Master's Thesis of Agriculture

Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

정향오일과 대기압 플라즈마 병용처리에 따른 병원성대장균과 황색포도상구균에 대한 시너지 살균효과 메커니즘 규명

February, 2020

Ji Hyun Yoo

Department of Agricultural Biotechnology

Graduate School

Seoul National University

Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

Advisor: Prof. Cheorun Jo, Ph.D.

Submitting a Master's Thesis of Agriculture

February, 2020

Department of Agricultural Biotechnology
Graduate School
Seoul National University

Ji Hyun Yoo

Confirming the Master's Thesis written by
Ji Hyun Yoo

February, 2020

정향오일과 대기압 플라즈마 병용처리에 따른 병원성대장균과 황색포도상구균에 대한 시너지 살균효과 메커니즘 규명

Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

지도교수 조 철 훈

이 논문을 농학석사학위논문으로 제출함

2020년 01월

서울대학교 대학원

농생명공학부 동물생명공학전공

유 지 현

유지현의 석사학위논문을 인준함

2020년 01월

위 원 장 _____ (인)

부위원장 _____ (인)

위　　원 _____ (인)

Abstract

Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

Ji Hyun Yoo

Department of Agricultural Biotechnology

Program in Animal Science and Biotechnology

Graduate School of Seoul National University

The synergistic bactericidal effect and its mechanism of clove oil and encapsulated atmospheric pressure plasma (EAP) against *Escherichia coli* O157:H7 and *Staphylococcus aureus* were investigated. The bactericidal effect of the combined treatment was also confirmed using inoculated beef jerky. For both pathogens, clove oil and EAP single treatments resulted in less than 3.0-log reductions, whereas the combined treatment resulted in more than 7.5-log reductions. The disc-diffusion assay and gas chromatography-mass spectrometry showed no changes in both the clear zone diameter and chemical composition of clove oil before and after the EAP treatment. Significant changes in cell membrane permeability and morphological shapes via the combined treatment of clove oil and EAP were

evidenced by UV absorption, confocal laser scanning microscopy, and transmission electron microscopy. The synergistic bactericidal effects of clove oil and EAP against both pathogens were also observed in inoculated beef jerky, but less for *S. aureus* due to differences in its outer cell membrane structure. These findings indicated that the synergistic bactericidal effects between clove oil and EAP may be due to the increased susceptibility of the bacteria to EAP by clove oil, rather than an alteration of antibacterial activity of clove oil itself via EAP.

Keywords: Encapsulated atmospheric pressure plasma, Clove oil, Synergistic bactericidal effect, *Escherichia coli* O157:H7. *Staphylococcus aureus*

Student Number: 2018-29879

Contents

Chapter II. Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

List of Tables

Chapter I.

Chapter II.

List of Figures

Chapter I.

Chapter II.

List of Abbreviations

A260	:	Absorbance value at 260 nm
A280	:	Absorbance value at 280 nm
ABTS	:	2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA	:	Analysis of variance
BPA	:	Baird-Parker agar
CAP	:	Cold atmospheric plasma
CLSM	:	Confocal laser scanning microscopy
CO	:	Clove oil
DNA	:-	Deoxyribonucleic acid
DPPH	:	1,1-Diphenyl-2-picrylhydrazyl
EAP	:	Encapsulated atmospheric pressure plasma
EI	:	Electron impact ionization
EMB	:	Eosin-methylene blue agar
EtOH	:	Ethanol
FDA	:	Food and drug administration
GC-MS	:	Gas chromatography-mass spectrometry

GRAS	:	Generally recognized as safe
MBC	:-	Minimum bactericidal concentration
MIC	:	Minimum inhibitory concentration
PBS	:	Phosphate buffer saline
PI	:	Propidium iodide
ROS	:	Reactive oxygen species
SAS	:	Statistical analysis system
SEM		Scanning electron microscopy
TBA	:-	2-Thiobarbituric acid
TEM	:	Transmission electron microscopy
TSA	:	Tryptic soy agar
TSB	:	Tryptic soy broth
UV	:	Ultraviolet Ray

Chapter I.

Literature review

1.1. Clove oil

1.1.1. Use of clove oil in food industry

Clove oil is an essential oil derived from buds, stems, and leaves of clove plants (*Syzygium aromaticum*) and has various functions including antiviral, antioxidant, antifungal, and antibacterial activity (Chaieb et al., 2007). Since consumers' demand for a natural product, the use of clove oil which is classified as generally recognized as safe (GRAS) by United States Food and Drug Administration (FDA) has increased in the food industry (Prakash et al., 2012).

The antioxidant activity of clove oil was demonstrated by Gülçin et al. (2012) using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and ferrous ions (Fe^{2+}) chelating activities, and ferric ions (Fe^{3+}) reducing power assay. Clove oil was found to be an effective antioxidant compared to the other antioxidants such as alpha-butylated hydoxy anisole, dibutyl hydroxyl toluene, tocopherol, and trolox when used at the same concentration (45 μg/mL). Therefore, the use of clove oil can prolong the shelf life of food by preventing lipid oxidation. For example, the lipid oxidation of the minced beef was measured by the 2-Thiobarbituric acid (TBA) method during

the storage period, and when stored for 9 d at 4℃, the TBA value of the clove oil-treated samples was half of that of the control group (Zengin and Baysal, 2015).

Clove oil is also utilized as antimicrobial agents that can effectively control food-borne pathogens and extend shelf life of foods (Voon et al., 2012) and many studies have demonstrated antimicrobial activity of clove oil. Clove oil not only inhibit *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *S. aureus* (Oussalah et al., 2007) *in vitro* but also effectively inactivate pathogens which are inoculated on foods (Jayasena and Jo, 2013).

1.1.2. Bactericidal mechanisms of clove oil

Since the major component of clove oil is eugenol, bactericidal mechanisms of clove oil or eugenol have been widely studied (Xu et al., 2016). Scanning electron microscopy (SEM) images confirmed that clove oil caused irreversible changes in the microstructure of microorganisms and damages cell membranes (Chami et al., 2005). Damaged cell membranes caused changes in membrane permeability and leakage of intracellular materials such as protein and DNA (Zhang et al., 2016). In addition, clove oil also inactivated the bacteria by affecting not only key enzymes such as α-ketoglutarate dehydrogenase and isocitrate dehydrogenase but also metabolites including succinic acid of the TCA cycle (Cui et al., 2018a). The bactericidal mechanism of essential oils is summarized and shown in Fig. 1.

When examined at the genetic level, eugenol inhibited biofilm formation by

down-regulating *icaD*, *sarA*, and *seA* genes of *S.aureus* (Yadav et al., 2015). Also, eugenol prevented bacterial adhesion, invasion, migration, and metabolism by reducing the expression of *Salmonella* Enteritidis virulence genes (Upadhyaya et al., 2013).

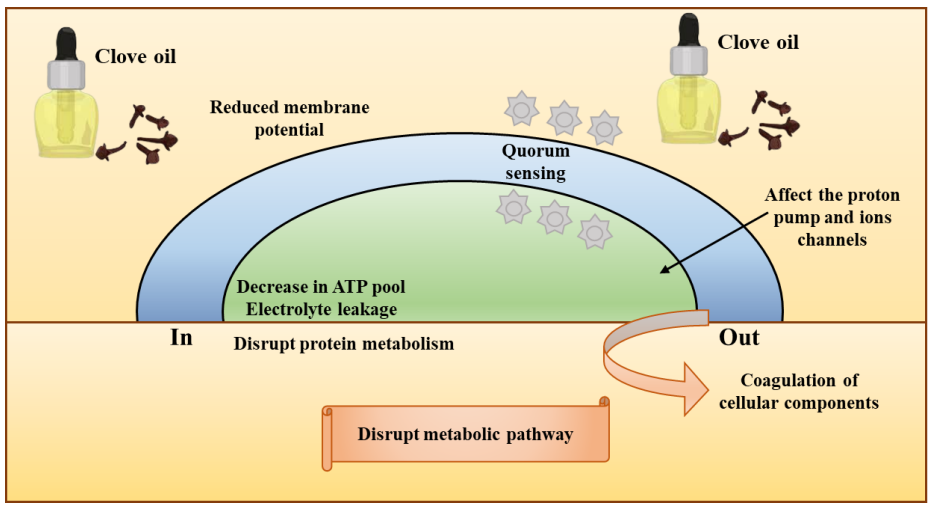

Fig. 1. Bactericidal mechanisms of essential oil (modified from Swamy et al., 2016).

1.1.3. Limitation of clove oil

Essential oils should be used in high concentrations to inactivate bacteria in foods. However, high concentrations of clove oil are expensive, potentially toxic, and have a strong aroma, degrading food quality (Prakash et al., 2015).

Additionally, Gram-positive bacteria such as *L. monocytogenes* and *S. aureus* do not have a lipopolysaccharide outer membrane, so they interact directly with the hydrophobic clove oil and inactivation is effective. However, Gram-negative bacteria such as *S.* Typhimurium and *E. coli* are relatively difficult to inactivate due to the outer membrane of lipopolysaccharide (Gyawali et al., 2015).

Therefore, clove oil needs to be used in combination with other antimicrobial agents at the lowest concentration as possible in a synergistic way to maintain the quality of foods and to inactivate both Gram-negative and Gram-positive pathogens (Martínez-Graciá et al., 2015).

1.2. Cold atmospheric plasma (CAP)

1.2.1. Use of CAP in food industry

CAP has recently emerged as a non-thermal technology in the food industry for the purpose of packaging, changing food properties, degradation of pesticide residues, and controlling microorganisms (Ekezie et al., 2017). The advantage of atmospheric pressure plasma is that, unlike low- or high-pressure plasma, it does not require a device to maintain pressure, so the operating cost is efficient (Park et al., 2000).

Various studies have been conducted on the application of CAP to food packaging, and Pankaj et al. (2014) reviewed that the properties of food packaging polymers, including polyethylene, polypropylene, and polyethylene terephthalate, were changed when CAP was treated. Also, Song et al. (2016) demonstrated the possibility of increasing the biodegradability of the polylactic acid film by CAP without causing negative changes in the film.

Thirumdas et al. (2017) demonstrated that reactive species produced by CAP treatment can alter the composition of starch molecules, resulting in changes to swelling, adhesion properties, water absorption, enzyme susceptibility, solubility, composition, structure, and thermal properties of starch. In addition, Lee et al. (2019) studied the changes in physicochemical properties of CAP-treated three cultivars of brown rice. Water binding capacity increased with increasing plasma treatment time irrespective of cultivars, but other characteristics showed differences according to cultivars. Also, Yong et al. (2017) manufactured pork jerky

with CAP treatment and confirmed the possibility of replacing sodium nitrite in the manufacturing of pork jerky considering color, pigment, lipid oxidation, and shear force.

Recently, CAP has been used for the degradation of residual pesticides in foods. For example, pesticides such as azoxystrobin, cyprodinil, fludioxonil and pyriproxyfende were effectively reduced after CAP treatment in strawberries (Misra et al., 2014).

Numerous studies have demonstrated that a wide range of microorganisms in foods are inactivated by treatment with CAP. Lee et al. (2017a) reviewed the reduction of both Gram-positive and Gram-negative pathogens in meat products including ham, chicken breast, bacon, pork loin, pork butt, beef loin, and beef jerky according to plasma treatment discharge gas, power, frequency, exposure time, type, and distance.

1.2.2. Bactericidal mechanisms of CAP

Plasma, which is ionized gas, is composed of antimicrobial substances including electrons, charged ions, reactive species, ultraviolet photons (An et al., 2019). The reactive species have been reported to chemically and physically damage cells by penetrating through microbial cell membranes and reacting with macromolecules such as lipids and proteins in cell membranes and DNA within cells (Fig. 2; Hertwig et al., 2018).

Reactive oxygen species (ROS) such as superoxide, H_2O_2, and OH· cause lipid oxidation (especially, polyunsaturated fatty acids) and protein modulation on bacterial membrane, causing damage to the cell membrane and lead to cell leakage (Montie et al., 2000). Also, oxidation of nucleic acids and amino acids by ROS causes bacterial damage. According to Perni et al. (2007), oxygen atoms generated in CAP mainly contribute to mutation of *E. coli* K-12. Morphological changes of *E. coli* after CAP treatment were achieved by SEM images, CAP-treated bacterial cell shown not only shrinkage, disruption, and damage of cell wall but also disruption and lysis of bacterial cell (Ulbin-Figlewicz et al., 2015). Additionally, agarose gel electrophoresis confirmed that DNA damage increased with increasing CAP treatment time (Lu et al., 2014).

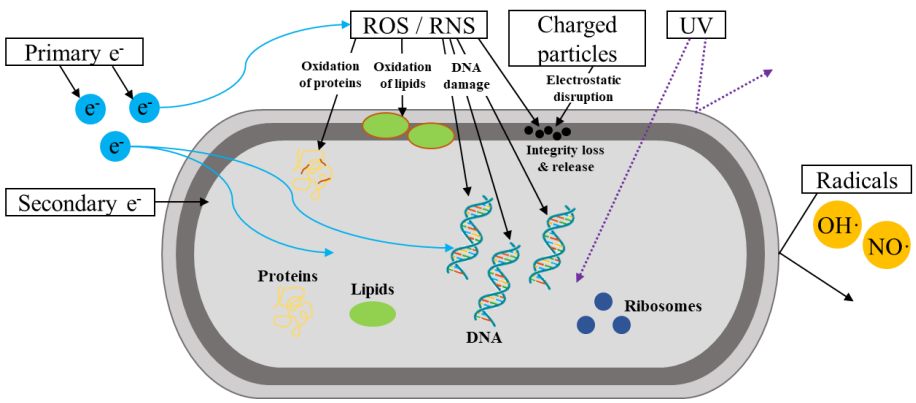

Fig. 2. Bactericidal mechanisms of cold atmospheric plasma (modified from Hertwig et al., 2018).

1.2.3. Limitation of CAP

Despite the numerous benefits of CAP mentioned above, there are some limitations. Firstly, since the efficiency of plasma treatment is greatly influenced by the states of the foods and the appearance of the surface, the bactericidal effect is different under the same conditions depending on the foods (Pinela and Ferreira, 2017). Although all conditions were the same, the effect of CAP on lettuce, cucumber, and carrot was different because roughness and hardness of samples' surfaces were different (Cui et al., 2018b).

Secondly, it is difficult to obtain a uniform bactericidal effect from plasma treatment because the variation according to microbial physicochemical state, survival time of active species, relative humidity, distance from the food, and sample exposure method is large (Surowsky et al., 2015). In detail, if the distance between the surface and the CAP is relatively close, it is mainly influenced by the reactive species with shorter half-life, but relatively long distance is affected by the reactive species with longer half-life (Stoffels et al., 2006).

Thirdly, the reactive species produced by the CAP reacts with the lipids in the food, resulting in lipid oxidation, which can reduce food quality (Gavahian et al., 2018). For example, TBA values of pork putt and beef loin increased significantly with longer CAP treatment time, because lipid oxidation was prompted by reactive species and produce byproducts including hexanal and malondialdehyde (Jayasena et al., 2015).

1.3. Synergistic effect

Synergistic effect means that when two or more single actions are used for one purpose, they achieve nonlinear cumulative effects (Elishalom, 2005). When clove oil was used in combination with lactic acid, it controlled the growth of microorganisms and lowered the TBA values, resulting in a synergistic effect in extending shelf life when compared with single treatment without changing the color and odor (Naveena et al., 2006). In addition, the combination of clove oil and chitosan showed a greater change in cell morphological changes, membrane damage, and intracellular leakage than in the single treatment, and showed synergistic effects on antifungal activity (Shao et al., 2015).

Not only clove oil but also CAP has been demonstrated synergistic effects in combination with various antimicrobial agents. For example, the combination of CAP with bacteriophage, a ubiquitous virus, was effective in removing *E. coli* O157: H7 biofilm from vegetables and caused greater changes in cell morphological and physiological properties than single treatment (Cui et al., 2018b). Furthermore, several studies have suggested the possibility of increased antimicrobial activity and more effective microbial inactivation in the combination of essential oil and CAP (Cui et al., 2016; Matan et al., 2014).

Chapter II.

Synergistic bactericidal effect and its mechanisms of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

*This study will be published in elsewhere as a fulfillment of Ji Hyun Yoo's M.S. program.

2.1. Introduction

As the consumption of meat products have increased continuously, the incidence of food poisoning is a constant problem (Feng et al., 2019). Pathogen contamination that causes food poisoning can occur during the production, processing, packaging, distribution, and consumption of meat products (Nerin et al., 2016). Among the pathogens, *Escherichia coli* O157:H7 and *Staphylococcus aureus* are the major bacteria that cause sitotoxism with serious abdominal pain,

diarrhea, fever, and vomiting in humans (Wei et al., 2018). Therefore, various efforts have been carried out to inactivate these microorganisms (Li and Farid, 2016). Since traditional thermal sterilization techniques negatively affect the nutritional value and sensory quality of meat products, interest in non-thermal sterilization has been increasing in the meat industry (Liao et al., 2017).

Although there have been several non-thermal sterilization techniques including pulsed light, ionizing radiation, hydrostatic pressure, high-power ultrasound, and ultraviolet light, there has been recent interest in cold atmospheric pressure plasma (Pinela and Ferreira, 2017). Plasma is the fourth state of matter and can inactivate microorganisms by forming reactive species including free radicals, electromagnetic fields, UV light, and charged particles (Yong et al., 2015). The advantages of cold atmospheric pressure plasma are that it does not affect the nourishment or quality of meat products compared to that of thermal sterilization, and is relatively inexpensive and easy to install against other non-thermal sterilization technologies (Lee et al., 2016). Numerous studies have successfully confirmed the possibility of inhibiting microorganisms by generating cold plasma through dielectric barrier discharge (DBD; Lee et al., 2017b).

Even though cold atmospheric pressure plasma has many advantages as mentioned above, there are some limitations in the use of plasma for completely controlling foodborne pathogens in foods (Hertwig et al., 2015; Surowsky et al., 2015). For example, Yong et al. (2017) studied the microbial safety of pork jerky inoculated with *S. aureus* and *Bacillus cereus* by atmospheric pressure DBD

plasma, but the resulting reduction was less than 1.0 Log CFU/g despite 60 min of plasma treatment (initial cell count: 5–6 Log CFU/g). Similarly, Bermúdez-Aguirre et al. (2013) reported that when tomatoes and cabbages were treated with atmospheric pressure cold plasma at 11.45 kV and 60 Hz for 10 min, both samples showed less than a 1-Log reduction of *E. coli* ATCC 11775 (initial cell count: 5 Log CFU/g). Therefore, to achieve effective microbial decontamination, efforts have been made to combine plasma with effective and safe antibacterial materials such as essential oils, bacteriophages, and lactic acid (Matan et al., 2014; Cui et al., 2018b; Trevisani et al., 2017).

Essential oils, which are hydrophobic liquids concentrated from plants, have been widely studied for their antimicrobial activity and efficacy because of the consumer's demand for natural food additives (Jayasena and Jo, 2013). Among the essential oils, clove oil classified as ‘generally recognized as safe (GRAS)’ by the FDA is used for its antifungal, antioxidant, and anti-aflatoxin effects (Prakash et al., 2012). Clove oil has also been demonstrated to effectively inactivate foodborne pathogens such as E. coli O157:H7, *S.* Typhimurium, *L. monocytogenes*, and *S. aureus* (Oussalah et al., 2007). However, due to its application cost, strong flavor, and the potential for toxicity at high concentrations, methods to effectively control microorganisms at low concentrations are needed (Shao et al., 2015). In this regard, some studies on synergistic bactericidal effects by combined treatment of cold atmospheric pressure plasma and essential oils have been conducted (Matan et al., 2014; Cui et al., 2016), but the mechanism has not been completely studied and elucidated.

Therefore, the purpose of this study was (1) to investigate the effects of the combined treatment of clove oil and cold atmospheric pressure plasma on the inactivation of *E. coli* O157:H7 and *S. aureus* to elucidate the mechanism of the synergistic bactericidal effect, and (2) to show the potential for application to beef jerky.

2.2. Materials and methods

2.2.1. Material, bacterial strain, and cultural methods

Clove oil extracted by steam distillation of clove buds, leaves, and stems was purchased from NOW Foods (IL, USA). The bacterial strains including the Gram-negative bacterium *E. coli* O157:H7 (NCCP 15739) and Gram-positive bacterium *S. aureus* (NCTC 7447) were obtained from National Culture Collection for Pathogen (Osong, Korea) and National Collection of Type Cultures (Colindale, England), respectively. Both bacterial cultures were stored in a deep freezer at -70℃ and cultivated in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 37℃ for 24 h. The cultures were centrifuged (2,265 ×g for 15 min at 4℃) using a refrigerated centrifuge (Continent 512R, Hanil Science Industrial Co. Ltd., Incheon, Korea) and washed twice with sterile 0.85% saline solution. The resulting pellets were re-suspended in sterile 0.85% saline solution to a final concentration of 108 to 109 CFU/mL (OD600 = 0.2).

2.2.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To determine the antibacterial activity of clove oil, the MICs and MBCs for clove oil against *E. coli* O157:H7 and *S. aureus* were determined using a method by Bajpai et al. (2013). Clove oil was added to TSB (10 mL) inoculated with 10^8 to 10^9 CFU/mL of bacterial cells to a final concentration of 0.0125, 0.025, 0.05, 0.1,

and 0.2%, respectively, and incubated at 37℃ for 24 h. The MIC of clove oil was determined as the lowest concentration that completely inhibited the growth of the inoculated bacteria. In addition, the MBC was obtained as the minimum concentration that inhibited the growth of colonies when bacterial suspensions that were incubated with different clove oil concentration were cultured on tryptic soy agar (TSA; Difco, Detroit, MI, USA) at 37℃ for 24 h.

2.2.3. Encapsulated atmospheric pressure plasma (EAP)

Encapsulated atmospheric pressure dielectric barrier discharge plasma was generated from a plasma apparatus that was used previously as described by Yong et al. (2015). For simplicity, the conductive copper tape attached to the wall of the cuboid's container (13.7×10.4×5.3 cm) was the power electrode, the ultrahigh molecular weight polyethylene sheet on it was the dielectric layer, and the copper tape on it served as the ground electrode. The samples were placed in a petri dish (60×15 mm) in the center of the container, and atmospheric air was utilized as a carrier gas. The discharge conditions were 2.2 kHz and 8.4 kVpp.

2.2.4. Bactericidal effect of clove oil and EAP

To investigate the bactericidal effect of clove oil, the clove oil was added to 10 mL of *E. coli* O157:H7 and *S. aureus*, respectively to the final concentration of MIC (0.05%) and reacted at 37℃ for 3 h under 120 rpm agitation. Separately, to

assess the bactericidal effect of EAP, the planktonic *E. coli* O157:H7 and *S. aureus* (10 mL) were placed in a petri dish (60×15 mm) and treated with EAP for 0, 1, 2, 3, and 4 min in the center of the container. The combined treatment was applied to develop a synergistic bactericidal effect, and planktonic *E. coli* O157:H7 and *S. aureus* (10 mL) were treated with the MIC of clove oil at 37℃ for 3 h followed by 4 min and 2 min of EAP treatment, respectively. After all treatments, the number of viable bacteria were analyzed using the viable plate counts method. The treated *E. coli* O157:H7 and *S. aureus* were serially diluted in sterile 0.85% saline, and 100 μL of both bacterial diluent was spread on TSA (Difco) plates. The agar plates were incubated for 24 h at 37℃, and then the total number of colonies were counted, which was expressed as Log CFU/mL.

2.2.5. Mechanism of synergistic bactericidal effect of clove oil and EAP

2.2.5.1. Disc-diffusion assay

The antibacterial activities of clove oil against *E. coli* O157:H7 and *S. aureus* were determined using the disc diffusion assay method (Devi et al., 2010). The cultures of both pathogens were adjusted to approximately 10^8 CFU/mL and spread (100 μL) on TSA (Difco), respectively. The sterile paper discs (Ø 8 mm; Advantec, Tokyo, Japan) were evenly sprinkled with 50 μL of the MIC (0.05%) of clove oil (NOW Foods) or 0.05% clove oil treated with EAP for 15 min. The paper disc was placed in the center of the agar and incubated at 37℃ for 24 h, and then the diameter of the clear zone was determined.

2.2.5.2. Gas chromatography-mass spectrometry (GC-MS)

The change in the composition of the clove oil after 15 min of EAP treatment was analyzed using a Thermo Trace 1300 GC (Thermo Scientific, MA, USA), equipped with a DB-5 MS capillary column (60 m×0.25 mm; film thickness, 25 μm) and an ISQ MS (Thermo Scientific, MA, USA). The mass selective detector was operated with the electron impact ionization (EI) method with a mass scan range from 35 to 550 amu at 70 eV and ion source temperature was 270℃. The carrier gas was helium at 1.0 mL/min, and a sample of 1.0 μL was injected using a 1:20 split ratio. Injector and MS transfer line temperatures were both 250℃. The oven temperature was initiated from 50℃ (hold of 5 min); raised to 65℃ (hold of 30 min) at 10℃/min; raised to 120℃ (hold of 10 min) at 5℃/min; raised to 120℃ (hold of 10 min) at 5℃/min; raised to 210℃ (hold of 10 min) at 5℃/min; and finally raised to 325℃ (hold of 10 min) at 20℃/min. Quantification was achieved by calculating the relative percent peak area, and then the components were identified by comparison of their mass spectra and retention indices with the NIST mass spectral search program.

2.2.5.3. Membrane permeability

The integrity of the bacterial membrane was determined by UV absorption using a spectrophotometer (X-ma 3100, Human Co. Ltd.). *E. coli* O157:H7 and *S. aureus* treated with clove oil, EAP, or clove oil combined with EAP were centrifuged at 12,000 ×g for 10 min, respectively (untreated samples were used as

control). Then, the leakage of nucleic acid and protein were analyzed by measuring the absorbance of supernatants at 260 and 280 nm (A260 and A280), respectively.

2.2.5.4. Confocal laser scanning microscopy (CLSM)

To evaluate the live/dead states of *E. coli* O157:H7 and *S. aureus*, the BacLight™ Live/Dead Bacterial viability kit (L-7012; Molecular Probes, Eugene, OR, USA) was used in this study. Bacterial cells treated with each treatment (control, clove oil, EAP, clove oil + EAP) were centrifuged at 8000 ×*g* for 5 min and resuspended in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Then, an equal volume of the two DNA-binding dyes, (1) SYTO 9 (green fluorescence) and (2) propidium iodide (PI, red fluorescence), were thoroughly mixed. The mixed reagent (3 μL) was added to 1 mL of the treated *E. coli* O157:H7 and *S. aureus*, and then incubated in a dark room for 20 min at room temperature. Each sample (5 μL) was then dropped onto a microscope slide (Paul Marienfeld GmbH & Co. KG, Laud-königshofen, Germany) and covered with cover glass (Marienfeld Superior). Live/dead cells were detected by CLSM (Leica TCS SP8 X, Wetzlar, Germany) using appropriate filters with excitation/emission wavelengths at 483/490–540 nm for SYTO 9 and excitation/emission wavelengths at 535/590–680 nm for PI. The green fluorescence (SYTO 9) and red fluorescence (PI) indicated that the bacterial membrane was either intact or damaged, respectively.

2.2.5.5. Transmission electron microscopy (TEM)

To determine the morphological changes after each treatment, TEM was performed. *E. coli* O157:H7 and *S. aureus* were untreated (control) or treated with 0.05% clove oil, EAP, and 0.05% clove oil + EAP, respectively, and centrifuged at 8,000 ×g for 10 min, and then resuspended in 0.1 M PBS (pH 7.4). The cell pellets were obtained by centrifugation at 8,000 ×g for 10 min and initially fixed overnight with Karnovsky's fixative at 4°C. After the primary fixation, each sample was washed three times with 0.05 M sodium cacodylate buffer for 10 min at room temperature, followed by post-fixation in 1% osmium tetroxide diluted in 0.05 M sodium cacodylate buffer for 2 h at 4℃. Then, all samples were washed three times with distilled water for 10 min at room temperature, and then the staining was performed using 0.5% uranyl acetated diluted in distilled water at 4℃ overnight. After staining, the samples were rinsed three times with distilled water and were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 3 times with 100% ethanol) for 10 min in each series. For transition, each sample was sequentially replaced with 100% propylene oxide twice for 15 min, propylene oxide and Spurr's resin (1:1) for 2 h, propylene oxide and Spurr's resin (1:2) for 2 h, and Spurr's resin for 24 h at 4°C, and Spurr's resin for 3 h at room temperature. The sample was polymerized for 24 h at 70℃ and sectioned using an ultramicrotome (MT-X, RMC, Tucson, AZ, USA). The TEM (JEM-1010, JEOL, Tokyo, Japan) at 80 kV was used to observe the microstructure of each bacterial cell.

2.2.6. Bactericidal effect of clove oil and EAP on beef jerky

2.2.6.1. Sample preparation and inoculation

The beef jerky was purchased from the local food market and cut to a size of 35×35 mm and weighed 5 g. *E. coli* O157:H7 and *S. aureus* were cultivated in TSB for 24 h at 37℃ and then centrifuged at 2265 ×g for 15 min in a refrigerated centrifuge (Continent 512R, Hanil Science Industrial Co. Ltd.). The bacterial pellets were washed twice using a sterile 0.85% saline, and 100 μL of each cell suspension was inoculated on beef jerky to a final concentration of 10^8 CFU/g and dried for 30 min at room temperature on a clean bench.

2.2.6.2. Bactericidal effects of clove oil and EAP on beef jerky

Inoculated beef jerky samples were treated with EAP for 0, 5, 10, and 15 min, respectively, to determine the optimal EAP treatment time. Independently, the samples were treated with clove oil (100 μL) dissolved in 95% ethanol (EtOH; Baker Analyzed Reagent, J.T. Baker Chemical Co., NJ, USA) to a final concentration of 0.50%, 1.00%, and 1.50%, respectively, and then dried for 2 h. Additionally, samples treated only with ethanol (J.T. Baker Chemical Co.) were used as a negative control. Finally, to determine the synergistic bactericidal effects of combined treatment of clove oil and EAP on beef jerky, the optimum EAP treatment time was subsequently used with the previous clove oil treatment (EtOH/0.5%/1.0%/1.5% clove oil + 15 min EAP).

2.2.6.3. Microbial analysis

Immediately after the treatment, each beef jerky sample was diluted with 45 mL of 0.85% sterile saline and blended using a stomacher (BagMixer 400, Interscience, St Nom, France) for 2 min. Then, the mixed samples were decimally diluted in saline aqueous solution. The eosin-methylene blue agar (EMB; Difco) and Baird-Parker agar (BPA; Difco) with egg yolk tellurite enrichment (Difco) were prepared for cultivation of *E. coli* O157: H7 and *S. aureus*, respectively. The selective agar plates were incubated overnight at 37℃, and all colonies were counted and expressed as Log CFU/g.

2.2.7. Statistical analysis

All experiments were conducted in three replicates. Statistical analysis was calculated using the Student's t-test and one-way Analysis of Variance (ANOVA). Significant differences among data were determined by Tukey's multiple test with SAS software version 9.4. (SAS Institute Inc., NC, USA) with the confidence level at $P<0.05$. The obtained results were reported as means and standard deviation.

2.3. Results and discussion

2.3.1. Bactericidal effect of individual treatment of clove oil and EAP

The MIC and MBC of clove oil against both *E. coli* O157:H7 and *S. aureus* were 0.05% and 0.10%, respectively. Zengin and Baysal (2015) also reported that the MIC of clove oil against *E. coli* O157:H7 and *S. aureus* was 0.05%. Therefore, the concentration of 0.05% was chosen for verification of the bactericidal effect of clove oil.

The reductions in *E. coli* O157:H7 and *S. aureus* by different EAP treatment times are shown in Fig. 1. As the EAP treatment time increased, the number of both pathogens decreased. The number of *E. coli* O157:H7 in the control group was 7.98 Log CFU/mL, and after 4 min of EAP treatment, the bacterial count was 6.20 Log CFU/mL which showed a significant bactericidal effect. The number of *S. aureus* in the control group was 9.50 Log CFU/mL, and significantly decreased to 7.98 Log CFU/mL after 2 min of EAP treatment, but no significant difference was found after 2, 3, and 4 min of EAP treatment. Pursuantly, the EAP treatment time of 4 min and 2 min was used for the *E. coli* O157:H7 and *S. aureus* suspension, respectively, to confirm the synergistic effect of the clove oil and EAP treatment. Also, Han et al. (2016) demonstrated that the number of viable *E. coli* and *S. aureus* cells decreased in proportion to the plasma treatment time.

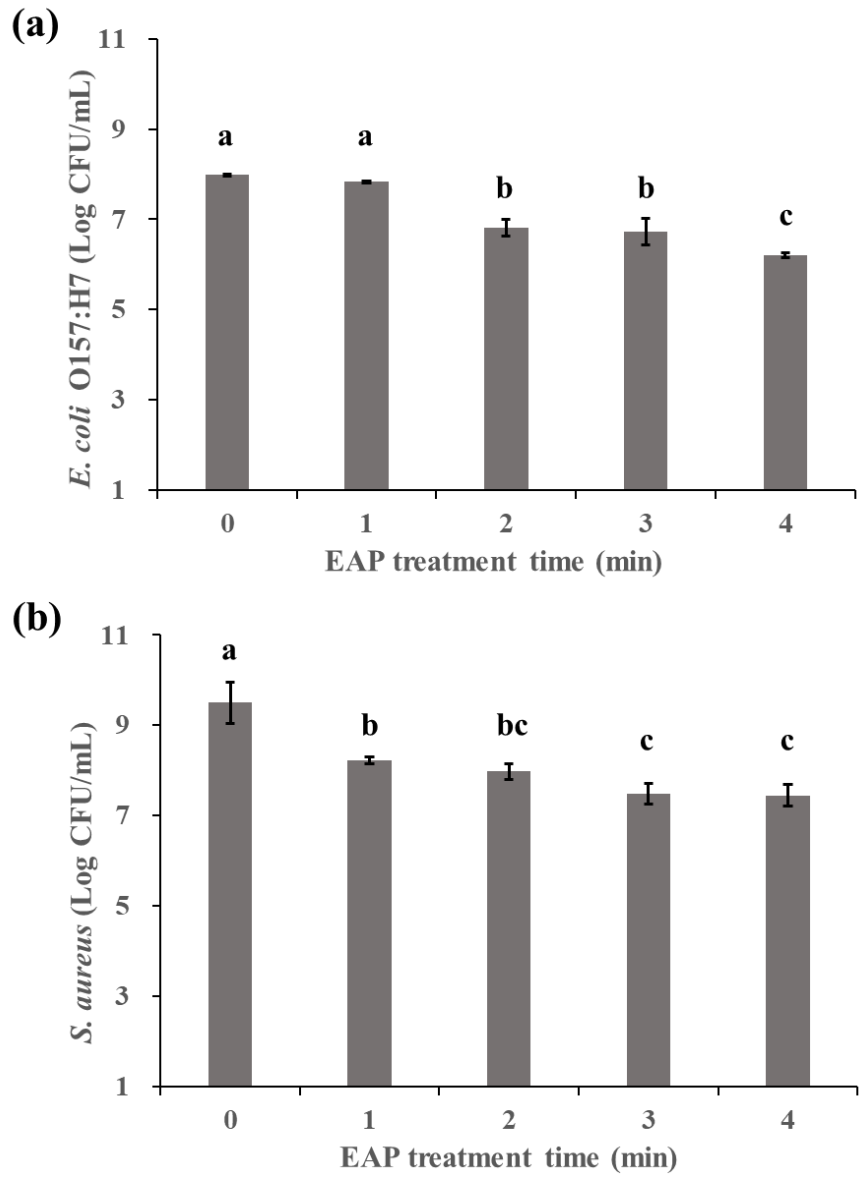

Fig. 1. The population (Log CFU/mL) of E. coli O157:H7 (a) and S. aureus (b) after different encapsulated atmospheric pressure plasma (EAP) treatment times (0, 1, 2, 3, and 4 min). Error bars indicate standard deviation. [a-c]Different letters indicate significant differences (P<0.05).

2.3.2. Bactericidal effects of the combined treatment of clove oil and EAP

The microbial inhibition patterns of the combined treatment of clove oil and EAP are depicted in Fig. 2. The initial microbial count of *E. coli* O157:H7 was 8.54 Log CFU/mL, and after the single treatment with clove oil (0.05%) or EAP (4 min), the microbial counts were 7.54 and 5.81 Log CFU/mL, respectively. However, when 0.05% clove oil and 4 min of EAP were combined-treated, the bacteria were completely controlled (detection limit: 10^1 CFU/mL). Moreover, in *S. aureus*, the initial microbial counts were 9.39 Log CFU/mL and decreased to 6.86 and 7.97 Log CFU/mL after the clove oil (0.05%) and EAP (2 min) single treatment, respectively. However, in the combined treatment, there were no viable bacterial cells observed, and the reduction was more than 8 Log CFU/mL. In conclusion, the combined treatment of clove oil and EAP showed considerable synergistic microbial inhibition effect against both *E. coli* O157:H7 and *S. aureus*.

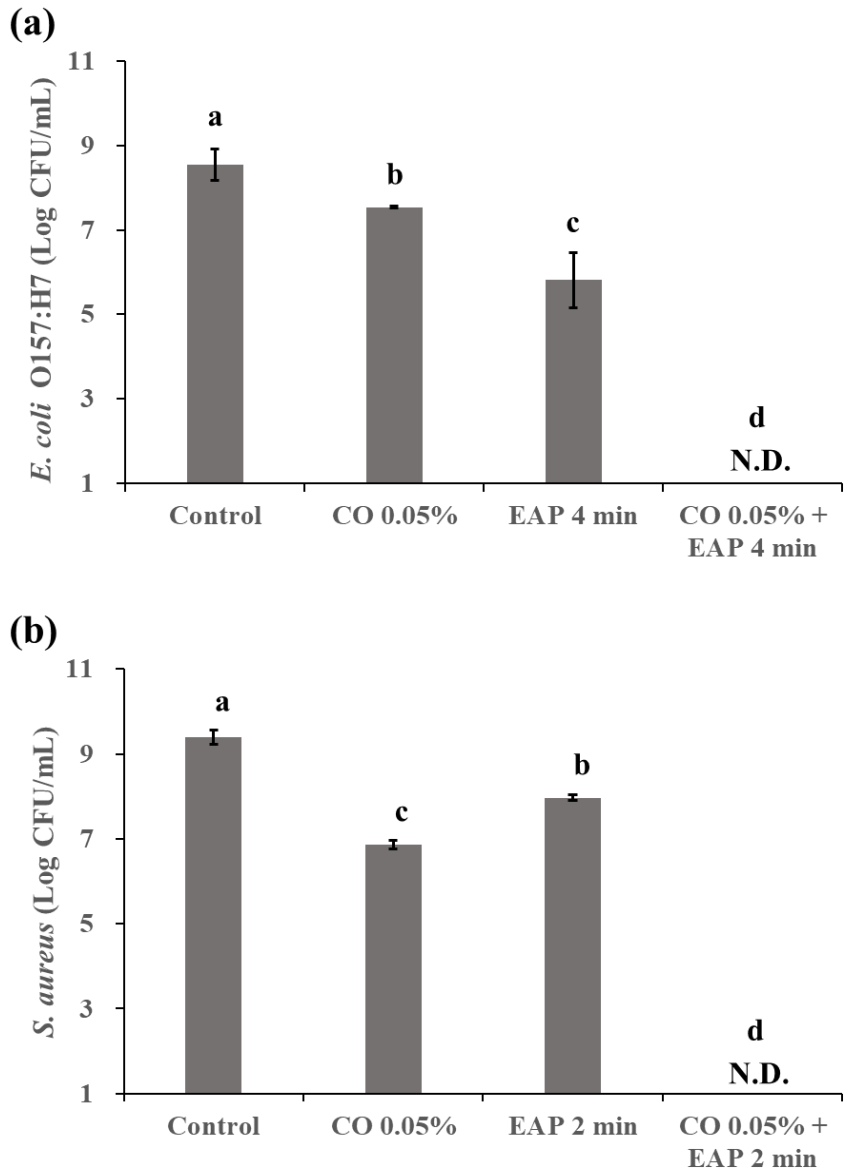

Fig. 2. The population (Log CFU/mL) of *E. coli* O157:H7 (a) and *S. aureus* (b) that survived after the single or combined treatment of 0.05% clove oil (CO) and encapsulated atmospheric pressure plasma (EAP). Error bars indicate standard deviation. [a-d]Different letters indicate significant differences (P<0.05).

2.3.3. Mechanism of the synergistic bactericidal effect of clove oil and EAP

2.3.3.1. Disc-diffusion assay

The disc-diffusion assay was performed to evaluate the changes in antibacterial activity of clove oil before and after the EAP treatment (Fig. 3). The clear zones of 0.05% clove oil against *E. coli* O157:H7 and *S. aureus* were 10.5 mm and 9.0 mm, respectively. After 15 min of EAP treatment on 0.05% clove oil, the clear zones against *E. coli* O157:H7 and *S. aureus* were 10.7 mm and 10.5 mm, respectively, which showed no significant difference before and after the EAP treatment on clove oil. However, Kim et al. (2014a) demonstrated that 20 min of plasma treatment to naringin, which had no microbial inhibition ability initially, increased the clear zone diameter from 8 mm to 16 mm in *E. coli* O157:H7 and from 8 mm to 18 mm in *S. aureus*. Therefore, it indicated that EAP treatment on 0.05% clove oil did not result in any changes in the antibacterial activity of the clove oil in the present study.

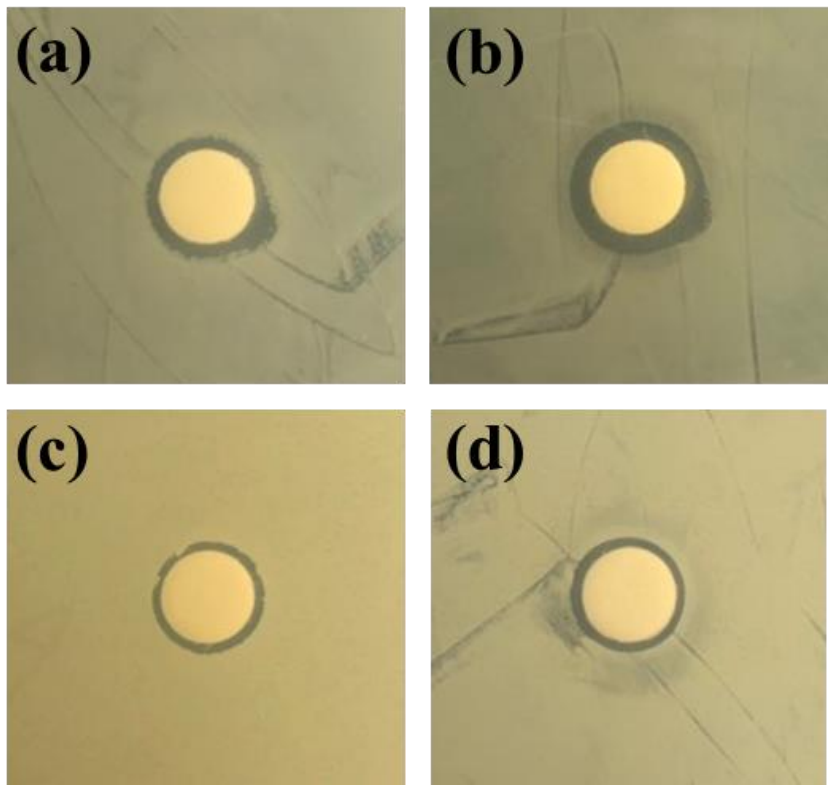

Fig. 3. Agar diffusion assay at the minimum inhibitory concentration (MIC) of 0.05% clove oil against *E. coli* O157:H7 and *S. aureus* before (a, c) and after (b, d) 15 min of encapsulated atmospheric pressure plasma (EAP) treatment.

2.3.3.2. GC-MS

The chemical composition of clove oil before and after the EAP treatment was analyzed using GC-MS to evaluate any possible chemical changes in clove oil by the EAP treatment (Table 1). Ten components accounting for more than 0.03% were identified, representing 98.24% and 97.88% of the total before and after the EAP treatment, respectively. The major component of clove oil was eugenol, which accounted for 88.90% and 91.57% before and after the EAP treatment, respectively. Eugenol, a phenolic compound, represent the component with the largest proportion in clove oil and plays a major role in its antibacterial activity (Jayasena and Jo, 2014). Although a few studies have reported that plasma alters the chemical structure and biological activity of natural materials (Kim et al., 2017), that of eugenol was not changed by the EAP treatment in this study. Additionally, cayrophyllene, the component with the second largest proportion followed by α-caryophyllene and caryophyllene oxide was considered. Only caryophyllene oxide showed a significant decrease after EAP treatment, but only in very small quantities. Considering the results of the paper disc diffusion assay and GC-MS analysis, we confirmed that EAP treatment cannot increase the antibacterial activity of clove oil itself.

Table 1. Changes in chemical composition of clove oil before and after EAP treatment[a,b].

Compound	Retention time	Pure clove oil	Pure clove oil + 15 min of EAP
		Peak Area (%)	
(3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one	14.17	0.04 ± 0.02	0.02 ± 0.01
Eugenol	65.95	88.90 ± 0.49	91.57 ± 1.00
α -Copaene	67.08	0.04 ± 0.01	0.03 ± 0.00
β-Caryophyllene	69.01	8.07 ± 0.46	5.21 ± 1.07
α-Caryophyllene	70.35	0.94 ± 0.02	0.69 ± 0.09
Seychellene	71.75	0.03 ± 0.01	0.04 ± 0.01
δ-Cadinene	72.38	0.07 ± 0.01	0.07 ± 0.01
1S,CIS-Calamenene	72.51	0.04 ± 0.00	0.04 ± 0.00
Caryophyllene oxide	74.53	0.11 ± 0.00	0.08 ± 0.01*
Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)-(CAS)	77.05	0.00 ± 0.00	0.12 ± 0.08
Total		98.24	97.88

[a]Data represent the mean ± standard deviation
[b]Student's *t*-test; *, $P<0.05$ with respect to the untreated control.

2.3.3.3 Membrane permeability

The leakage of cellular nucleic acid and protein indicates the integrity of the cell membrane (Jenie et al., 2008). Fig. 4 shows changes with respect to the A260 and A280 values; the untreated bacteria (control) showed the lowest A260 and A280 values for both *E. coli* O157:H7 and *S. aureus*, which indicated that there were relatively lower amounts of extracellular nucleic acids and proteins than that of clove oil or/and EAP-treated bacteria.

In *E. coli* O157:H7, the A260 value significantly increased after the clove oil and EAP combined treatment, which indicated that the leakage of nucleic acid was the highest among the treatments. However, no significant difference was found between clove oil and EAP single treatments, which showed a different pattern when compared with the results of viable counts.

In both bacterial strains, the A280 value showed only a significant difference in the clove oil and EAP combined treatment. Zhang et al. (2016) reported that as the concentration of clove oil and reaction time increased, 260 nm-absorbing material increased via changes in the trans-membrane transport processes of *S. aureus*. Cold plasma has also been shown to impair the cell membrane integrity of *E. coli* and *L. monocytogenes* as treatment time increases, as indicated by increased A260 and A280 values (Lu et al., 2014). Similarly, the combination of chitosan and clove oil against *Penicillium digitatum*, which induces citrus green mold, resulted in higher A260 values than either control or single treatments with either of the 2 substances (Shao et al., 2015).

Therefore, the combined treatment of clove oil and EAP synergistically damaged the cell membrane, increased the permeability of the cell membrane, and consequently resulted in more leakage of nucleic acids and proteins.

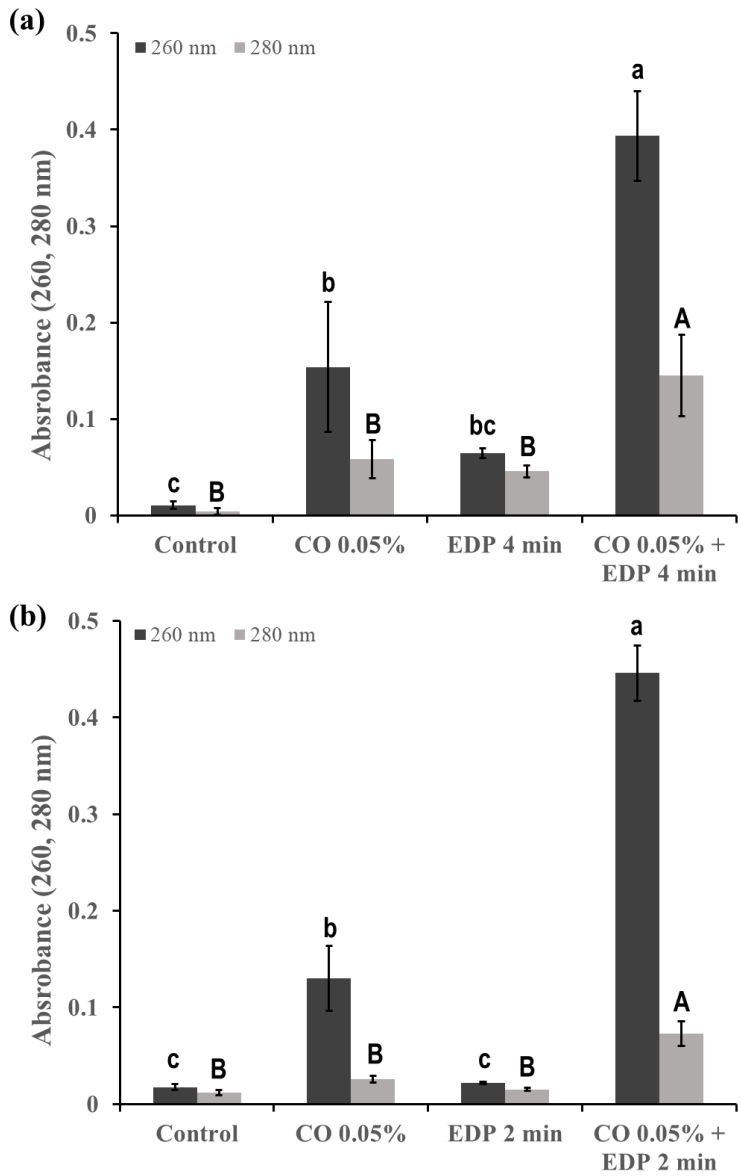

Fig. 4. The changes in absorbance values of intracellular nucleic acid (260 nm) and protein (280 nm) leakage from *E. coli* O157:H7 (a) and *S. aureus* (b) resulting from single or combined treatment of 0.05% clove oil (CO) and encapsulated atmospheric pressure plasma (EAP).

2.3.3.4. CLSM

The viability related to the membrane integrity of *E. coli* O157:H7 and *S. aureus* treated with clove oil or/and EAP were analyzed by CLSM (Fig. 5). The green-fluorescence stain SYTO9 is permeable through both live and dead bacterial membranes, while the red-fluorescence stain PI is permeable only through damaged cytoplasmic membranes (Stiefel et al., 2015). For both pathogens, the control group with intact bacterial membranes generally showed green fluorescence by SYTO 9 reacting with DNA, except for a few naturally dead bacteria. After the clove oil single treatment, some bacterial cytoplasmic membranes were damaged, reacted with the PI, and showed red fluorescence. Similar to the clove oil single treatment, some bacterial cells showed green fluorescence, and others showed red fluorescence even after the EAP single treatment. These results were also confirmed when plasma was treated on *E. coli* O157:H7 biofilm, unlike the control group that showed bacteria with only green fluorescence, the membrane was damaged and the number of bacteria with red fluorescence increased (An et al., 2019). However, when a combined treatment of clove oil and EAP was used, all bacterial cells showed red fluorescence. Therefore, the combined treatment of clove oil and EAP was found to cause severe damage to the cytoplasmic membrane.

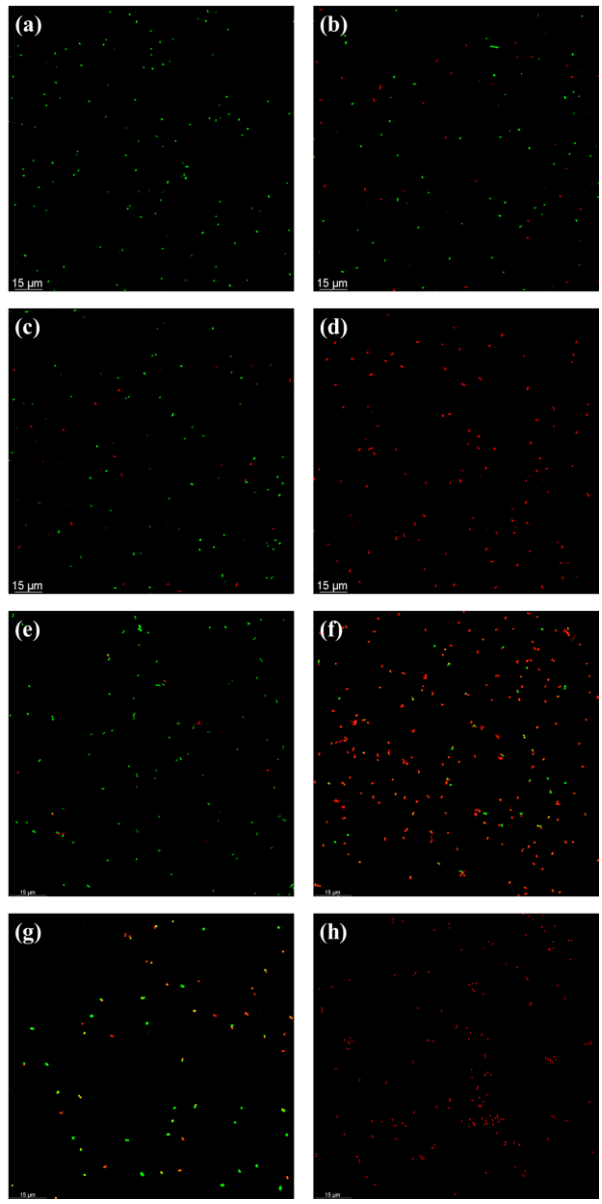

Fig. 5. Confocal laser scanning microscopy images of *E. coli* O157:H7 (a–d) and *S. aureus* (e–f) with different treatments. (a, e) Untreated control; (b, f) 0.05% clove oil; (c) encapsulated atmospheric pressure plasma (EAP) for 4 min; (d) 0.05% clove oil + EAP for 4 min; (g) EAP for 2 min; (h) 0.05% clove oil + EAP for 2 min.

2.3.3.5. TEM

TEM images confirmed that *E. coli* O157:H7 and *S. aureus* subjected to clove oil or/and EAP treatment were morphologically changed (Fig. 6). The control group of both bacterial cells were uniformly distributed, displayed an intact cell wall and membrane, and internal constituents were evenly arranged and clearly visible. After unitarily treated with clove oil or EAP, bacterial cells were partially damaged, and various levels of cell alterations were observed. Although clove oil changed the cytoplasmic density and resulted in cell wall degradation, there were still some viable cells (Lucas et al., 2012). Plasma also resulted in the physical lysis of cells, resulting in more severe cytosol leakage, debris, and membrane damage, which were less uniform as the plasma treatment time increased (Timmons et al., 2018). However, after the combined treatment with clove oil and EAP, the morphological shapes of both bacterial strains were severely mutated and the damage was at its worst. Both *E. coli* O157:H7 and *S. aureus* showed uneven cell membranes, broken cell walls, and loss of internal constituents.

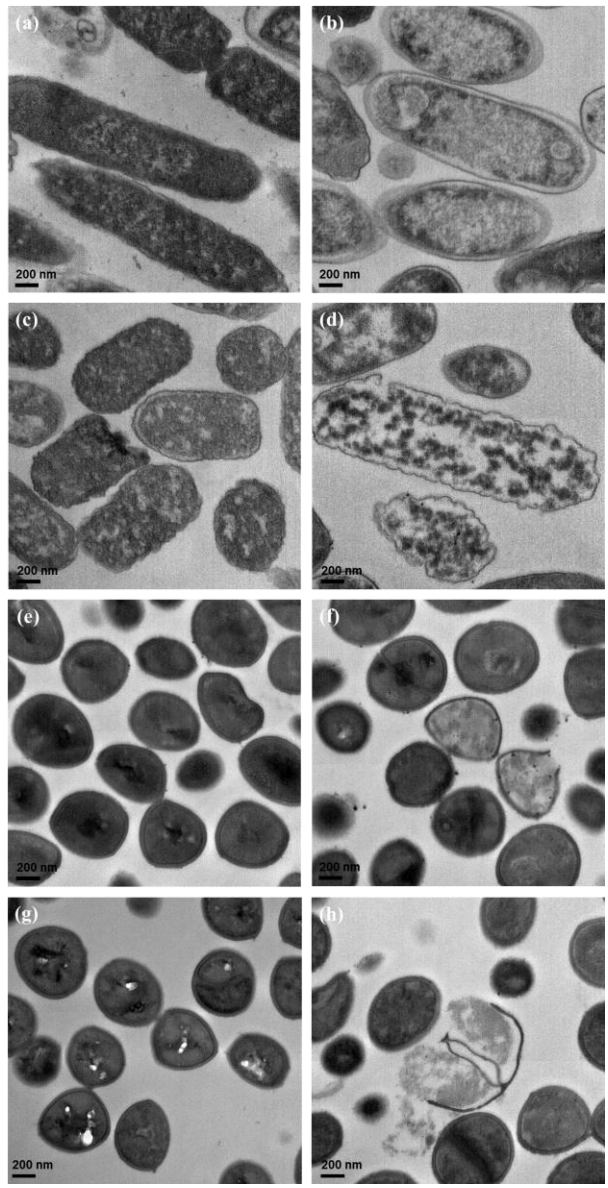

Fig. 6. Transmission electron microscopy images of *E. coli* O157:H7 (a-d) and *S. aureus* (e-f) with different treatments. (a, e) Untreated control; (b, f) 0.05% clove oil; (c) encapsulated atmospheric pressure plasma (EAP) for 4 min; (d) 0.05% clove oil + EAP for 4 min; (g) EAP for 2 min; (h) 0.05% clove oil + EAP for 2 min.

2.3.4. Bactericidal effects of clove oil and EAP on beef jerky

The bactericidal effects of clove oil and EAP on beef jerky were evaluated to confirm whether the synergistic bactericidal effect between them was also presented in food as well as in planktonic bacteria. To set an optimum EAP treatment time, the inoculated beef jerkies were treated with different EAP time (0, 5, 10, and 15 min) (Fig. 7a). The number of *E. coli* O157:H7 cells was 7.78, 7.74, 7.29, and 6.88 Log CFU/g, and the number of *S. aureus* cells was 8.47, 7.75, 7.49, and 7.00 Log CFU/g in beef jerky after it was treated with EAP for 0, 5, 10, and 15 min, respectively. Since a significant bactericidal effect was observed in both bacterial strains after 15 min of EAP treatment, 15 min was set as the optimum EAP treatment time for the beef jerky. Since beef jerky has a rough surface and is in a solid state, a longer treatment time of EAP is required to achieve a similar bactericidal effect as that observed for planktonic bacteria because the bactericidal effect of EAP is immensely affected by surface properties (Lee et al., 2011). For example, Kim et al. (2014b) studied the bactericidal effect against *S. aureus* via DBD plasma. Even though the agar plate experiment showed about a 4-Log reduction after 120 s of plasma treatment (initial cell count: 10^6–10^7 CFU/mL), it required about 600 s of treatment time to result in similar levels of bactericidal effect in beef jerky (initial cell count: 10^6–10^7 CFU/g). The synergistic bactericidal effect was observed after EAP treatment with different concentrations of clove oil on the beef jerky (Fig. 7b–c). When beef jerky inoculated with *E. coli* O157:H7 was treated with different concentrations of clove oil (EtOH, 0.5, 1.0, and 1.5%), only a 1.75 Log CFU/g reduction was observed at the highest clove oil

concentration. However, when the EAP treatment was used for 15 min after exposing the beef jerky to the same concentration (EtOH, 0.5, 1.0, and 1.5%) of clove oil, the reduction at the concentration of 1.0% was more than 7 Log CFU/g, which showed a tremendous bactericidal effect, resulting in non-detectable levels (detection limit: 10^1 CFU/g). Moreover, in beef jerkies inoculated with *S. aureus*, the synergistic microbial inhibitory effect was observed when EAP was combined with clove oil, although the bactericidal effect of clove oil alone was inadequate. However, the bactericidal effect was less in beef jerky inoculated with Gram-positive *S. aureus* compared to that of beef jerky inoculated with Gram-negative *E. coli* O157:H7 because reactive oxygen species generated by EAP have a limitation to damage the envelop of Gram-positive *S. aureus* with a thicker peptidoglycan layer than Gram-negative *E. coli* O157:H7 (Han et al., 2016).

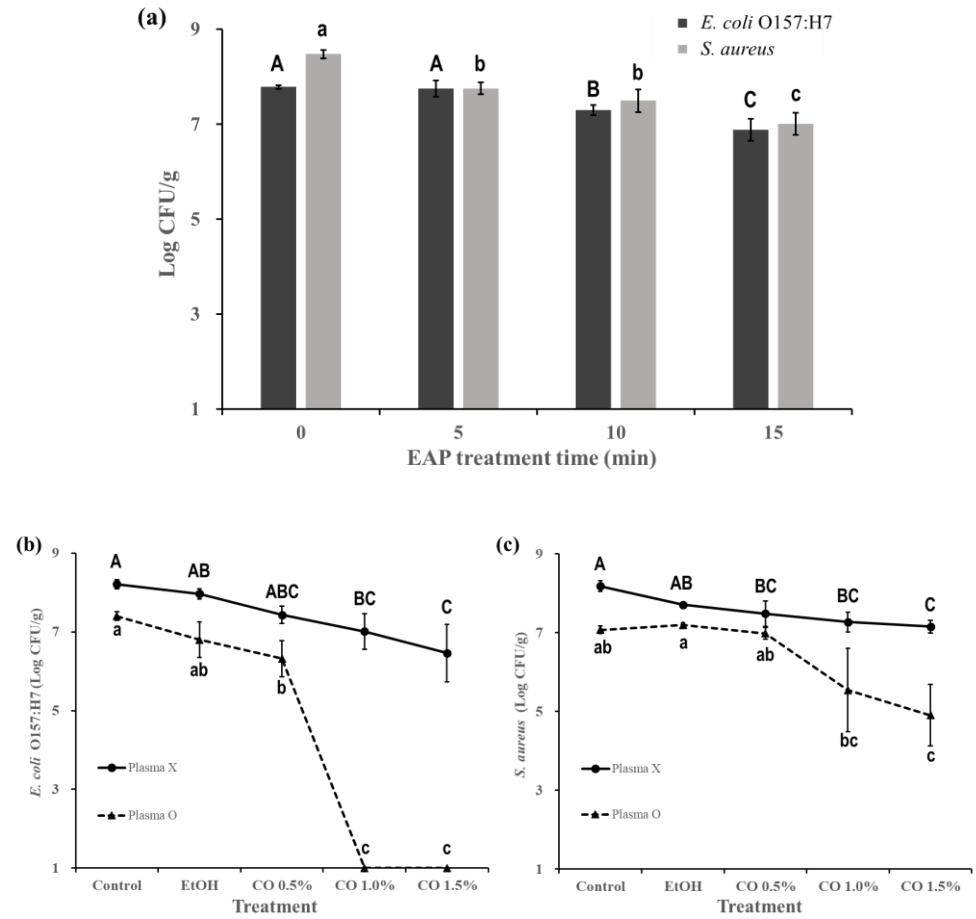

Fig. 7. The population (Log CFU/g) of different encapsulated atmospheric pressure plasma (EAP) treatment times (0, 5, 10, and 15 min) against *E. coli* O157:H7 and *S. aureus* on beef jerky (a). The surviving population (Log CFU/g) of *E. coli* O157:H7 (b) and *S. aureus* (c) after exposure to various concentrations of clove oil (CO) with or without EAP on beef jerky are shown. Error bars indicate standard deviation. $^{A-C}$Different letters differ significantly (P<0.05). $^{a-c}$Different letters differ significantly (P<0.05).

2.4. Conclusion

Encapsulated atmospheric pressure dielectric barrier discharge plasma combined with clove oil displayed synergistic bactericidal effects against *E. coli* O157:H7 and *S. aureus* compared to that of each single treatment. This synergistic bactericidal effect is the result of more serious morphological changes of cells, damage to cell membranes, increased membrane permeability, and more leakage of intracellular materials, rather than the alteration of the antimicrobial activity and chemical composition of clove oil by plasma treatment. In addition, the possibility of enhancing the bactericidal effect in food through the combined treatment of clove oil and plasma was demonstrated using beef jerky.

References

An, J. Y., Yong, H. I., Kim, H. J., Park, J. Y., Lee, S. H., Baek, K. H., Choe, W., & Jo, C. (2019). Estimation of inactivation effects against *Escherichia coli* O157: H7 biofilm by different plasma-treated solutions and post-treatment storage. *Applied Physics Letters*, 114, 073703.

Bajpai, V. K., Sharma, A., & Baek, K. H. (2013). Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. *Food Control*, *32*, 582-590.

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.

Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A. B., Rouabhia, M., Mahdouani, K., & Bakhrouf, A. (2007). The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. *Myrtaceae*): a short review. *Phytotherapy research*, *21*, 501-506.

Chami, F., Chami, N., Bennis, S., Bouchikhi, T., & Remmal, A. (2005). Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *19*, 405-408.

Cui, H., Bai, M., Yuan, L., Surendhiran, D., & Lin, L. (2018b). Sequential effect of phages and cold nitrogen plasma against *Escherichia coli* O157: H7 biofilms

on different vegetables. *International journal of food microbiology*, *268*, 1-9.

Cui, H., Ma, C., Li, C., & Lin, L. (2016). Enhancing the antibacterial activity of thyme oil against *Salmonella* on eggshell by plasma-assisted process. *Food Control*, *70*, 183-190.

Cui, H., Zhang, C., Li, C., & Lin, L. (2018a). Antimicrobial mechanism of clove oil on *Listeria monocytogenes*. *Food control*, *94*, 140-146.

Devi, K. P., Nisha, S. A., Sakthivel, R., & Pandian, S. K. (2010). Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *Journal of ethnopharmacology*, *130*, 107-115.

Ekezie, F. G. C., Sun, D. W., & Cheng, J. H. (2017). A review on recent advances in cold plasma technology for the food industry: Current applications and future trends. *Trends in Food Science & Technology*, *69*, 46-58.

Elishalom Yechiel. (2005). *Delivery System Handbook for Personal Care and Cosmetic Products*.

Feng, X., Jo, C., Nam, K. C., & Ahn, D. U. (2019). Impact of electron-beam irradiation on the quality characteristics of raw ground beef. *Innovative Food Science & Emerging Technologies*, *54*, 87-92.

Gavahian, M., Chu, Y. H., Khaneghah, A. M., Barba, F. J., & Misra, N. N. (2018). A critical analysis of the cold plasma induced lipid oxidation in foods. *Trends in Food Science & Technology*, *77*, 32-41.

Gülçin, İ., Elmastaş, M., & Aboul-Enein, H. Y. (2012). Antioxidant activity of clove oil–A powerful antioxidant source. *Arabian Journal of chemistry*, *5*, 489-

499.

Gyawali, R., Hayek, S. A., & Ibrahim, S. A. (2015). Plant extracts as antimicrobials in food products: Mechanisms of action, extraction methods, and applications. *Handbook of natural antimicrobials for food safety and quality*, 49-68.

Han, L., Patil, S., Boehm, D., Milosavljević, V., Cullen, P. J., & Bourke, P. (2016). Mechanisms of inactivation by high-voltage atmospheric cold plasma differ for *Escherichia coli* and *Staphylococcus aureus*. *Applied and Environmental Microbiology*, *82*, 450-458.

Hertwig, C., Meneses, N., & Mathys, A. (2018). Cold atmospheric pressure plasma and low energy electron beam as alternative nonthermal decontamination technologies for dry food surfaces: a review. *Trends in Food Science & Technology*, *77*, 131-142.

Hertwig, C., Reineke, K., Ehlbeck, J., Knorr, D., & Schlüter, O. (2015). Decontamination of whole black pepper using different cold atmospheric pressure plasma applications. *Food Control*, *55*, 221-229.

Jayasena, D. D., Kim, H. J., Yong, H. I., Park, S., Kim, K., Choe, W., & Jo, C. (2015). Flexible thin-layer dielectric barrier discharge plasma treatment of pork butt and beef loin: Effects on pathogen inactivation and meat-quality attributes. *Food microbiology*, *46*, 51-57.

Jayasena, D. D., & Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends in Food Science & Technology*, *34*, 96-108.

Jayasena, D. D., & Jo, C. (2014). Potential application of essential oils as natural

antioxidants in meat and meat products: a review. *Food reviews international*, *30*, 71-90.

Jenie, B. S. L., Priosoeryanto, B. P., Syarief, R., & Rekso, G. T. (2008). Mode of action Temu kunci (*Kaempferia pandurata*) essential oil on *E. coli* K1. 1 cell determined by leakage of material cell and salt tolerance assays. *HAYATI Journal of Biosciences*, *15*, 56-60.

Kim, H. J., Yong, H. I., Park, S., Kim, K., Kim, T. H., Choe, W., & Jo, C. (2014a). Effect of atmospheric pressure dielectric barrier discharge plasma on the biological activity of naringin. *Food chemistry*, *160*, 241-245.

Kim, J. S., Lee, E. J., Choi, E. H., & Kim, Y. J. (2014b). Inactivation of *Staphylococcus aureus* on the beef jerky by radio-frequency atmospheric pressure plasma discharge treatment. *Innovative food science & emerging technologies*, *22*, 124-130.

Kim, T. H., Lee, J., Kim, H. J., & Jo, C. (2017). Plasma-induced degradation of quercetin associated with the enhancement of biological activities. *Journal of agricultural and food chemistry*, *65*, 6929-6935.

Lee, H. J., Jung, H., Choe, W., Ham, J. S., Lee, J. H., & Jo, C. (2011). Inactivation of *Listeria monocytogenes* on agar and processed meat surfaces by atmospheric pressure plasma jets. *Food Microbiology*, *28*, 1468-1471.

Lee, H., Yong, H. I., Kim, H. J., Choe, W., Yoo, S. J., Jang, E. J., & Jo, C. (2016). Evaluation of the microbiological safety, quality changes, and genotoxicity of chicken breast treated with flexible thin-layer dielectric barrier discharge plasma. *Food science and biotechnology*, *25*, 1189-1195.

Lee, J. H., Woo, K. S., Yong, H. I., Jo, C., Lee, S. K., Lee, B. W., Lee, Y. Y., Lee, B., & Kim, H. J. (2019). Physicochemical properties of brown rice according to the characteristics of cultivars treated with atmospheric pressure plasma. *Journal of Cereal Science*, *87*, 138-142.

Lee, J., Lee, C. W., Yong, H. I., Lee, H. J., Jo, C., & Jung, S. (2017b). Use of atmospheric pressure cold plasma for meat industry. *Korean journal for food science of animal resources*, *37*, 477-485.

Lee, J. W., Song, Y. S., Park, Y. R., Ryu, S. M., Jeon, H. W., & Eom, S. H. (2017a) Sterilization of Food-Borne Pathogenic Bacteria by Atmospheric Pressure Dielectric Barrier Discharge Plasma. *Journal of Food Hygiene and Safety*, *32*, 222-227.

Liao, X., Liu, D., Xiang, Q., Ahn, J., Chen, S., Ye, X., & Ding, T. (2017). Inactivation mechanisms of non-thermal plasma on microbes: A review. *Food Control*, *75*, 83-91.

Li, X., & Farid, M. (2016). A review on recent development in non-conventional food sterilization technologies. *Journal of Food Engineering*, *182*, 33-45.

Lucas, G. C., Alves, E., Pereira, R. B., Perina, F. J., & Souza, R. M. D. (2012). Antibacterial activity of essential oils on *Xanthomonas vesicatoria* and control of bacterial spot in tomato. *Pesquisa Agropecuária Brasileira*, *47*, 351-359.

Lu, H., Patil, S., Keener, K. M., Cullen, P. J., & Bourke, P. (2014). Bacterial inactivation by high-voltage atmospheric cold plasma: influence of process parameters and effects on cell leakage and DNA. *Journal of applied microbiology*, *116*, 784-794.

Martínez-Graciá, C., González-Bermúdez, C. A., Cabellero-Valcárcel, A. M., Santaella-Pascual, M., & Frontela-Saseta, C. (2015). Use of herbs and spices for food preservation: advantages and limitations. *Current opinion in food science*, *6*, 38-43.

Matan, N., Nisoa, M., & Matan, N. (2014). Antibacterial activity of essential oils and their main components enhanced by atmospheric RF plasma. *Food Control*, *39*, 97-99.

Misra, N. N., Pankaj, S. K., Walsh, T., O'Regan, F., Bourke, P., & Cullen, P. J. (2014). In-package nonthermal plasma degradation of pesticides on fresh produce. *Journal of hazardous materials*, *271*, 33-40.

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

Naveena, B. M., Muthukumar, M., Sen, A. R., Babji, Y., & Murthy, T. R. K. (2006). Improvement of shelf-life of buffalo meat using lactic acid, clove oil and vitamin C during retail display. *Meat Science*, *74*, 409-415.

Nerín, C., Aznar, M., & Carrizo, D. (2016). Food contamination during food process. *Trends in food science & technology*, *48*, 63-68.

Oussalah, M., Caillet, S., Saucier, L., & Lacroix, M. (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157: H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control*, *18*, 414-420.

Pankaj, S. K., Bueno-Ferrer, C., Misra, N. N., Milosavljević, V., O'donnell, C. P., Bourke, P., Keener, K.M., & Cullen, P. J. (2014). Applications of cold plasma technology in food packaging. *Trends in Food Science & Technology*, *35*, 5-17.

Park, J., Henins, I., Herrmann, H. W., Selwyn, G. S., Jeong, J. Y., Hicks, R. F., Shim, D., & Chang, C. S. (2000). An atmospheric pressure plasma source. *Applied Physics Letters*, *76*, 288-290.

Perni, S., Shama, G., Hobman, J. L., Lund, P. A., Kershaw, C. J., Hidalgo-Arroyo, G. A., Penn, C. W., Deng, X. T., Walsh, J. L., & Kong, M. G. (2007). Probing bactericidal mechanisms induced by cold atmospheric plasmas with *Escherichia coli* mutants. *Applied Physics Letters*, *90*, 073902.

Pinela, J., & Ferreira, I.C. (2017). Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables: Trends aiming at quality and safety. *Critical reviews in food science and nutrition*, *57*, 2095-2111.

Prakash, B., Kedia, A., Mishra, P. K., & Dubey, N. K. (2015). Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities–Potentials and challenges. *Food Control*, *47*, 381-391.

Prakash, B., Singh, P., Mishra, P.K., & Dubey, N.K. (2012). Safety assessment of *Zanthoxylum alatum* Roxb. essential oil, its antifungal, antiaflatoxin, antioxidant activity and efficacy as antimicrobial in preservation of *Piper nigrum* L. fruits. *International journal of food microbiology*, *153*, 183-191.

Shao, X., Cao, B., Xu, F., Xie, S., Yu, D., & Wang, H. (2015). Effect of postharvest

application of chitosan combined with clove oil against citrus green mold. *Postharvest Biology and Technology*, *99*, 37-43.

Song, A. Y., Oh, Y. A., Roh, S. H., Kim, J. H., & Min, S. C. (2016). Cold oxygen plasma treatments for the improvement of the physicochemical and biodegradable properties of polylactic acid films for food packaging. *Journal of food science*, *81*, E86-E96.

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., & Ren, Q. (2015). Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC microbiology*, *15*, 36.

Stoffels, E., Gonzalvo, Y. A., Whitmore, T. D., Seymour, D. L., & Rees, J. A. (2006). A plasma needle generates nitric oxide. *Plasma Sources Science and Technology*, *15*, 501.

Surowsky, B., Schlüter, O., & Knorr, D. (2015). Interactions of non-thermal atmospheric pressure plasma with solid and liquid food systems: a review. *Food Engineering Reviews*, *7*, 82-108.

Swamy, M. K., Akhtar, M. S., & Sinniah, U. R. (2016). Antimicrobial properties of plant essential oils against human pathogens and their mode of action: an updated review. *Evidence-Based Complementary and Alternative Medicine*.

Thirumdas, R., Kadam, D., & Annapure, U. S. (2017). Cold plasma: an alternative technology for the starch modification. *Food Biophysics*, *12*, 129-139.

Timmons, C., Pai, K., Jacob, J., Zhang, G., & Ma, L.M. (2018). Inactivation of *Salmonella enterica*, Shiga toxin-producing *Escherichia coli*, and *Listeria monocytogenes* by a novel surface discharge cold plasma design. *Food Control*,

84, 455-462.

Trevisani, M., Berardinelli, A., Cevoli, C., Cecchini, M., Ragni, L., & Pasquali, F. (2017). Effects of sanitizing treatments with atmospheric cold plasma, SDS and lactic acid on verotoxin-producing *Escherichia coli* and *Listeria monocytogenes* in red chicory (radicchio). *Food Control*, *78*, 138-143.

Ulbin-Figlewicz, N., Jarmoluk, A., & Marycz, K. (2015). Antimicrobial activity of low-pressure plasma treatment against selected foodborne bacteria and meat microbiota. *Annals of microbiology*, *65*, 1537-1546.

Upadhyaya, I., Upadhyay, A., Kollanoor-Johny, A., Darre, M., & Venkitanarayanan, K. (2013). Effect of plant derived antimicrobials on Salmonella Enteritidis adhesion to and invasion of primary chicken oviduct epithelial cells in vitro and virulence gene expression. *International journal of molecular sciences*, *14*, 10608-10625.

Voon, H. C., Bhat, R., & Rusul, G. (2012). Flower extracts and their essential oils as potential antimicrobial agents for food uses and pharmaceutical applications. *Comprehensive Reviews in Food Science and Food Safety*, -55.

Wei, C., Zhong, J., Hu, T., & Zhao, X. (2018). Simultaneous detection of *Escherichia coli* O157: H7, *Staphylococcus aureus* and *Salmonella* by multiplex PCR in milk. *3 Biotech*, *8*, 76.

Xu, J. G., Liu, T., Hu, Q. P., & Cao, X. M. (2016). Chemical composition, antibacterial properties and mechanism of action of essential oil from clove buds against *Staphylococcus aureus*. *Molecules*, *21*, 1194.

Yadav, M. K., Chae, S. W., Im, G. J., Chung, J. W., & Song, J. J. (2015). Eugenol: a phyto-compound effective against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain biofilms. *PLoS One*, *10*, e0119564.

Yong, H.I., Kim, H.J., Park, S., Alahakoon, A.U., Kim,K., Choe, W., & Jo, C. (2015). Evaluation of pathogen inactivation on sliced cheese induced by encapsulated atmospheric pressure dielectric barrier discharge plasma. *Food microbiology*, *46*, 46-50.

Yong, H.I., Lee, S.H., Kim, S.Y., Park, S., Park, J., Choe, W., & Jo, C. (2017). Color development, physiochemical properties, and microbiological safety of pork jerky processed with atmospheric pressure plasma. *Innovative Food Science & Emerging Technologies*, *53*, 78-84.

Zengin, H., & Baysal, A.H. (2015). Antioxidant and antimicrobial activities of thyme and clove essential oils and application in minced beef. *Journal of food processing and preservation*, *39*, 1261-1271.

Zhang, Y., Liu, X., Wang, Y., Jiang, P., & Quek, S. (2016). Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control*, *59*, 282-289.

Zhang, Y., Wang, Y., Zhu, X., Cao, P., Wei, S., & Lu, Y. (2017). Antibacterial and antibiofilm activities of eugenol from essential oil of *Syzygium aromaticum* (L.) Merr. & LM Perry (clove) leaf against periodontal pathogen *Porphyromonas gingivalis*. *Microbial pathogenesis*, *113*, 396-402.

Summary in Korean

정향오일과 대기압 플라즈마 병용처리에 따른 병원성대장균과 황색포도상구균에 대한 시너지 살균효과 메커니즘 규명

유지현
서울대학교 대학원
농생명공학부 동물생명공학전공

육류 제품의 소비가 계속하여 증가함에 따라 식중독 발생은 지속적으로 문제가 되고 있다. 식중독을 유발하는 병원균 오염은 육류 제품의 생산, 가공, 포장, 유통 및 소비를 포함하는 모든 과정에서 발생할 수 있다. 병원성대장균과 황색포도상구균은 인간에게 심각한 복통, 설사, 발열 및 구토를 동반하는 식중독을 발생시키는 주요 박테리아이다. 따라서 이러한 병원균을 제어하기 위해 다양한 노력들이 이루어지고 있다. 전통적인 가열 미생물 제어 기술은 육류 제품의 영양가 및 관능 품질에 부정적인 영향을 미치기 때문에 육류 산업에서 비가열 미생물 제어 기술에 대한 관심이 높아지고 있다.

광펄스, 방사선 조사, 초고압, 초음파 및 자외선 조사를 포함하여 여러 가지 비가열 미생물 제어 기술이 있지만 설치에 고비용이 발생하고

소비자의 부정적인 인식 때문에 최근 비교적 새로운 기술인 저온 대기압 플라즈마의 활용 가능성이 주목을 받고 있다. 플라즈마는 물질의 제 4상태로, 자유 라디칼, 전자기장, 자외선 및 하전 입자를 포함한 활성종을 생성하여 미생물을 비활성화 시킬 수 있다. 저온 대기압 플라즈마의 장점은 가열살균 기술에 비해 육제품의 영양이나 품질에 부정적인 영향을 미치지 않으면서 다른 비가열살균 기술에 비해 상대적으로 설치비용이 저렴하고 작동이 쉽다는 것이다. 유전체 장벽 방전을 통해 저온 플라즈마를 발생시켜 미생물을 제어 할 수 있는 가능성이 다양한 연구를 통해 확인되었다.

저온 대기압 플라즈마는 앞서 언급 된 바와 같이 많은 이점을 갖지만, 식품에서 식중독 발생 병원균을 완전히 제어하기에는 한계가 있다. 따라서, 효과적으로 식품내 유해 미생물을 제거하기 위해 에센셜오일, 박테리오파지 및 젖산과 같은 효과적이고 안전한 항박테리아제와 플라즈마를 병용처리 하여 미생물 제어 효과를 증진시키고자 하는 노력들이 이루어져왔다.

에센셜오일 중에서 정향오일은 미국 FDA로부터 'generally recognized as safe (GRAS)'로 승인되었으며 항진균, 항산화 및 항 아플라톡신 등의 효과가 있다고 밝혀져 있다. 그러나, 고농도로 사용될 때 많은 비용이 발생하고 특유의 강한 향 및 독성 가능성의 문제가 있다. 따라서, 저농도의 정향오일과 저온 대기압 플라즈마의 병용처리를 통해

살균효과를 증진시키고자 하는 일부 연구가 수행되었지만, 그 메커니즘은 완전히 밝혀지지 않았다.

따라서, 본 연구의 목적은 (1) 정향오일과 저온 대기압 플라즈마(EAP)의 병용처리를 통한 병원성대장균과 황색포도상구균에 대한 시너지 살균효과와 그 메커니즘을 밝히고, (2) 육포에 적용하여 식품에서의 적용가능성을 확인하는 것이었다.

두 병원균에 있어서, 정향오일과 EAP의 단일처리는 3.0−log 이하의 살균효과를 가졌지만, 병용처리시 7.5−log 이상의 살균효과를 보였다. 한천내확산법 및 가스 크로마토그래프 질량분석계로 확인한 결과, EAP 처리 전후 정향오일의 미생물 생장 저해 직경과 화학적 구성에서 유의적인 차이를 나타내지 않았다. 하지만, 정향오일과 EAP의 병용처리시 세포의 막투과성 및 형태학적 모양의 현저한 변화가 분광광도계, 공초점레이저주사현미경 및 투과전자현미경에 의해 입증되었다. 두 병원균이 접종된 육포에서도 정향오일과 EAP의 병용처리에 의한 시너지 살균효과가 관찰되었지만 외부 세포막 구조의 차이로 인해 황색포도상구균에서의 살균효과가 더 적었다. 결론적으로 정향오일과 EAP 병용처리시 플라즈마가 정향오일의 항균 활성을 증가시킨 것이 아닌, 정향오일이 박테리아의 세포막과 세포벽에 일차적 손상을 가하여 박테리아의 플라즈마에 대한 감수성을 증가시켜 시너지 살균 효과를 나타내는 것으로 사료된다.