하였으며 이는 산성조건에서 분해되어 최종 수산화라디칼, 이산화질소 및 일중항산소의 형태로 미생물의 살균에 유의적인 영향을 미쳤을 것으로 판단된다. 이에 대한 결과는 플라즈마 활성 초산의 방치에 따른 과산화 질산염의 농도 및 방치시간에 따른 살균결과에서도 확인할 수 있었다.

두번째 실험 결과, 닭 스킨 살균실험에서 초산의 농도가 증가함에 따라 플라즈마 활성 초산의 살균효과도 증진되는 것을 확인하였으며, 최적 조건을 확인한 0.8% (v/v) 농도의 초산을 30분 플라즈마를 방전시켜 만든 플라즈마 활성 초산을 닭 가슴살 및 닭 북채에 10분 처리하였을 때, 대조군 대비 각각 2.33 및 2.75 log CFU/g 감균 효과를 확인하였다. 이를 통해 플라즈마 활성 초산은 닭 스킨뿐만 아니라 닭 가슴살 및 닭 북채에서도 같은 농도의 일반 초산 (AA)보다 더 높은 살균력을 나타내는 것을 확인하였다.

또한 이화학적 특성 분석 중 pH 및 지질 산패도 (TBARS) 분석 결과, 닭 가슴살 및 닭 북채에서 대조군 대비 처리군(0.8% AA 및 PAAA)에서 값이 유의적으로 감소했지만 AA와 PAAA 처리군들 사이에서 유의적인 차이를 확인할 수 없었다. 색도의 경우, 닭 가슴살 및 닭 북채에서 백색도 (L^*)는 증가하였지만 적색도 (a^*) 및 황색도 (b^*)값이 감소하였다. 닭북채에서는 대조군을 포함한 각 처리군 간 유의적인 차이를 확인할 수 없었다. 이는 각 식육에 존재하는 헴 색소(Heme pigments)및 형태의 구성 차이에 기인한 것으로 사료되며, 처리군에 따른 결과의 차이는 초산 처리에 의한 Oxymyoglobin의 영향이 어느정도 작용했을 것으로 판단된다. 전자현미경 분석 결과, 대조군을 포함한 각 처리군 간 눈에 띄는 형태적 변화를 확인할 수 없었지만, 대조군 대비 처리군에서 미세한 기공들이 확인되었다. 하지만, 마찬가지로 AA와 PAAA 처리군들 사이에서 유의적인 차이를 확인할 수 없었다. 또한 휘발성 향 화합물의 결과에서도 닭 가슴살 및 닭 북채 모두 AA와 PAAA 처리군들 사이에서 유의적인 변화를 관찰할 수 없었다. 따라서 플라즈마 활성 초산은 계육에서 일반 초산 대비 이화학적 변화가 거의 없는 반면 살균력이 더 높은 것이 확인되었다.

본 연구 결과들을 통해 플라즈마 활성 초산은 유망한 기술로서 식품 산업에서의 효과적인 살균제의 역할을 담당할 수 있을 것으로 판단되지만, 산업적 응용 잠재력을 더 높이기 위한 추가적인 연구가 더욱 뒷받침이 되어야 할 것으로 사료된다.

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