Mustafa Kemal Üniversitesi Tarım Bilimleri Dergisi, 29 (2), 606-621
Mustafa Kemal University Journal of Agricultural Sciences, 29 (2), 606-621
https://dergipark.org.tr/tr/pub/mkutbd
e-ISSN: 2667-7733

ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Evaluation of ozone effectiveness against Gram-positive and Gram-negative pathogens using different methods

Gram-pozitif ve Gram-negatif patojenlere karşı ozon etkinliğinin farklı yöntemlerle değerlendirilmesi

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ARTICLE INFO

Article history:
Recieved / Geliş: 14.02.2024
Accepted / Kabul: 08.06.2024

Keywords:
Ozone
Staphylococcus aureus
Listeria monocytogenes
Salmonella enteritidis
Escherichia coli

Anahtar Kelimeler:
Ozon
Staphylococcus aureus
Listeria monocytogenes
Salmonella enteritidis
Escherichia coli

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ABSTRACT

Ozone attracts great attention due to its strong oxidative properties, antimicrobial activity, easy applicability, operating costs almost negligible, lack of chemicals in its use, highly effective and environmentally friendly application. In this study, two Gram (+) and two Gram (-) bacterial cultures, known as pathogens, were used to examine the effect of ozone gas on the growth of bacterial cultures. The samples were treated with ozone at different flow rates (4, 5, and 6 mg/L) and durations (1, 5, 10, 15, and 20 min) with different application parameters (pathogen bacteria, distilled water, and the mixture of distilled water and pathogen bacteria) and the number of viable cells was determined after the procedure. Among the methods applied we found that the direct application of ozone to the bacteria is the most effective in preventing/destroying bacterial growth. Also, it was determined that the growth of pathogenic microorganisms decreased as the flow rate and ozone contact time enhanced.

ÖZET

Ozon, güçlü oksidatif özelliği, antimikrobiyal aktivitesi, kolay uygulanabilirliği, işletme maliyetlerinin yok denecek kadar az olması, kullanımında kimyasal madde içermemesi, oldukça etkili ve çevre dostu bir uygulama olması nedeniyle büyük ilgi görmektedir. Bu çalışmada ozon gazının bakteri kültürlerinin üremesi üzerindeki etkisini incelemek amacıyla patojen olarak bilinen iki Gram (+) ve iki Gram (-) bakteri kültürü kullanılmıştır. Numuneler, farklı uygulama parametreleriyle (patojen bakterilere, distile suya ve patojen bakteri ve distile su karışımına) farklı akış hızlarında (4, 5 ve 6 mg/L) ve sürelerde (1, 5, 10, 15 ve 20 dakika) ozonla muamele edilmiştir ve muamele işleminden sonra canlı hücre sayıları belirlenmiştir. Uygulama yöntemleri arasında ozonun bakterilere doğrudan uygulanmasının bakteri üremesini önleme/yok etmede en etkili yöntem olduğu belirlenmiştir. Ayrıca akış hızı ve ozonla temas süresi arttıkça patojen mikroorganizmaların üremesinin azaldığı belirlenmiştir.

Cite/Atıf Çınar Acar, B. (2024). Evaluation of ozone effectiveness against Gram-positive and Gram-negative pathogens using different methods. *Mustafa Kemal Üniversitesi Tarım Bilimleri Dergisi, 29* (2), 606-621. https://doi.org/10.37908/mkutbd.1437244

INTRODUCTION

The ozone molecule, consisting of three oxygen atoms, has a molecular weight of 48 g/mol at 1 atm pressure, a bond angle of 116.8° and a bond length of 1.278 Å, -111.9°C boiling and -192.7°C melting point. It exists as a gas at room temperature and normal pressure. Ozone gas appears bluish when produced from dry air at room temperature but is colorless when made with high-purity oxygen (Lindsley et al., 2016; Botondi et al., 2023).

Ozone can attack various cell membrane components of microorganisms, such as the cell wall, cytoplasm, endospore coatings, virus capsids, and viral envelopes (Khadre et al., 2001; Patil, 2012). The double bonds of unsaturated fatty acids are susceptible to ozone (Guzel-Seydim et al., 2004). Regarding its high oxidation potential and ability to pass through biological membranes, ozone oxidizes the cellular components of the cell wall and several biological molecules, such as enzymes, proteins, DNA, and RNA, after entering the bacterial cell (Hunt & Mariñas, 1997). Since its mechanism of action is cell destruction, it has a different effect than other disinfectants.

Ozone has an impact in two different ways, either directly or indirectly. In direct effect, the ozone molecule reacts with organic or inorganic substances. In direct reactions, ozone is a dipole with electrophilic and nucleophilic features (Viebahn-Haensler & León Fernández, 2021). When a double bond in a molecule reacts with ozone (O_3), an unstable compound called ozonide is formed. It breaks apart if this ozonide reacts with an acidic solution (like water containing dissolved H+ ions). Depending on the structure of the original molecule, the broken-down pieces can be various products. These products include simple carbonyl compounds (aldehydes or ketones), molecules with both positive and negative charges (zwitterions), hydrogen peroxide (H_2O_2), and fragments of carboxylic acids. In indirect effect, as a result of ozone decomposition by reacting with organic matter, radicals, especially hydroxyl radicals, are formed. In indirect reactions, three phases can be defined: activation, propagation, and termination. In the way with an activator [hydroperoxide radical (HO2•)], the decomposition of ozone is accelerated, and at a pH>4.8 (corresponding to the pKa of the radical), the radical forms the anion superoxide, which triggers chain propagation. This reacts with ozone and forms the hydroxyl radical (von Gunten, 2003; Botondi et al., 2023). Ozone reacts with oxidizable cellular components such as double bonds, sulfhydryl groups, and phenolic rings, and thus inactivates microorganisms through cell damage and leads to death of the microorganism.

Ozone kills bacteria (bactericidal effect) by damaging their DNA. While the exact details are still being studied (Khanashyam et al., 2022), we know ozone reacts with fats (lipids) in the bacteria's outer shell. This reaction creates harmful molecules that travel inside the bacteria and damage its genetic material. The main difference between Gram-positive and Gram-negative bacteria lies in the structure of their cell walls. Gram-positive bacteria have a thicker cell wall, while Gram-negative bacteria have a thinner wall with an additional outer membrane. This difference might influence the rate at which ozone can reach and damage the cell membrane in each type. Ozone's strong oxidizing power is known to destroy the cell walls and membranes of both Gram-positive and Gram-negative bacteria, as well as fungi (Celiberti et al., 2006; Azarpazhooh & Limeback, 2008). The mechanism of inactivation against microorganisms by ozone is achieved in two ways: i) oxidation of amino acid and sulfhydryl groups of peptides, proteins, and enzymes to generate small peptides during ozone exposure, ii) oxidation of polyunsaturated fatty acids with the formation of acid peroxides (McHugh, 2015; Dubey et al., 2022; Epelle et al., 2023).

In the case of bacterial cell walls and membranes, ozone oxidizes several components, especially unsaturated fatty acids, enzymes, glycoproteins, and glycolipids at the membrane level, leading to modification of the permeability of the cell membrane and, finally, cell disintegration (Botondi et al., 2023). Ozone applications can be used in various industries such as agriculture, paper, food, and paint industries, in clinical medicine in order to improve water quality, to minimize harmful nitrites and organic carbons in water, to preserve freshness and extend the shelf life of foods, to disinfect closed work areas and spring waters (Calunga et al., 2012; Epelle et al., 2023).

Depending on the ozone application type, different instruments and systems are used. These instruments enable the effective production, distribution, and control of ozone. Ozone generators, ozone generator control systems,

ozone monitors, ozone diffusers, ozone cabins, and rooms are some instruments used in ozone applications. The flow rate of the ozone treatments can vary depending on the application and apparatus in which ozone gas is produced or distributed. The flow rate is adjusted according to the volume of the media, the targeted ozone concentration, and application requirements (Guo et al., 2019; Radjabov et al., 2019).

This study used a closed ozone generator system that can produce ozone gas with corrosion-resistant electrodes in air and water. It aims to examine the bacteriocidal/bacteriostatic effects of ozone gas applied with different parameters (duration, flow rate) and application methods (pathogen bacteria, distilled water, and the mixture of distilled water and pathogen bacteria) on pathogenic microorganisms. No study has been found that involves the application of ozone gas directly to pathogenic microorganisms or combining the effect of ozonated water on pathogenic microorganisms and ozone application to water-containing microorganisms.

The study investigated the efficacy of ozone gas in combating foodborne pathogens. Ozone gas was applied to pathogenic microorganisms using an ozone generator under varying flow rates and exposure durations. Three distinct methods were utilized to evaluate the effectiveness of ozone treatment in hindering the growth of foodborne pathogens. Each method involved exposing pathogenic microorganisms to ozone gas under varying conditions of flow rate and duration. The most effective method for growth inhibition was determined based on the results obtained from all three approaches.

MATERIALS and METHODS

Microorganisms and media

In this study, Gram (+) *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, Gram (-) *Salmonella enteritidis* ATCC 13076, *Escherichia coli* O157:H7, which were in the stock culture collection of Gazi University, Faculty of Science, Department of Biology, Biotechnology Laboratory, were used. While the nutrient medium (Merck) was used as a liquid medium to cultivate the bacteria, their cultivation was carried out in selective nutrient media specific to each bacterium for live cell counts to be determined after ozone application. The Gram-positive and Gram-negative pathogenic bacteria and culture medium used in the study are listed in Table 1 (Rusenova et al., 2022; Tavassoli et al., 2022; Sadeq et al., 2024).

To prepare the bacteria for ozone treatment (pre-activation), 2% of the bacteria from the frozen stock (stored at -30°C) was added to a nutrient-rich liquid broth. This mixture was then incubated at 37°C for 24 hours. This growth step was repeated twice in fresh broth to ensure a good supply of active bacteria for the ozone application.

Ozone production

For ozone production, a dielectric barrier discharge (DBD) type ozone generator (Avem IT, Turkey) (Fig. 1) was used. It was achieved by changing the flow rate and application time parameters to obtain high ozone efficiency. The ozone generator used in the study and the reactor of the instrument was given in Figure 2.

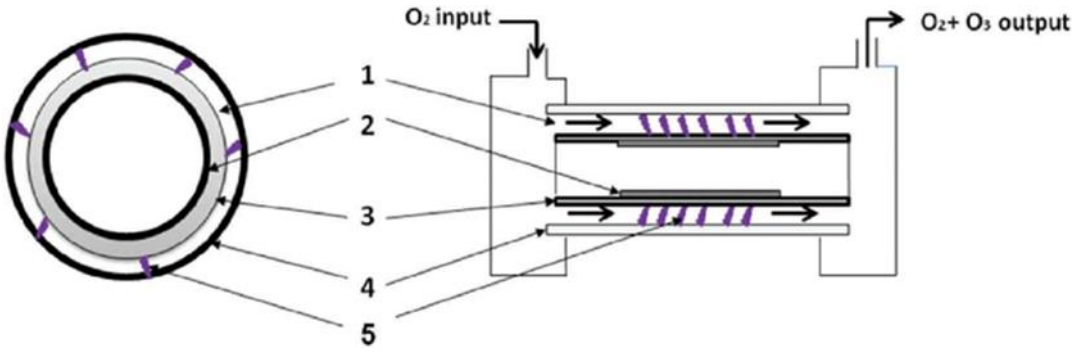

Figure 1. Dielectric barrier discharge (DBD) generator system (1 and 5: ozone formation, 2: inner electrodes, 3: dielectric quartz glass, 4: outer electrodes)

Şekil 1. Dielektrik bariyerli deşarj (DBD) jeneratör sistemi (1 ve 5: ozon oluşumu, 2: iç elektrotlar, 3: dielektrik kuvars camı, 4: dış elektrotlar)

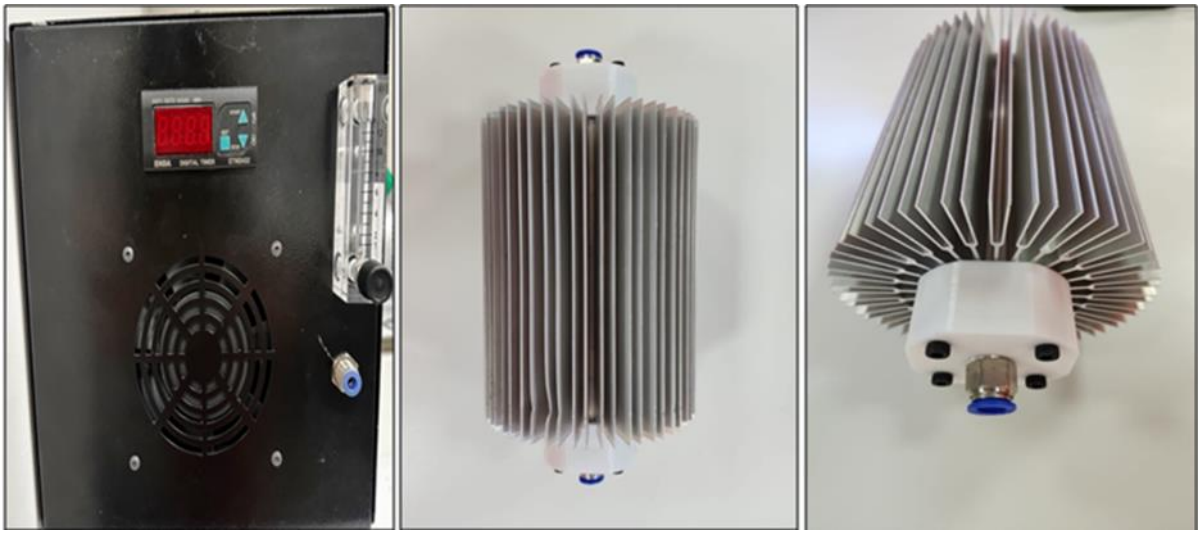

Figure 2. The ozone instrument used in the study and the reactor of the instrument

Şekil 2. Çalışmada kullanılan ozon jeneratörü ve cihazın reaktörü

Experimental procedure

The samples were treated with ozone gaseous at different flow rates (4, 5, and 6 mg L^{-1}) and times (1, 5, 10, 15, and 20 min) using three various procedures (pathogen bacteria, distilled water, and the mixture of distilled water and pathogen bacteria). Then, the number of viable cells was determined after the procedure. Also, bacteria without ozone treatment were used as the control group.

Effects of gaseous ozone exposure on pathogen bacteria

After the microorganisms were grown twice in the medium, their optical densities were adjusted to McFarland 5 ($1.5x10^9$ log cfu mL^{-1}) and 10 ($3.0x10^9$ log cfu mL^{-1}) to determine the effect of ozone gas on the growth of microorganisms at different densities. To determine the concentration of the bacteria, 100 microliters were taken. This sample then underwent serial dilutions (10^{-5}) in test tubes containing sterile salt solution (0.875% sodium chloride, NaCl, Merck). Finally, the diluted samples were plated onto a specific solid growth medium suitable for that particular type of bacteria. Ozone gas was for 1, 5, 10, 15, 20 and 20 min and with a flow rate of 4,5 or 6 mg L^{-1} to the petri dishes where pathogenic bacteria were inoculated. Then, the petri was incubated at 37 °C for 24 hours, and was monitored for visual colonies. The number of colonies growing in petri dishes were calculated according to the formula below.

Number of live bacteria (cfu mL^{-1})= (Number of colonies Dilution factor)/Bacteria inoculated in the petri dish (mL) **Eq.(1)**

Dilution factor= 1 / Dilution ratio **Eq.(2)**

Treatment of distilled water with ozone gaseous

Distilled water (100 mL) was treated with ozone at different times (1, 5, 10, 15, and 20 min) and flow rate (4, 5, and 6 mg L^{-1}). Microorganisms were serially diluted by a factor of 10^{-5}, after their densities were adjusted to McFarland 5 and 10. Equal amounts of water and bacterial culture were mixed (1:1), and spread was inoculated on a suitable solid medium for each bacteria. After the procedure, viable cells were counted after incubating the petri dishes at 37 °C for 24 hours.

Application of ozone gaseous to a mixture of distilled water and bacteria

Since direct ozone gas treatment of pathogenic microorganisms was found to be more effective in terms of bactericidal activity than ozone gas treatment of distilled water, an alternative method was investigated. This involved applying ozone to a mixture of distilled water and bacteria (1:1 ratio) and evaluating its effectiveness. Microorganisms were serially diluted by a factor of 10^{-5}, after their densities were adjusted to McFarland 5 and 10. Samples were taken from distilled water and bacterial culture at a ratio of 1:1. The mixture was treated with ozone gas at different times (1, 5, 10, 15, and 20 min) and flow rates. Cultivation was carried out on solid media specific to each bacterium for live cell counting. After inoculating in a petri dish, the samples were incubated at 37 °C for 24 hours.

Statistical analysis

All studies were carried out in three parallel and three repetitions. Data obtained from these studies are presented as the mean of these replicates ± standard deviation (SD). SPSS Inc. Software (version 22.0, SPSS Inc., Chicago, IL) was used for statistical analyses. The significance of the relationship between ozone gas applied at different flow rates and times and the number of living cells was determined by two-way ANOVA analysis, Tukey's post hoc test, and using GraphPad Prism (www.graphpad.com) software. Results were considered significant where $P < 0.05$.

RESULTS and DISCUSSIONS

In our study, to determine the bacteriocidal/bacteriostatic effect of ozone gas on the growth of microorganisms, gaseous ozone exposure on pathogen bacteria, distilled water and a mixture of distilled water and pathogenic bacteria at different flow rates (4, 5, and 6 mg L^{-1}) and durations (1, 5, 10, 15 and 20 min). In the study, the effect of ozone gas on *S. aureus* ATCC 25923, *L. monocytogenes* ATCC 7644, *S. enteritidis* ATCC 13076 and *E. coli* O157:H7 was examined (Table 1).

Table 1. Pathogenic bacteria and culture medium
Çizelge 1. Patojenik bakteri ve kültür ortamı

Bacteria	Medium (Merck)
S. aureus ATCC 25923	Mannitol Salt Phenol Red Agar
L. monocytogenes ATCC 7644	Listeria Enrichment Agar
S. enteritidis ATCC 13076	Salmonella Shigella Agar
E. coli O157:H7	Eosin Methylene Blue Agar

Direct ozone treatment of the pathogen microorganisms eliminated bacterial growth at all flow rates (4, 5, and 6 mg L^{-1}) for exposure times of 10, 15, and 20 minutes, with one exception: *E. coli* O157:H7 showed some growth. However, a lower amount of growth was detected in the 1st and 5th minutes compared to the control group (Table 2).

Table 2. Number of live cells (log cfu/mL) as a result of ozone treatment to Gram (+) and Gram (-) pathogen bacteria
Çizelge 2. Gram (+) ve Gram (-) patojen bakterilere ozon uygulaması sonucu oluşan canlı hücre sayısı (log kob mL^{-1})

Bacteria Name	Bacteria Density (McFarland)	4 mg/L					5 mg/L					6 mg/L					Control (Bakteria) log cfu/mL
		Time (Minute)															
		1 [a]	5 [x]	10	15	20	1 [b]	5 [y]	10	15	20	1 [c]	5 [z]	10	15	20	
L. monocytogenes ATCC 7644	5	7.35±3.3 [b,c]	7.13±1.8 [y,z]	-	-	-	7.21±2.3 [a,c]	7.02±2.0 [x,z]	-	-	-	7.09 ±0.8 [a,b]	6.50±1.3 [x,y]	-	-	-	7.89±2.7
	10	7.76±2.4 [b,c]	7.44±1.1 [y,z]	-	-	-	7.52±1.7 [a,c]	7.01±2.2 [x,z]	-	-	-	7.49±0.9 [a,b]	6.14±1.6 [x,y]	-	-	-	8.72±3.0
E. coli O157:H7	5	7.40±2.0 [b,c]	7.28±1.0 [y,z]	6.90±1.5	-	-	7.35±1.4 [a,c]	7.19±1.7 [x,z]	-	-	-	7,30±2,8 [a,b]	6.98±2,0 [x,y]	-	-	-	7.53 ±1.4
	10	7.55±1.7 [b,c]	7.19±2.1 [y,z]	-	-	-	7.30±2.0 [a,c]	6.96±2.5 [x,z]	-	-	-	7.14±3,5 [a,b]	6.68±0,5 [x,y]	-	-	-	7.81±2.7
S. aureus ATCC 25923	5	7.49±3.0 [b,c]	-	-	-	-	7.33±1.5 [a,c]	-	-	-	-	7.20±1.7 [a,b]	-	-	-	-	8.08±2.9
	10	7.18±1.4 [b,c]	6.78±0.6	-	-	-	6.90±1.8 [a,c]	-	-	-	-	6.53±2.5 [a,b]	-	-	-	-	8.74±2.1
S. enteridis ATCC 13076	5	7.62±2.8 [b,c]	7.33±1.6 [y,z]	-	-	-	7.41±1.4 [a,c]	7.17±0.8 [x,z]	-	-	-	7.30±2.1 [a,b]	7.00±0.9 [x,y]	-	-	-	8.12±1.7
	10	7.69± 3.5 [b,c]	7.52± 1.4 [y,z]	-	-	-	7.41±2.0 [a,c]	7.02±0.6 [x,z]	-	-	-	7.36± 1.7 [a,b]	6.17± 1.1 [x,y]	-	-	-	8.65±4.0

-: No reproduction observed

±: presented as standard deviation.

[a,b,c ve x,y,z] Values expressed with different letters in the same column are statistically significant at the $p<0.05$ level according to the Tukey test.

Among the tested bacteria, *E. coli* O157:H7 exhibited the lowest reduction in live cells (1.73%) when treated with ozone for 1 minute at the lowest concentration (4 mg L^{-1}) and initial bacterial density (McFarland 5). Conversely, *S. aureus* ATCC 25923 showed the highest decrease in live cells (27.92%) under the strongest treatment conditions (6 mg L^{-1} ozone, McFarland 10) (Table 3).

Table 3. Percentage (%) decrease in pathogen bacterial cell viability after direct ozone treatment to pathogen bacteria

Çizelge 3. Patojen bakterilere doğrudan ozon uygulanmasından sonra patojen bakteri hücre canlılığında azalma yüzdesi (%)

Bacteria Name	Bacteria Density (McFarland)	Decrease in Bacterial Cell Viability (%)					
		4 mg/L		5 mg/L		6 mg/L	
		1 min [a]	5 min [x]	1 min [b]	5 min [y]	1 min [c]	5 min [z]
L. monocytogenes ATCC 7644	5	6.84 [b,c]	10.66 [y,z]	8.62 [a,c]	11.03 [x,z]	10.14 [a,b]	17.62 [x,y]
	10	11.01 [b,c]	14.68 [y,z]	13.76 [a,c]	19.61 [x,z]	14.11 [a,b]	29.59 [x,y]
E. coli O157:H7	5	1.73 [b,c]	3.32 [y,z]	2.39 [a,c]	4.52 [x,z]	3.05 [a,b]	7.30 [x,y]
	10	3.33 [b,c]	7.94 [y,z]	6.53 [a,c]	14.34 [x,z]	8.58 [a,b]	14.47 [x,y]
S. aureus ATCC 25923	5	7.30 [b,c]	-	9.28 [a,c]	-	10.89 [a,b]	-
	10	17.85 [b,c]	22.43	21.05 [a,c]	-	27.92 [a,b]	-
S. enteridis ATCC 13076	5	6.16 [b,c]	9.73 [y,z]	8.74 [a,c]	11.70 [x,z]	10.10[a,b]	16.00 [x,y]
	10	11.09 [b,c]	13.06 [y,z]	14.34 [a,c]	18.84 [x,z]	17.53 [a,b]	28.67 [x,y]

[a,b,c ve x,y,z] Values expressed with different letters in the same column are statistically significant at the $p<0.05$ level according to the Tukey test.

Ozonated water at different flow rates and times was mixed with bacterial culture at the same rate, and the number of viable cells was determined. According to the analysis results, as the applied flow rate and ozone contact time increased, a decrease in the viability of the bacteria was observed ($P < 0.05$). Even with the increase in ozone flow rate and exposure time, some bacteria remained viable and continued to grow (Table 4).

Table 4. Number of living cells as a result of treatment of pathogen bacteria + ozone distilled water (log cfu mL^{-1})
Çizelge 4. Patojen bakteri + ozonlu distile su uygulaması sonucu oluşan canlı hücre sayısı (log kob mL^{-1})

Bacteria Name	Bacteria Density (McFarland)	4 mg/L					5 mg/L					6 mg/L					Control (Bakteria) log cfu/mL
		Time (Minute)															
		1 ^{a}	5 ^{x}	10^{i}	15 ^{v}	20 *	1 ^{b}	5 ^{y}	10 ^{ii}	15 ^{vi}	20 $^{\&}$	1 ^{c}	5 ^{z}	10 ^{iii}	15 ^{vii}	20 $^{\#}$	
L. monocytogenes ATCC 7644	5	7.82±1.2 ^{b,c}	7.73±0.9 ^{y,z}	7.31±1.7 ^{ii,iii}	6.96±2.1 ^{vi,vii}	6.70± 0,7 $^{\&,\#}$	7.61±0.5 ^{a,c}	7.42±2.3 ^{x,z}	7.00±1.4 ^{i,iii}	6.49±1.8 ^{v,vii}	6.17 ±0.4 $^{*,\#}$	7.50±1.3 ^{a,b}	7.28±2.1 ^{x,y}	6.96±3.0 ^{i,ii}	6.38±1.7 ^{v,vi}	6.02±1.4 $^{*,\&}$	7.89± 2.7
	10	8.60±0.6 ^{b,c}	8.29±1.1 ^{y,z}	7.58±1.8 ^{ii,iii}	7.24±1.4 ^{vi,vii}	7.03 ± 1,3 $^{\&,\#}$	8.37±0.6 ^{a,c}	8.10±0.9 ^{x,z}	7.44±1.7 ^{i,iii}	7.12±2.2 ^{v,vii}	6.51±1.8 $^{*,\#}$	8.16±0.6 ^{a,b}	7.88±1.4 ^{x,y}	7.31±1.1 ^{i,ii}	6.77±0.6 ^{v,vi}	6.24±1.2 $^{*,\&}$	8.72±3.0
E. coli O157:H7	5	7.48±1.2 ^{b,c}	7.39±1.8 ^{y,z}	7.16±0.6 ^{ii,iii}	6.71±1.0 ^{vi,vii}	6.43±1.2 $^{\&,\#}$	7.40±1.6 ^{a,c}	7.28±0.5 ^{x,z}	6.94±1.2 ^{i,iii}	6.51±2.0 ^{v,vii}	6.27±1.4 $^{*,\#}$	7.29±0.8 ^{a,b}	7.04±1.1 ^{x,y}	6.69±0.3 ^{i,ii}	6.40±0.9 ^{v,vi}	6.19±1.5 $^{*,\&}$	7.53 ±1.4
	10	7.70±1.3 ^{b,c}	7.61±0.9 ^{y,z}	7.28±1.2 ^{ii,iii}	6.91±1.7 ^{vi,vii}	6.65±0.4 $^{\&,\#}$	7.60±1.0 ^{a,c}	7.36±1.3 ^{x,z}	7.09±0.8 ^{i,iii}	6.77±1.2 ^{v,vii}	6.46±0.3 $^{*,\#}$	7.52±2.0 ^{a,b}	7.26±1.1 ^{x,y}	7.01±0.9 ^{i,ii}	6.67±0.5 ^{v,vi}	6.03±1.2 $^{*,\&}$	7.81±2.7
S. aureus ATCC 25923	5	7.78± 2.5 ^{b,c}	7.61± 1.0 ^{y,z}	7.27± 1.6 ^{ii,iii}	6.70± 0.5 ^{vi,vii}	6.44± 0.8 $^{\&,\#}$	7.65 ± 1.4 ^{a,c}	7.30 ± 0.3 ^{x,z}	6.71± 1.4 ^{i,iii}	6.26± 0.5 ^{v,vii}	6.05± 1.0 $^{*,\#}$	7.41 ± 1.1 ^{a,b}	7.19 ± 1.8 ^{x,y}	6.65 ± 0.5 ^{i,ii}	6.31± 0.9 ^{v,vi}	5.97 ± 0.4 $^{*,\&}$	8.08±2.9
	10	8.37±1.6 ^{b,c}	8.04±0.9 ^{y,z}	7.39±1.8 ^{ii,iii}	7.03±1.1 ^{vi,vii}	6.81±0.6 $^{\&,\#}$	8.17±1.4 ^{a,c}	7.89±1.2 ^{x,z}	7.32±0.9 ^{i,iii}	6.74±0.3 ^{v,vii}	6.60±1.1 $^{*,\#}$	7.98±1.9 ^{a,b}	7.80±1.4 ^{x,y}	7.31±1.1 ^{i,ii}	6.87±0.8 ^{v,vi}	6.40±1.2 $^{*,\&}$	8.74±2.1
S. enteridis ATCC 13076	5	7,90±2.0 ^{b,c}	7.75±1.3 ^{y,z}	7.46±1.1 ^{ii,iii}	7.19±1.7 ^{vi,vii}	6.85±0.6 $^{\&,\#}$	7.79±0.9 ^{a,c}	7.53±1.4 ^{x,z}	7.27±0.5 ^{i,iii}	6.98±0.6 ^{v,vii}	6.65±1.0 $^{*,\#}$	7.61±1.7 ^{a,b}	7.39±2.1 ^{x,y}	7.02±1.4 ^{i,ii}	6.77±0.8 ^{v,vi}	6.42±1.6 $^{*,\&}$	8.12±1.7
	10	8.38±0.6 ^{b,c}	8.06±1.2 ^{y,z}	7,54±1.5 ^{ii,iii}	7.19±0.7 ^{vi,vii}	6.98±1.1 $^{\&,\#}$	8.18±2.1 ^{a,c}	7.81±1.7 ^{x,z}	7.33±1.2 ^{i,iii}	7.02±0.3 ^{v,vii}	6.86±0.9 $^{*,\#}$	7.91±1.6 ^{a,b}	7.60±0.2 ^{x,y}	7.11±0.8 ^{i,ii}	6.80±1.4 ^{v,vi}	6.59±1.1 $^{*,\&}$	8.65±4.0

^{a,b,c} ^{x,y,z} ^{i,ii,iii} ^{v,vi,vii} $^{*,\&,\#}$ Values expressed with different letters in the same column are statistically significant at the p<0.05 level according to the Tukey test.

The minimum reduction in the live cell numbers of bacteria combined in the same ratio with the water sample to which ozone was applied in the shortest time (1 min) was *E. coli* O157:H7 with 0.66% (7.48 log cfu mL^{-1}) at 4 mg L^{-1} flow rate and McFarland 5. The highest decrease in live cells was observed in *S. aureus* ATCC 25923 with 8.70% (7.98 log cfu mL^{-1}) at a 6 mg L^{-1} flow rate and McFarland 10 (Table 5).

Table 5. Percentage (%) decrease in pathogen bacterial cell viability after treatment of pathogen bacteria + ozonated distilled water

Çizelge 5. Patojen bakteri + ozonlanmış distile su uygulamasından sonra patojen bakteri hücre canlılığında azalma yüzdesi (%)

Bacteria Name	Bacteria Density (McFarland)	Decrease in Bacterial Cell Viability (%)														
		4 mg/L					5 mg/L					6 mg/L				
		1 min a	5 min x	10 min i	15 min v	20 min *	1 min b	5 min y	10 min ii	15 min vi	20 min &	1 min c	5 min z	10 min iii	15 min vii	20 min #
L. monocytogenes ATCC 7644	5	0.8 9 b,c	2.0 3 y,z	7.35 ii,iii	11.7 9 vi,vii	15.0 8 &,#	3.5 5 a,c	5.9 6 x,z	11.2 8 i,iii	17.7 4 v,vii	21.8 0 *,#	4.9 4 a,b	7.73 x,y	11.7 9 i,ii	19.1 4 v,vi	23.7 0 *,&
	10	1.3 8 b,c	4.9 3 y,z	13.0 7 ii,iii	16.9 7 vi,vii	19.3 8 &,#	4.0 1 a,c	7.1 1 x,z	14.6 8 i,iii	18.3 5 v,vii	25.3 4 *,#	6.4 2 a,b	9.63 x,y	16.1 7 i,ii	22.3 6 v,vi	28.4 4 *,&
E. coli O157:H7	5	0.6 6 b,c	1.8 6 y,z	4.91 ii,iii	10.8 9 vi,vii	14.6 1 &,#	1.7 3 a,c	3.3 2 x,z	7.84 i,iii	13.5 5 v,vii	16.7 3 *,#	3.1 9 a,b	6.51 x,y	11.1 6 i,ii	15.0 1 v,vi	21.6 5 *,&
	10	1.4 1 b,c	2.5 6 y,z	6.79 ii,iii	11.5 2 vi,vii	14.8 5 &,#	2.6 9 a,c	5.7 6 x,z	9.22 i,iii	13.3 2 v,vii	17.2 9 *,#	3.7 1 a,b	7.04 x,y	10.2 4 i,ii	14.6 0 v,vi	22.7 9 *,&
S. aureus ATCC 25923	5	3.7 1 b,c	5.8 2 y,z	10.0 2 ii,iii	17.0 8 vi,vii	20.3 0 &,#	5.3 2 a,c	9.6 5 x,z	16.9 6 i,iii	22,5 2 v,vii	25.1 2 *,#	8.2 9 a,b	11.0 1 x,y	17.8 8 i,ii	21.9 1 v,vi	26.1 1 *,&
	10	4.2 3 b,c	8.0 1 y,z	15.4 5 ii,iii	19.5 7 vi,vii	22.0 8 &,#	6.5 2 a,c	9.7 3 x,z	16.2 5 i,iii	22.8 8 v,vii	24.4 9 *,#	8.7 0 a,b	10.7 6 x,y	16.3 6 i,ii	21.4 0 v,vi	26.7 7 *,&
S. enteridis ATCC 13076	5	2.7 1 b,c	4.5 6 y,z	8.13 ii,iii	11.4 5 vi,vii	15.6 4 &,#	4.0 6 a,c	7.2 7 x,z	10.4 7 i,iii	14.0 4 v,vii	18.1 0 *,#	6.2 8 a,b	8.99 x,y	13.5 5 i,ii	16.6 3 v,vi	20.9 4 *,&
	10	3.1 2 b,c	6.8 2 y,z	12.8 3 ii,iii	16.8 8 vi,vii	19.3 0 &,#	5.4 3 a,c	9.7 1 x,z	15.2 6 i,iii	18.8 4 v,vii	20.6 9 *,#	8.5 5 a,b	12.1 4 x,y	17.8 0 i,ii	21.3 9 v,vi	23.8 2 *,&

a,b,c x,y,z i,ii,iii v,vi,vii *,&,# Values expressed with different letters in the same column are statistically significant at the $p<0.05$ level according to the Tukey test.

In another application method, ozone gas was applied to the mixture of distilled water and bacteria at flow rates of 4, 5, and 6 mg L^{-1} for varying contact times: 1, 5, 10, 15, and 20 minutes. No bacterial growth was observed after 15 and 20 minutes of ozone exposure. However, bacteria were still present after 1, 5, and 10 minutes of treatment (Table 6).

Table 6. Number of viable cells as a result of ozone treatment to the mixture of pathogen bacteria + distilled water (log cfu mL^{-1})

Çizelge 6. Patojen bakteri+distile su karışımına ozon uygulaması sonucu oluşan canlı hücre sayısı (log kob mL^{-1})

Bacteria Name	Bacteria Density (McFarland)	4 mg/L					5 mg/L					6 mg/L					Control (Bakteria) log cfu /mL
		Time (Minute)															
		1 ^{a}	5 ^{x}	10 $_{i}$	15	20	1 ^{b}	5 ^{y}	10 $_{ii}$	15	20	1 ^{c}	5 ^{z}	10 $_{iii}$	15	20	
L. monocytogenes ATCC 7644	5.0	7.58±1. 3 ^{b,c}	7.37±1.6 ^{y,z}	6.91±2.0 $^{ii, iii}$	-	-	7.38±1.8 ^{a,c}	7.29±2.4 ^{x,z}	6.56±1.3 $^{i, iii}$	-	-	7.24±0.7 ^{a,b}	7.01±1.2 ^{x,y}	6.17±1.9 $^{i, ii}$	-	-	7.89± 2.7
	10.0	8.38±1.5 ^{b,c}	8.06±1.8 ^{y,z}	7.41±0.4 $^{ii, iii}$	-	-	8.20±1.1 ^{a,c}	7.79±1.3 ^{x}	7.11±1.6 $^{i, iii}$	-	-	8.01±1.4 ^{a,b}	7.75±1.9 ^{x}	7.02±0.5 $^{i, ii}$	-	-	8.72±3.0
E. coli O157:H7	5.0	7.47±0.4 ^{b,c}	7.33±0.8 ^{y,z}	6.95±1.3 $^{ii, iii}$	-	-	7.32±0.5 ^{a,c}	7.26±0.9 ^{x,z}	6.77±1.2 $^{i, iii}$	-	-	7.30±1.4 ^{a,b}	7.03±1.0 ^{x,y}	6.45±0,7 $^{i, ii}$	-	-	7.53 ±1.4
	10.0	7.64±1.3 ^{b,c}	7.42±0.5 ^{y,z}	7.00±1.6 ^{ii,iii}	-	-	7.51±1.0 ^{a,c}	7.14±1.4 ^{x,z}	6.83±0.8 $^{i, iii}$	-	-	7.28±1.2 ^{a,b}	6.93±0.3 ^{x,y}	6.61±0.7 $^{i, ii}$	-	-	7.81±2.7
S. aureus ATCC 25923	5.0	7.67± 2.0 ^{b,c}	7,49 ± 1,3 ^{y,z}	7.09 ± 1,1 $^{ii, iii}$	-	-	7.57 ± 2.1 ^{a,c}	7.07 ± 0.7 ^{x,z}	6.41± 1.4 $^{i, iii}$	-	-	7.40 ± 1.3 ^{a,b}	6.93 ± 1.7 ^{x,y}	6.40 ± 1.4 $^{i, ii}$	-	-	8,08±2,9
	10.0	8.01±1.0 ^{b,c}	7.69±0.5 ^{y,z}	7.16±0.9 $^{ii, iii}$	-	-	7.77±1.3 ^{a,c}	7.31±1.5 ^{x,z}	6.69±0.6 $^{i, iii}$	-	-	7.52±1.1 ^{a,b}	7.16±0.4 ^{x,y}	6.62±1.2 $^{i, ii}$	-	-	8.74±2.1
S. enteridis ATCC 13076	5.0	7.71±1.7 ^{b,c}	7.57±1.3 ^{y,z}	7.08±0.4 $^{ii, iii}$	-	-	7.55±1.5 ^{a,c}	7,36±2.0 ^{x,z}	6,94±0.9 $^{i, iii}$	-	-	7.40±0.7 ^{a,b}	7.23±1.1 ^{x,y}	6.76±1.3 $^{i, ii}$	-	-	8.12±1.7
	10.0	8.08±0.7 ^{b,c}	7.73±0.5 ^{y,z}	7.31±1.9 $^{ii, iii}$	-	-	7.80±1.2 ^{a,c}	7,52±1.5 ^{x,z}	7,04±0.3 $^{i, iii}$	-	-	7.55±2.1 ^{a,b}	7.39±0.9 ^{x,y}	6.93±1.4 $^{i, ii}$	-	-	8.65±4.0

^{a,b,c} ^{x,y,z} ^{i,ii,iii} Values expressed with different letters in the same column are statistically significant at the $p<0.05$ level according to the Tukey test.

In the study when ozone was applied to the mixture of distilled water and bacteria for the shortest time (1 min), the lowest decrease in the live cell numbers of bacteria was observed in E. coli O157:H7 with 0.80% (7.47 log cfu mL^{-1}) at 4 mg L^{-1} flow rate and McFarland 5. The highest decrease in viable cells was determined in *S. aureus* ATCC

25923 with 13.96% (7.52 log cfu mL^{-1}) at a 6 mg L^{-1} flow rate and McFarland 10 (Table 7). When the general applications were evaluated, *E. coli* O157:H7 was the least affected among the pathogenic bacteria used in all three applications.

Table 7. Percentage (%) decrease in pathogen bacterial cell viability after ozone treatment to the mixture of pathogen bacteria+distilled water

Çizelge 7. Patojen bakteri+distile su karışımına ozon uygulaması sonrası patojen bakteri hücre canlılığında azalma yüzdesi (%)

Bacteria Name	Bacteria Density (McFarland)	Decrease in Bacterial Cell Viability (%)								
		4 mg/L			5 mg/L			6 mg/L		
		1 min [a]	5 min [x]	10 min [i]	1 min [b]	5 min [y]	10 min [ii]	1 min [c]	5 min [z]	10 min [iii]
L. monocytogenes ATCC 7644	5	3.93 [b,c]	6.59 [y,z]	12.42 [ii,iii]	6.46 [a,c]	7.60 [x,z]	16.86 [i,iii]	8.24 [a,b]	11.15 [x,y]	21.80 [i,ii]
	10	3.90 [b,c]	7.57 [y,z]	15.02 [ii,iii]	5.96 [a,c]	10.67 [x,z]	18.46 [i,iii]	8.14 [a,b]	11.12 [x,y]	19.50 [i,ii]
E. coli O157:H7	5	0.80 [b,c]	2.66 [y,z]	7.70 [ii,iii]	2.79 [a,c]	3.59 [x,z]	10.09 [i,iii]	3.05 [a,b]	6.64 [x,y]	14.34 [i,ii]
	10	2.18 [b,c]	4.99 [y,z]	10.37 [ii,iii]	3.84 [a,c]	8.58 [x,z]	12.55 [i,iii]	6.79 [a,b]	12.70 [x,y]	15.36 [i,ii]
S. aureus ATCC 25923	5	5.07 [b,c]	7.30 [y,z]	12.25 [ii,iii]	6.31 [a,c]	12.50 [x,z]	20.67 [i,iii]	7.73 [a,b]	14.23 [x,y]	20.79 [i,ii]
	10	8.35 [b,c]	12.01 [y,z]	18.07 [ii,iii]	11.09 [a,c]	16.36 [x,z]	23.45 [i,iii]	13.96 [a,b]	18.07 [x,y]	24.25 [i,ii]
S. enteridis ATCC 13076	5	5.05 [b,c]	6.77 [y,z]	12.81 [ii,iii]	7.02 [a,c]	9.36 [x,z]	14.53 [i,iii]	8.87 [a,b]	10.96 [x,y]	16.75 [i,ii]
	10	6.59 [b,c]	10.64 [y,z]	15.49 [ii,iii]	9.83 [a,c]	13.06 [x,z]	18.61 [i,iii]	12.72 [a,b]	14.57 [x,y]	19.88 [i,ii]

[a,b,c] [x,y,z] [i,ii,iii] Values expressed with different letters in the same column are statistically significant at the $p<0.05$ level according to the Tukey test.

Maintaining product quality and safety is of great importance. Since ozone application effectively reduces microbial contamination of the products without causing a negative impact on their visual, textural, and nutritional quality, it can be used in industry applications (Brodowska et al., 2018). Ozone offers several advantages over other disinfectants: low operating costs, no unpleasant odors in treated water or air, chemical-free application, broad-spectrum disinfection against bacteria, viruses, and parasites, reduced chemical waste, effectiveness against insects, and ease of use (Glowacz et al., 2015; Brodowska et al., 2018; Chun et al., 2023).

Ozone applications are an environmentally friendly and residue-free technique with widespread use in the inhibition of pathogenic microorganism growth, food spoilage, agricultural biotechnology, and environmental contamination, food spoilage, agricultural biotechnology, and environmental contamination. Thus, ozone applications have the potential to be an alternative to traditional methods due to their important advantages, such as high effect in a short time and low cost (Pandiselvam et al., 2019; Sivaranjani et al., 2021; Khanashyam et al., 2022).

Ozone, a powerful disinfectant, decomposes into hydroxyl, hydroperoxide, and superoxide radicals. Ozone decomposition into these free radicals provides short-lived compounds with strong oxidation potential (Mouele et

al., 2021). Direct reaction with ozone also includes oxidation. Through these oxidation reactions, ozone provides a powerful and broad-spectrum effect that neutralizes many pathogenic bacteria such as *S. enteritidis, E. coli* O157:H7, *L. monocytogenes, Shigella dysenteriae, Clostridium botulinum*, as well as various yeasts, fungi, viruses, parasites, and molds (Niveditha et al., 2021).

In this study, it aims to extend the products' shelf life without deteriorating their smell, taste, and appearance, preventing the risk of contamination and spoilage in foods by hindering the development of pathogenic bacteria.

Unlike other studies, ozone applications were not applied directly to food products (beef, turkey and chicken meat, tomatoe, cucumber, lettuce, pepper, cherry, strawberry, etc.) (Novak & Yuan, 2003; Al-Haddad et al., 2005; Alexandre et al., 2011; Coll Cárdenas et al., 2011; Alexopoulos et al., 2013; Alwi & Ali, 2014; Kanaan, 2018; Ayrancı, 2020). İnstead they were used against some pathogenic bacteria. Thus, it is thought that ozone-treated water can be used in agricultural applications such as fighting diseases and increasing plant resistance. It will lead to further studies in agricultural biotechnology.

Ozone can be applied in various forms, such as gas, water, and oil. Ozone gas has an inactivating effect on various microorganisms (bacteria, fungi, yeast, viruses, etc.), ozonated water can be used in the treatment of wounds, burns, and infections, and ozonated oil has the potential to create products using plant extracts (Suh et al., 2019).

In our study, it was determined that there was a decrease of %1.73-29.59 in the viability of microorganisms by applying exposure of ozone gaseous directly to the pathogen microorganism, while after ozone application to the bacteria+ozonated water sample and the combination of water sample and bacteria, the number of living organisms decreased by % 0.66-28.44 and % 0.80-24.25. When the data obtained from the study were compared with the results of other studies, it was determined that all three applied methods exhibited generally bactericidal effects. Considering the three different methods applied. Ozone application directly to the bacteria was the most effective method in preventing/destroying bacterial growth, while the application of ozone applied to the water sample and combined with the bacteria had the least effect.

Poultry, chicken, seafood, and dairy products are food products that can quickly spoil due to bacterial contamination. The primary goal of food manufacturers is to protect consumers from pathogenic microorganism contamination while extending the product's shelf life without compromising its quality. A study examining the combined effects of ozone gas and freeze-drying on chicken found that the shelf life of chicken meat treated with 0.6 mg/L ozone for 10 minutes was above the prescribed limit. According to the research, ozone treatment, freeze-drying, and vacuum packaging only extended the shelf life of chicken meat by four months (Cantalejo et al., 2016). Giménez et al. (2021) applied 280 mg O_3 m^{-3} ozone gas for 5-10 minutes at half-hour intervals during the cooling of beef and found that it was quite effective in reducing *L. monocytogenes*. The level of bacteria in the sample was around 2 log CFU g^{-1} before ozone. Immediately after the ozone treatment, the amount of *L. monocytogenes* decreased by about tenfold (close to one log CFU g^{-1}). Novak and Yuan (2004) treated the beef surface with ozone and cooked it at 45-75°C. They reported a 1-2 log cfu g^{-1} decrease in *Clostridium perfringens* viability and a small decrease in the number of spores. In another study, where turkey breast meat was treated with ozone for 8 hours, it was reported that a log decrease of 2.9, 2.3, and 1.9 was observed in the counts of yeast, mold, Enterobacteriaceae and aerobic mesophilic bacteria, respectively (Ayrancı et al., 2020). Yuk et al. (2007) applied 5 mg kg^{-1} ozone for 5 minutes to *E. coli* O157:H7 and *L. monocytogenes* bacteria and reported a 1.09 log and 0.94 log reduction in bacteria, respectively. When the same bacteria were treated with 3 mg kg^{-1} ozone combined with 1% citric acid and kept for 1 minute, a 2.31 log and 1.84 log decrease in viability was observed, respectively. In another study, ozone gas (160 g m^{-3}) and then heat treatment were applied to eggs containing *Salmonella enterica* serovar enteritidis, and it was reported that pathogenic microorganisms were significantly neutralized and differences in the visual quality of the eggs were observed (Perry & Yusuf 2013). Nie et al. (2020), treated freshly cut cabbage in aqueous ozone (2 mg kg^{-1}) containing sodium metasilicate (0.4%) for 2 minutes. They reported that after 12 days of storage, a log 3.33 decrease in *E. coli* O157:H7 viability was observed compared to the control group. In another

study, ozone was applied to wheat grains with and without pearls, and it was reported that the dough strength and degree of pearlization of wheat grain flours increased after the treatment (Zhang et al., 2021).

The effectiveness of the ozone treatment method may vary depending on ozone concentration, flow rate, exposure time, and target organism to be inactivated (Pandiselvam et al., 2022). We found that as the flow rate and ozone treatment time increased, the development of pathogenic microorganisms decreased or even could be prevented entirely. Additionally, it was determined that the study's Gram (+) microorganisms were more vulnerable to ozone application than Gram (-) microorganisms. This difference is mainly due to the difference in the cell wall structure of the bacteria, and their viability decreases due to more cell destruction (Khanashyam et al., 2022).

In conclusion, with the increasing consumer demand for fresh, safe, high-quality, and nutritious foods, ozone applications have become remarkably interesting in recent years for several industries (Sarron et al., 2021; Islam et al., 2022; Monica et al., 2024). Ozone applications reduce contamination and extend the shelf life without negatively affecting the visual, textural, and nutritional quality of the products used in the food and agriculture industries. Ozone applications are one of the methods preferred instead of using biological and chemical product treatments (pesticide, herbicide, insecticide, etc.), which are applied to prevent/reduce microbial contamination in agricultural products (Ibanoğlu, 2023). Ozone application is an important process to counter the spread of disease-causing microorganisms and ensure food processing safety particularly in agriculture. In this study, three different ozone applications were tested to eliminate pathogens. All three applications tested reduced the viability of pathogenic microorganisms that cause adverse effects such as unpleasant odor, taste, and food poisoning. Therefore, the three procedures (pathogen bacteria, distilled water, and the mixture of distilled water and pathogen bacteria) used in the study may be used effectively in the fight against pathogenic microorganisms.

STATEMENT OF CONFLICT OF INTEREST

The author declare no conflict of interest for this study.

AUTHOR'S CONTRIBUTIONS

Berat ÇINAR ACAR took part in the planning, execution, supervision, evaluation, and writing of the research.

STATEMENT OF ETHICS CONSENT

Ethical approval is not applicable, because this article does not contain any studies with human or animal subjects.

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