International Journal of Agricultural Technology 2024 Vol. 20(2):697-710
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Antimicrobial potential of gaseous ozone against *Salmonella* Thyphimurium and *Escherichia coli* O157:H7 contaminated on Bird Eye Chili (*Capsicum frutescens* L.)

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Phakawan, J. and Tepsorn, R. (2024). Antimicrobial potential of gaseous ozone against *Salmonella* Thyphimurium and *Escherichia coli* O157:H7 contaminated on Bird eye Chili (*Capsicum frutescens* L.). International Journal of Agricultural Technology 20(2):697-710.

Abstract The optimum condition of using ozone gas to decontaminate *S.* Typhimurium and *E. coli* O157:H7 on Tryptic Soy Agar (TSA) presented at 5 °C. The ozone gas fumigation inhibited *S*. Typhimurium and *E. coli* O157:H7 at *ca*. 4.00 Log_{10} within 15 and 30 min, respectively. Time-kill assay was used to determine the antimicrobial overtime. It was indicated that at 5 °C after ozone gas fumigation, the population of *S.* Typhimurium and *E. coli* O157:H7 at *ca*. 3.00 Log_{10} was completely inhibited within 2 and 4 min, respectively. The efficiency of ozone gas affected the reduction of *S.* Typhimurium and *E. coli* O157:H7 contaminated on Bird Eye chili in both *ca*. 6.00 Log_{10} and *ca*. 3.0 Log_{10} reductions within 10, 15 and 20 min at 5, 27 and 55°C after ozone gas fumigation respectively. Physical changes of Bird Eye chili after ozone gas fumigation was observed that L*, a* and b* increased slightly when the fumigation time increased and temperature decreased and the most colour difference was found at 5 °C.

Keywords: Ozone, Vapour, *Salmonella* Thyphimurium, *Escherichia coli* O157:H7, Bird Eye chili

Introduction

Bird Eye chilies (*Capsicum annuum* L.) are herbaceous plant and spice worldwide. People commonly consume because of their unique flavour, colour, and pungency. Bird Eye chili was eaten either fresh or powdered in numerous cultures (Rico *et al*., 2010). Fresh forms frequently got contaminated with different microbes, such as diseases that were harmful to food. During cultivation, harvesting, processing, shipping, and storage, contamination might happen. Fresh chili and its variations have been shown to harbor foodborne

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pathogens such *Salmonella* sp. (Jeong *et al*., 2010, Sardiñas *et al*., 2011), *Bacillus cereus* (Jeong *et al*., 2010), and *Escherichia coli* (Gómez-Aldapa *et al*., 2014). Over 400 persons have contracted *Salmonella* sp. contaminated red chili in the last 20 years (Hanning *et al*., 2009). According to the research, foods with high or low moisture content could harbor *Salmonella* sp. (CDC, 2008, CDC, 2010, Beuchat *et al*., 2013, FDA, 2013). For these reasons, widespread outbreaks of salmonellosis have been linked to both fresh and dried chillies. According to multiple studies (Cravioto *et al*., 1991, Estrada-Garcia *et al*., 2005, Estrada-Garcia *et al*., 2009, Nataro and Kaper, 1998, Paniagua *et al*., 2007, Trabulsi *et al*., 2002), *E. coli* was a major cause of diarrhea in both developed and developing nations. During the growing and harvesting process, fresh vegetables may get cross-contaminated with *E. coli*, which had been isolated from a range of animal species (Levine, 1987, Nagy and Fekete, 1999, Kaper *et al*., 2004, Caprioli *et al*., 2005, Cortes *et al*., 2005).

Decontaminating fresh fruit with chemical sanitizers and washing had become a common postharvest practice to reduce contamination and infection risk, but the issue with these sanitizers was their limited ability to improve antibacterial efficacy (Balaguero *et al*., 2015; Gil *et al*., 2009; Ruiz-Cruz *et al*., 2007; Singh *et al*., 2002). The various technologies had been recognized and assessed in order to prevent issues with contamination reduction efficiency that did not meet the goal of food safety (Goodburn and Wallace, 2013). Overall, the required concentration of aqueous sanitizers could only achieve a decrease of less than 2.0 logarithmic units. According to Zheng *et al.* (2013), target bacteria might have innated to protection characteristics that prevented aqueous chemicals from penetrating the cells, explaining the low efficacy of aqueous sanitizers. To lessen microbial contamination on chili, several sanitizing treatments had been used (Schweiggert *et al*., 2007). These therapies were not without restrictions, though. Thus, to reduce the pathogen contamination of fresh chili, innovative treatments and technologies had to be successful. To guarantee that new food technologies serve the intended goal, development was necessary.

Ozone was a material that oxidizes strongly. Food sector usage authorized by the FDA (Brodowska *et al*., 2018). The food industry could use ozone, both gaseous and aqueous, as a food additive while storing and processing different types of food (US FDA, 2001). In order to reduce spoilage and foodborne pathogens, water dissolved ozone was frequently used for washing and rinsing food, mostly fresh produce, during the postharvest period (Brodowska *et al*., 2018, Glowacz *et al*., 2015). Ozonization water could also be utilized in water treatment systems or for surface sanitation. Furthermore, disinfection could also be accomplished using gaseous ozone (Horvitz and Cantalejo, 2014). Research had demonstrated that gaseous ozone may effectively inactivate foodborne

pathogens that were polluted on fresh produce (Daş *et al*., 2006, Trinetta *et al*., 2011, Fan, 2021). The findings showed that gaseous ozone treatments at 300 and 1500 µL L^{-1} for 8 min were able to inactivate over 99.99% of *Staphylococcus aureus* and *Escherichia coli* (Kowalski *et al*., 1998). Gaseous ozone at 10,000 µL L^{-1} for 30 minutes under partial vacuum showed the reduction potential as 4.2 and 2.8 Log_{10} CFU in the instance of *Salmonella* sp. (Selma *et al*., 2008a, b). Furthermore, according to Akata *et al.* (2015), ozonation therapy (5.3 mg L^{-1} for 60 min) inactivated *Salmonella* sp., *E. coli* O157:H7, and *L. monocytogenes* by 3.61, 3.41, and 2.80 log. The effectiveness of gaseous ozone against foodborne infections had been the subject of several studies; however, the frangible and tiny sample of Bird Eye chili had not been examined. The objective was to ascertain the gaseous ozone antibacterial capacity against Bird Eye chili-contaminated *S.* Typhimurium and *E. coli* O157:H7.

Materials and methods

Chemical and microbiological media

Peptone, Tryptic Soya Broth (TSB), and Tryptic Soy Agar (TSA) were acquired from Difco (Dico, USA). From QRÑC (QRëC, New Zealand), acetic acid was used.

Preparation of test organism

The *E. coli* O157:H7 and *S.* Typhimurium ATCC 13311 used in this investigation were received from the Thai Ministry of Public Health, Department of Medical Science. Cultures were maintained at -18°C. After being activated in Tryptic Soy Broth (Difco, USA), those were incubated for eighteen hours at 37°C. The ultimate bacterial concentration of the working inoculum was 7.00-8.00 Log_{10} CFU/mL.

Corona discharge gaseous ozone generation process

The corona discharge method produced gaseous ozone (Figure 1). Along the pipe leading to the fumigation chamber, the ozone gas dispersed. In order to get fresh ozone gas during the fumigation time, the ozone tube was linked to a distillation system at the end. 800 mg/h of gaseous ozone was generated.

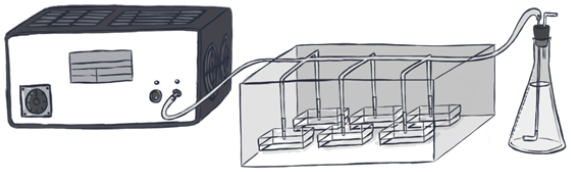

Figure 1. Schematic illustration represented the corona discharge gaseous ozone generation process fumigation chamber

Preperation of contaminated Bird Eye Chili

Before the experiment, fresh Bird Eye chili (*Capsicum frutescens* L.), which were utilized as the food model, were kept at 10°C after being bought from TaLadThai, the largest wholesale market of Thailand. In order to protect the chilies from the effects of natural flora, 1000 ppm of sodium hypochlorite solution was used for washing. After rinsing the test specimens in sterile distilled water to get rid of any leftover sodium hypochlorite, they were dried in a laminar flow cabinet for fifteen minutes while maintaining sterility. To achieve a final concentration of either 4.00 Log_{10} CFU/mL or 7.00 Log_{10} CFU/mL, 100 mL of *S.* Typhumurium and *E. coli* O157:H7 were produced separately in 0.1% peptone water. The test organism was introduced into the prepared Bird Eye chilies using the immersion technique, resulting in a final population of 4.00 Log_{10} CFU/g as the low inoculum and 6.00 Log_{10} CFU/g as the high inoculum. For fifteen minutes, the infected Bird Eye chilies were dried in a laminar flow hood.

In vitro susceptibility to gasous ozone by agar overlay method

Using the agar overlay technique, the sensitivity of *S.* Typhimurium and *E. coli* O157:H7 to gasous ozone was ascertained *in vitro*. To create a high level of inoculum, 0.1 mL of test organism suspension containing approximately 7.00 Log_{10} CFU/mL was temporarily transferred on TSA, whereas 0.1 mL containing approximately 4.00 Log_{10} CFU/mL was utilized as a low level inoculum. The fumigation chamber was aseptically filled with the contaminated surface (Figure 1). The production of gaseous ozone occurred at a steady 800 mg/hr pace. Gaseous ozone was applied to the ozone fumigation at intervals of 0, 5, 10, 15, 20, 25, and 30 minutes. For twenty-four hours, all fumigated agar plates were incubated at 37°C. The remaining populations were tallied. Temperature effects were measured at 5°C and 55°C.

In vitro susceptibility to gasous ozone by time killing analysis

The antimicrobial properties of gaseous ozone were assessed *in vitro* using a modified version of the Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics (EN 1040). The results showed that the gaseous ozone was effective in destroying *S.* Typhimurium and *E. coli* O157:H7 against time or time killing analysis. The two test organisms were generated independently in 100 mL of phosphate buffer, with the high level population at about 8.00 Log_{10} CFU/mL and the low level population at around 5.00 Log_{10} CFU/mL.

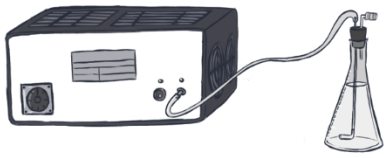

Figure 2. Schematic illustration represented the *in vitro* susceptibility to gasous ozone by Time Killing Analysis

The gaseous ozone generator was aseptically linked to the fumigant flask (Figure 2). The suspensions of *E. coli* O157:H7 and *S.* Typhimurium were removed after 0, 2, 4, 6, 8, 10, 20, and 30 minutes, respectively. TSA was utilized as the enumeration medium, and serial dilutions were carried out. After 24 hours of incubation at 37°C, the population of organisms on each plate was determined using Log_{10} CFU/mL. The effect of temperature on the antibacterial potential was ascertained, as previously mentioned.

Gaseous ozone effects on fresh Bird Eye chili contaminated with S. Typhimurium and E. coli O157:H7

With the use of synthetically infected Bird Eye chili, the challenge test was examined. In a fumigation ozone chamber, the prepared infected Bird Eye chilies were subjected to gaseous ozone for 0,5,10,15, 25, and 30 minutes. Then, using the spread plate approach, the remaining populations of *S.* Typhimurium and *E. coli* O157:H7 were investigated. After 24 hours of incubation at 37°C, the population of organisms on each plate was determined using Log_{10} CFU/g. It was also established how temperature affected things. A quality test was conducted concurrently on the uncontaminated Bird Eye chili to examine the impact of

gaseous ozone on color changes following the ozone fumigation procedure. Equation (1) was utilized in the computation of the ΔE.

$$\Delta \boldsymbol{E} = [(\Delta \boldsymbol{L}^*)^2 + (\Delta \boldsymbol{a}^*)^2 + (\Delta \boldsymbol{b}^*)^2]^{1/2} \quad \textbf{(1)}$$

Statistical analysis

Experiments were designed as a complete randomized design. The SPSS software package was used to do an analysis of variance on the data and compare the treatment mean using Duncan's New Multiple Range Test at a significance threshold of $p \leq 0.05$.

Results

In vitro susceptibility to gasous ozone by agar overlay method

The percentage was decreased *S.* Typhimurium and *E. coli* O157:H7 at contamination levels of 2.00 and 4.00 Log_{10} CFU/cm^2 in all three gaseous ozone fumigation settings at temperatures of 5, 27, and 55°C. The findings demonstrated that ozone treatment at 5°C was able to reduce the number of *E. coli* O157:H7 by 4-log reduction within 15 minutes at a contamination level of 4.00 Log_{10} CFU/cm^2 (Figure 3A).

In less than 30 minutes, an ozone fumigation procedure at 27°C was able to reduce the amount of *E. coli* O157:H7 by 4-log reduction. Fumigation with ozone at temperatures of 5, 27, and 55°C was shown to be able to reduce the number of *E. coli* O157:H7 for 2-log reduction after 30 minutes at a contamination level of 2.00 Log_{10} CFU/cm^2 (Figure 3B). Figure 3C showed the decrease in *S.* Typhimurium at the 4.00 Log_{10} CFU/cm^2 contamination level. The findings showed that after 30 minutes, ozone fumigation at 5°C decreased the population of *S.* Typhimurium by 4.00 logarithmic units. Figure 3D showed the *S.* Typhimurium population at a contamination level of 2.00 Log_{10} CFU/cm^2. It was discovered that ozone fumigation at 55°C resulted in a 2-log drop in *S.* Typhimurium counts in just ten minutes. Within 30 minutes, the amount of *S.* Typhimurium 2-log decrease was lowered by fumigant at 5 and 27°C.

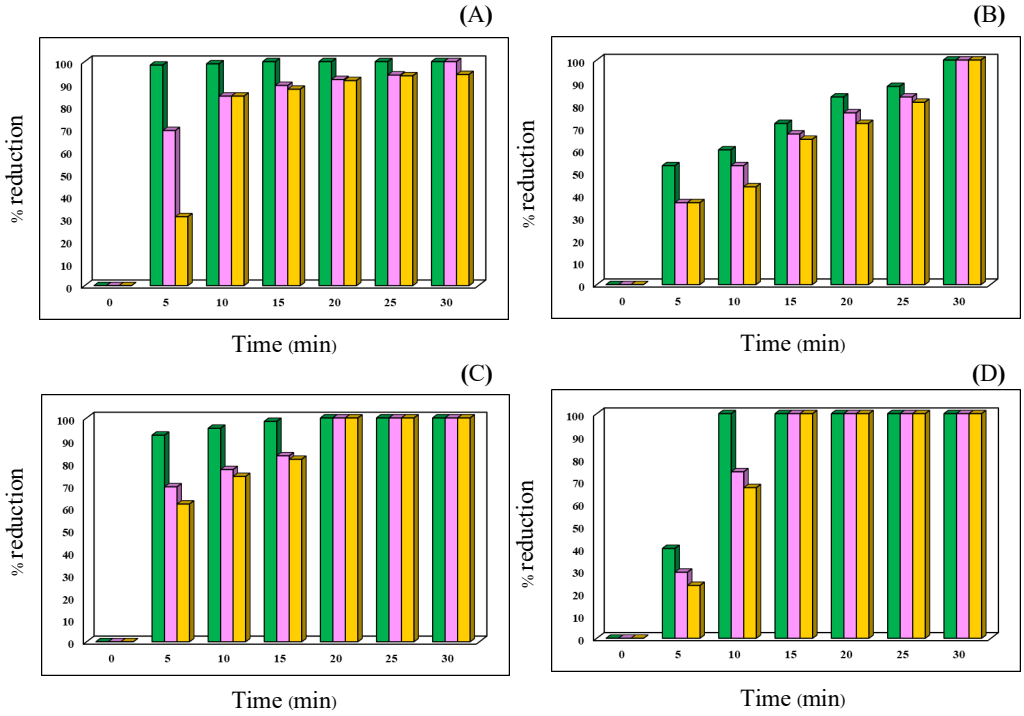

Figure 3. Reduction ***(%)*** of *S.* Typhimurium **(**C, D**)** and *E. coli* O157:H7 **(**A, B**)** using the agar overlay technique during gaseous ozone fumigation at various temperatures (■; 5°C, ■; 27°C, ■; 55°C) (High level inoculums A and C; low level inoculums B and D**)**

In vitro susceptibility to gasous ozone by time killing analysis

The quantity of *S.* Typhimurium and *E. coli* O157:H7 was decreased at the contamination level of 3.00 Log_{10} CFU/mL and at the contamination level of 6.00 Log_{10} CFU/mL at various temperatures of 5, 27, and 55°C (Figure 4).

According to the findings, the amount of *E. coli* O157:H7 was reduced to 4**.**35 Log_{10} CFU/mL after 30 minutes of gaseous ozone fumigation at 5°C. The amount of *E. coli* O157:H7 was decreased to 4.45 Log_{10} CFU/mL at 27°C and 4.49 Log_{10} CFU/mL at 55°C. Figure 4B shows the low of pollutants at 3.00 Log_{10} CFU/mL. It was discovered that ozone fumigation at 5°C decreased the quantity of *E. coli* O157:H7 for a 3-log drop in just 4 minutes. Within six minutes, the number of *E. coli* O157:H7 was reduced as a 3**-**log reduction at 27 and 55°C. The findings showed that after 30 minutes of ozone fumigation at 5°C, the amount of *S.* Typhimurium at a contamination level of 6.0 Log_{10} CFU/mL **(**Figure 4C**)** was

decreased to 5.16 Log_{10} CFU/mL. Following fumigant at 27°C, the quantity of *S.* Typhimurium was decreased to 5.32 Log_{10} CFU/mL and to 5.24 Log_{10} CFU/mL at 55°C. The antimicrobial effectiveness rervealed against a low-level population of *S.* Typhimurium at 3.00 Log_{10} CFU/mL (Figure 4D). Within two minutes, ozone fumigation at 5°C decreased the *S.* Typhimurium population by a factor of three. In just 4 minutes, the population had a 3-log drop when under ozone fumigation at 27 and 55°C.

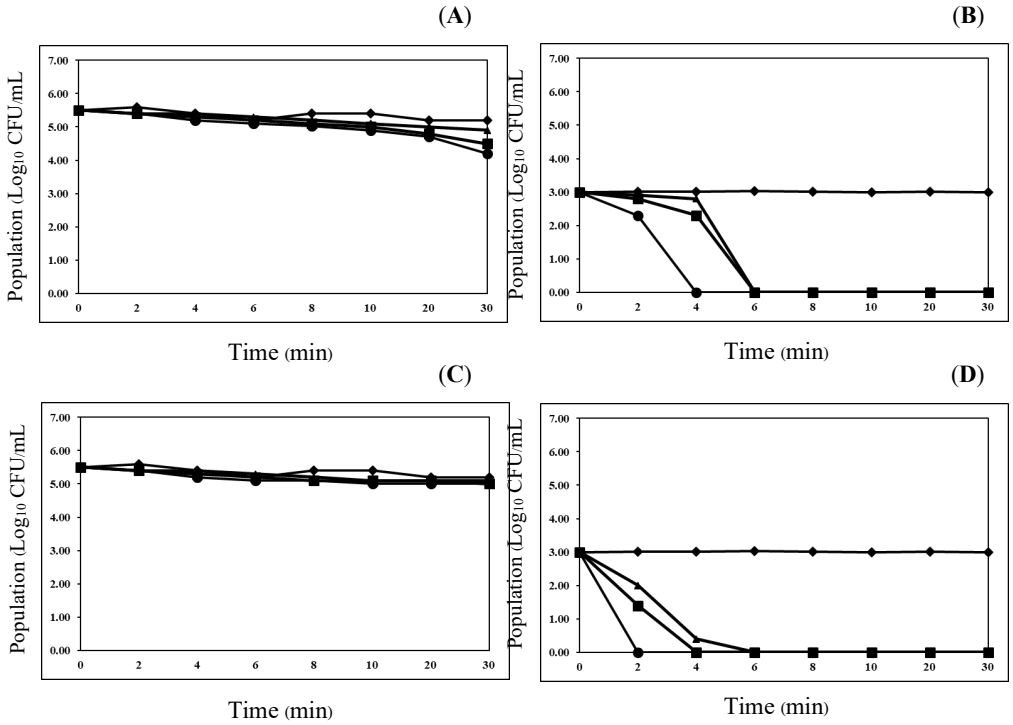

Figure 4. The population of *S.* Typhimurium **(**B, D**)** and *E. coli* O157:H7 **(**A, C**)** at various temperatures **(**◆**:** control, ▲**:** 55°C, ■**:** 27°C, ●**:** 5°C**)** during interaction with gaseous ozone

Gaseous ozone effects on fresh Bird Eye chili contaminated with S. Typhimurium and E. coli O157:H7

In all three conditions, fumigate, ozone at 5, 27, and 55 °C, it demonstrated a decrease in the quantity of *E. coli* O157:H7 and *S.* Typhimurium contaminated on Bird Eye chili at the contamination level of 6.00 Log_{10} CFU/g and 3.00 Log_{10} CFU/g (Figure 5). At 5°C, ozone fumigation led to a 6-log reduction in *E. coli* O157:H7 and a 3-log reduction in *S.* Typhimurium in just 10 minutes. Under

ozone fumigant at 27°C, the number of *E. coli* O157:H7 was decreased by 6-and 3-log reductions in 15 minutes, and at 55°C, the number of *E. coli* O157:H7 was decreased by 6-and 3-log reductions in 20 minutes. After being ozonated for 30 minutes at various temperatures, the color values of the Bird Eye chili revealed that the L*, a*, and b* values differed considerably from the control samples (Table 1). Furthermore, a comparison between temperatures revealed a considerable difference in the L* and a* values. There was no change in the b* values between the temperatures, however the L* a* values rose as the temperature dropped. As a result, it appeared that the ozone fumigation caused the Bird Eye chili to become somewhat browner.

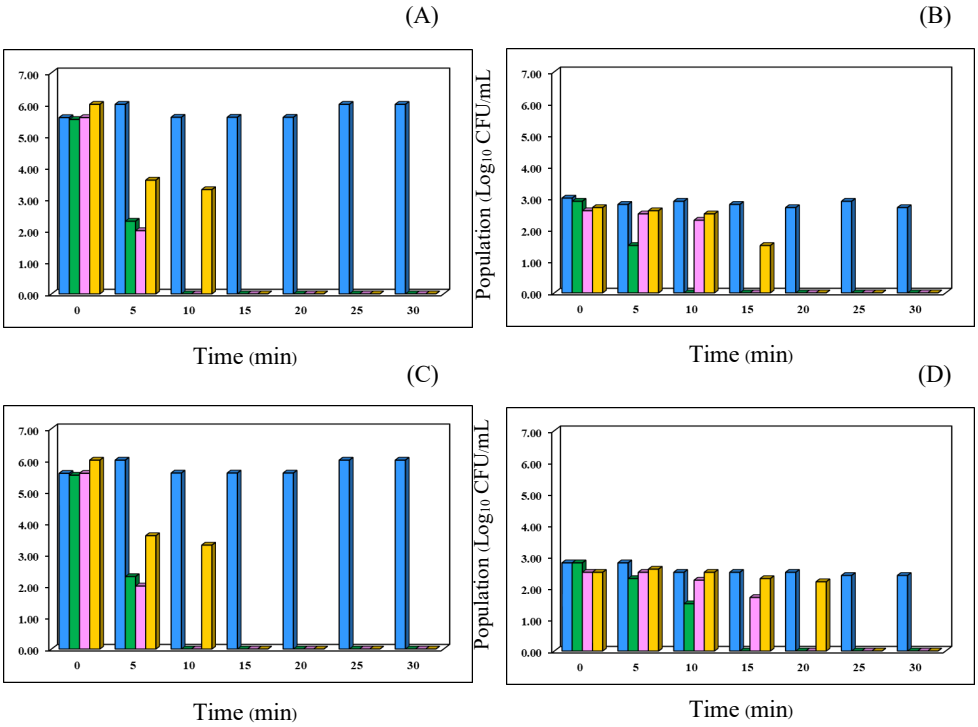

Figure 5. Total amount of *S.* Typhimurium (B: 6.00 Log_{10} CFU/g contamination, D: 3.00 Log_{10} CFU/g contamination) and *E. coli* O157:H7 (A: 6.0 Log_{10} CFU/g contamination, C: 3.00 Log_{10} CFU/g contamination) (■; control, ■; 5°C, ■; 27°C, ■; 55°C)

Table 1. L*, a*, b* and ΔE of Bird Eye chili after gaseous ozone fumigation process

Temperature	Colour parameter			
(°C)	L*	a*	b*	ΔE
5±2	39.64±0.14	-8.34±0.11	32.85±0.02	2.41±0.06
27±2	39.08±0.07	-8.66±0.08	35.87±0.02	1.80±0.08
55±2	38.23±0.07	-9.16±0.06	32.89±0.02	1.74±0.06

Discussion

This study examined the antibacterial activity of gaseous ozone against food models and *E. coli* O157:H7 and *S.* Typhimurium *in vitro*. The ozone reductions reported in this experiment were consistent with those previously reported by authors. These findings demonstrated that when ozone fumigation was carried out at a low temperature and great efficiency was seen, gaseous ozone could effectively eradicate both *S.* Typhimurium and *E. coli* O157:H7. The phenomena were caused by the ozone boiling point. It was a given that ozone expressed itself more actively at low temperatures than it did at high ones. It had been noted that the ozone fumigation system's ability to effectively eradicated *S.* Typhimurium and *E. coli* O157:H7 increased at lower temperatures. Because gaseous ozone decomposed into reactive oxygen species (ROS) including OH, OOH, and H_2O_2, it caused oxidative stress that deactivated bacterial cells (Forney, 2003). Protection enzymes like catalase, which were generated by bacteria, regularly neutralized these oxidative substances to shield the cells (Buchmeier *et al*., 1995). The equilibrium between the defense enzyme and oxidative stress was upset by high oxidative stress. Reactive oxygen species accumulated as a result, attacking proteins, lipids, and DNA more often in cells (Hunt and Marinas, 1999). Neutrophils used this oxidative mechanism to eliminate the pathogen (Bortolussi *et al*., 1987, Buchmeier *et al.*, 1995). Inconsistent cell structure on bacterial cells identified ozone oxidative damage. The oxidation actions of ozone and its induced reactive oxygen species (ROS) on lipid and protein molecules might be the cause of the uneven structure. Ozone-induced lipid peroxidation produced lipid hydroperoxides (LOOH), which in turn set off a chain reaction that degraded lipids. This led to cell wall rupture, cellular leakage, excessive nutrition loss, and cell death. It also decreased membrane integrity and increased fluidity. It also upset the osmotic equilibrium of the cell. By oxidizing sulfhydryl groups and amino acids in proteins or enzymes, ozone also rendered bacterial cells inactive (Al-Haddad *et al*., 2005, Torlak *et al*., 2013). As a result, the proteins broke down into smaller peptides, underwent

conformational changes, and eventually became inactive (Torlak *et al*., 2013). According to Torlak *et al.* (2013), ozone also damaged bacterial DNA and introduced mutations that changed genetic coding. The study's findings about the antibacterial activity of gaseous ozone validated its potential for fruit sanitization without the need for water, hence removing the substantial risk of cross-contamination in the aqueous treatment**.** Since the gaseous ozone treatment was unaffected by the pH of the water used in the aqueous treatment and the presence of organic matter, it had been demonstrated to be more stable than the ozone in the aqueous treatment.

By The most efficient method for lowering the quantity of *S*. Typhimurium and *E. coli* O157:H7 was ozone fumigation at 5°C. Furthermore, the duration of fumigation affected how well ozone inhibited bacteria. Nonetheless, during the experiment, the temperature needs to be kept consistent. The colour of Bird Eye chili changed as a result of ozone fumigation. Comparing the L* a* b* values to the control samples revealed differences. It had a little more brown to it, but colour changes were not discernible. Additionally, the most colour shift was seen after ozone treatment at 5°C.

Acknowledgements

The authors would like to express their sincere appreciation to Thammasat University Center of Excellence in Food Science and Innovation for financially supporting.

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(Received: 30 September 2023, Revised: 4 January 2024, Accepted: 9 January 2024)