Journal of Nutrition and Food Security
Shahid Sadoughi University of Medical Sciences
School of Public Health
Department of Nutrition
Nutrition & Food Security Research Center

eISSN: 2476-7425 pISSN: 2476-7417 JNFS 2023; 8(4): 587-596 Website: jnfs.ssu.ac.ir

Efficacy of Ozonized Water Spray for the Sanitization of Food Contact Surfaces Contaminated with E. Coli and S. Aureus as an Eco-Friendly Asepsis Approach

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ARTICLE INFO

ORIGINAL ARTICLE

Article history:
Received: 28 Feb 2022
Revised: 22 May 2022
Accepted: 22 May 2022

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ABSTRACT

Background: Cross-contamination between food, equipment, and contact surfaces during food processing may occur with some pathogenic bacteria, such as *E. coli* and *S. aureus*. This study was conducted to investigate the bactericidal potential of ozonized water spray in food contact surfaces (plastic, glass, steel, and ceramic) for the inactivation of *E. coli* and *S. aureus* isolated from traditional yogurt. **Methods**: Two isolated bacteria were tested for antibiotic resistance and the strains were exposed to ozonized water with concentration of 4 mg/l for 0, 2, and 10 minutes and after drying on food contact surfaces (FCS). **Results**: *E. coli* and *S. aureus* were defined as multidrug-resistant. Ozonized water inhibited two bacteria growth on the FCS after two minutes. On plastic, glass, and ceramic surfaces, *E. coli* was more sensitive than *S. aureus*, while *S. aureus* on steel surface was more sensitive than *E. coli*. **Conclusion:** Based on the results, it is recommended to use the ozonized water spray at 4 mg/l concentration to disinfect surfaces in contact with food, especially glass and ceramic surfaces.

Keywords: *Ozonized water; Sanitization; Food contact surfaces; E. Coli; S. Aureus*

Introduction

According to reports of World Health Organization (WHO), about 600 million people are involved in food-borne diseases, and 420,000 die each year due to contaminated food. Various pathogenic microorganisms may contaminate food contact surfaces (FCS). Contaminated FCS can be responsible for such incidences (World Health Organization, 2019). Several factors cause contamination of FCS, including cross-contamination, workers' hands and clothing, raw food, materials of packaging, lack of proper washing, and disinfection (Mohammed *et al.*, 2018). Mohammed et al. reported that 13 (26%) FCS of restaurants were contaminated with *E. coli* (Mohammed *et al.*, 2018). In a study, 63 *S. aureus* samples were isolated from food industry surfaces such as dairy, meat, and seafood (Gutiérrez *et al.*, 2012).

Biofilms of *S. aureus* and *E. coli* are important causes of food poisoning and contamination, causing risk for consumers' health. Biofilms are a

This paper should be cited as: Rahmani Z, Sharafati Chaleshtori R, Abdoli Arani A, Taghizadeh M. ***Efficacy of Ozonized Water Spray for the Sanitization of Food Contact Surfaces Contaminated with E. Coli and S. Aureus as an Eco-Friendly Asepsis Approach.*** *Journal of Nutrition and Food Security (JNFS)*, 2023; 8(4): 587-596.

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[DOR: 20.1001.1.24767417.2023.8.4.9.1]
[DOI: 10.18502/jnfs.v8i4.14008]

natural bacterial lifestyle; first, the cells attach to FCS, and then they start to reproduce and discharge a stable matrix of extracellular polymeric substances in which cells are involved (Yin *et al.*, 2019). Therefore, they are a major problem in many sections of the food industry.

There are many hygienic practices related to preventing microbial contamination of FCS. The Food Safety Modernization Act (FSMA) was approved for a preventative food safety system in the USA. Sanitization or disinfection indicates the reduction of the number of microorganisms to a safe level. Various methods such as thermal, mechanical, chemical, physical, and biological methods have been classified for the disinfection of FCS (Torres Dominguez *et al.*, 2019). Recent investigations have focused on the production of sanitizing solutions with less toxic effects. Jiménez-Pichardo *et al.* demonstrated that the aqueous solution of NaOH on stainless steel plates followed by rinsing with an aqueous chlorine compound could be recommended as an alternative sanitizing method (Jiménez-Pichardo *et al.*, 2016). Also, the effect of ClO2 to inactivate food-borne pathogens on FCS indicated that surface hydrophobicity such as silicon, rubber, Teflon®, stainless steel, and PVC is a major factor compared to the inhibition of bacterial growth by ClO2 gas from the surface (Park and Kang, 2017).

One of the most promising anti-microorganism agents in the list of sanitizers is ozone gas, with a half-life of about 1 hour at room temperature. Gas of ozone can be used as an antimicrobial compound with a powerful oxidizer activity against viruses, bacteria, and protozoa. Nonetheless, the gaseous form of O3 can be toxic. Therefore, O3 can be dissolved in water to reduce its toxicity, and maintain its sanitizing effect (Santos *et al.*, 2021). Previous studies have shown that aqueous O3 effectively reduced several pathogenic microorganisms, including viruses, bacteria, and fungi (Giuroiu *et al.*, 2020, Nakamura *et al.*, 2018). Due to the absence of harmful disinfectant residues after ozone processing by exposure to gaseous ozone or rinsing in ozonated water, its use in the fruit and vegetable industries has been strongly recommended (Botondi *et al.*, 2021).

However, despite the economic and environmental benefits and the high effectiveness of ozone in disinfecting food products, it should be essential to note that this processing does not damage the nutrition facts, appearance, and shelf life of food products. One of the factors increasing food contaminations is the contamination of the FCS in preparing vegetables and meat products. Disinfection of these surfaces using chemicals such as alcohol and chlorine in addition to environmental disadvantages can lead to toxic compounds and detrimental effects on the quality and taste of food products.

The present study investigates antibacterial activities of ozonized water spray on the sanitization of FCS contaminated with *E. coli* and *S. aureus* in the use of ozone-containing water in cleaning equipment surfaces for pre-operational and in-process sanitation.

Materials and Methods

Preparation of materials and bacteria: In this experimental study, four materials with many applications in the food industry were used, including plastic, metal, glass, and ceramic. These surfaces for each material were prepared with dimensions of 10 x 10 cm and sterilized in an autoclave for 15 minutes at 121 °C immediately before use.

For isolation and identification of *S. aureus* and *E.coli*, 10 g of traditional yogurt was added to 90 mL of nutrient broth (Merck Co., Darmstadt, Germany) and homogenized for one minute. From the homogenate, 0.1 ml of aliquots was spread over the surface of the Baird-Parker agar plate (Merck Co., Darmstadt, Germany) supplemented with 5% Egg Yolk Tellurite (Merck Co., Darmstadt, Germany) and the MacConkey as well as EMB agar (Merck Co., Darmstadt, Germany), respectively, in duplicate following incubation for 24-48 hr at 37 °C. Following incubation, *S. aureus* and *E. coli* suspected colonies (up to five colonies) were selected and confirmed using biochemical

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tests such as Gram stain, catalase, coagulase, DNase, oxidase, triple sugar iron agar, and IMViC tests (Chaleshtori *et al.*, 2017, Rahimi, 2013).

After isolation of strains, antibiotic susceptibility testing was carried out according to the Clinical and Laboratory Standards Institute (CLSI) by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (MHA) (Merck Co., Darmstadt, Germany) (Clinical and laboratory standards institiute, 2014). A bacterial suspension with turbidity equivalent to 0.5 (McFarland standard) (1.5×10^8 CFU/mL) was prepared in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The bacterial suspension was uniformly inoculated with a sterile swab stick on the surface of MHA, and then five antibiotic disks were placed for each plate and incubated at 35 °C for 24 hr. Inhibition zones on agar plates were measured after 24 hr, and the results were recorded according to interpretive criteria provided by CLSI. The isolates of bacteria were tested against a panel of seventeen antibiotic discs (HiMedia Laboratories Pvt. Ltd, Mumbai, India): ampicillin (10 µg), amoxicillin (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), oxacillin (1 µg), penicillin (10 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (10 µg), vancomycin (30 µg), nitrofurantoin (300 µg), methicillin (5 µg), norfloxacin (30 µg), kanamycin (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), Ceftazidime (30 µg), (10 µg) and cefoxitin (30 µg).

Preparation of ozonized water: In the present study, the ozone generator PLASOZI made by Plasma Fanavaran Kavir Company (Kashan, Iran) was used to produce ozonized water. This system uses cold plasma technology to produce ozone and other oxidants active species from the ambient air as feed gas by a dielectric barrier discharge (DBD) structure. DBD reactors operate at atmospheric pressure and are made of two electrodes, at least one coated with a dielectric. The reactor configuration can be flat or cylindrical depending on its application. A high voltage AC or pulse is applied to the electrodes, which results from instantaneous electron emission from the cathode. These electrons pass through two electrodes as a filament. When this group of electrons reaches the dielectric coating of the opposite electrode, it spreads on its surface and reduces the local electric field, thereby limiting electric current, and preventing uncontrolled discharge progression and arc generation. The electrons reach very high energies by accelerating in the strong electric field while passing through. By colliding with oxygen molecules, these energetic electrons can break down molecular bonds and turn them into oxygen atoms. The combination of atomic oxygen with oxygen molecules leads to the formation of ozone. In the air-cooled ozone generator PLASOZI, ambient air was drawn into the device by a pump. By applying pulse high voltage (15 kV and 20 kHz), an electrical discharge was performed, the air was ionized and passed from the gas phase to the plasma phase. The created plasma contained ozone as the most efficient active species. Ozone is only partially soluble in water. In order to efficiently transfer ozone to the solution, it was necessary to inject ozone into the water in the form of fine bubbles. A bubble diffuser was used to transfer ozone to water and create 4 mg/L ozonized water. The half-life of ozone in water is much shorter than in air. The solubility of ozone and its half-life in water is inversely proportional to water temperature. On the other hand, to apply the experiment results in the food industry and surface disinfection, it was decided to ozonize the treated municipal water at room temperature, and the resulting solution was tested as a bacterial substance. In the present study, the water temperature was 27 °C, and its pH was 3.56.

Contamination and sanitization of FCS with ozonized water: This study evaluated the effectiveness of spraying ozonized water (4 mg/l) against *S. aureus* and *E. coli* in three exposure times (2, 6, 10 minutes, and after drying). The positive control group was a spray of normal saline solution on FCS impregnated with *S. aureus* and *E.*

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coli. For contamination of FCS, 1 mL of each bacterial suspension with turbidity equivalent to 1.5×10^5 CFU/ml in sterile normal saline (0.9%) was spread on the FCS with a sterile swab at room temperature for 15 min. Then, each FCS sample was sprayed with 2 ml of the ozonized distilled water at an initial concentration of 4 mg/l. After 0, 2, 6, and 10 minutes, it was dried with a sterile swab thoroughly and then cultured on Baird-Parker and MacConkey agar. The plates were incubated at 37 °C for 48 h. All the results were expressed as the number of CFU/ m^2 (César *et al.*, 2012).

Data analysis: The obtained results based on CFU/m^2 were converted into a $\log_{10}$ form and subjected to an analysis of variance (ANOVA) followed by the Tukey test to compare multiple means. P-value < 0.05 was considered to indicate a statistically significant difference. The data were analyzed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) and Excel software version 2013 (Microsoft, Redmond, WA, USA). Log_{10} reduction factor (RF) was also calculated using Equation 1 (Megahed *et al.*, 2018):

$$\log_{10} RF = \log_{10} Control - \log_{10} Treated$$

Results

The resistance pattern of *S. aureus* and *E. coli* isolates to antibiotics tested in this study is shown in **Table 1**.

The spray of aqueous O3 at a concentration of 4 ppm reduced *S. aureus* and *E. coli* within 2 minutes of exposure in plastic, glass, steel, and ceramic surfaces. The results showed that the exposure time of aqueous O3 had a significant effect on *S. aureus* and *E. coli* on all surfaces (P<0.05).

In plastic, glass, and ceramic surfaces, the concentration of aqueous O3 demonstrated a higher antibacterial activity against *E. coli* compared to *S. aureus*, while *S. aureus* was more susceptible on steel surface (**Figure 1**). In plastic surface, the mean $\log_{10}$ reduction in *S. aureus* counts and killing percentage increased after drying aqueous O3 (3.42 log CFU/m^2 and 59.8%, respectively), compared to 2 minutes of exposure (0.95 logs CFU/m^2 and 16.75%, respectively). Also, the mean $\log_{10}$ reduction in *E. coli* counts and killing percentage increased after drying aqueous O3 (5.34 log CFU/m^2 and 100%, respectively), compared to 2 minutes exposure (1.35 log CFU/m^2 and 25.29%, respectively) (**Figure 2**).

In steel surface, the mean $\log_{10}$ RF increased numerically at 2, 6, and 10 minutes for *S. aureus* (2.44, 2.84, and 5.62 log CFU/m^2, respectively), compared to *E. coli* (1.32, 1.43, and 1.98 log CFU/m^2, respectively). Aqueous O3 at concentration 4 ppm after 2 minutes exposure killed 100% *E. coli* on the glass surface, while *S. aureus* at 10 minutes exposure only killed 54.19% (**Figure 2**).

According to the results, the concentration of aqueous O3 is considered the most powerful antibacterial against *S. aureus* and *E. coli* on the ceramic surface. After 2 minutes of exposure, approximately 50% of the reduction in *S. aureus* and *E. coli* counts was observed, while killing percentage at 6 and 10 minutes for *S. aureus* and *E. coli* was 100%, respectively (**Figure 2**).

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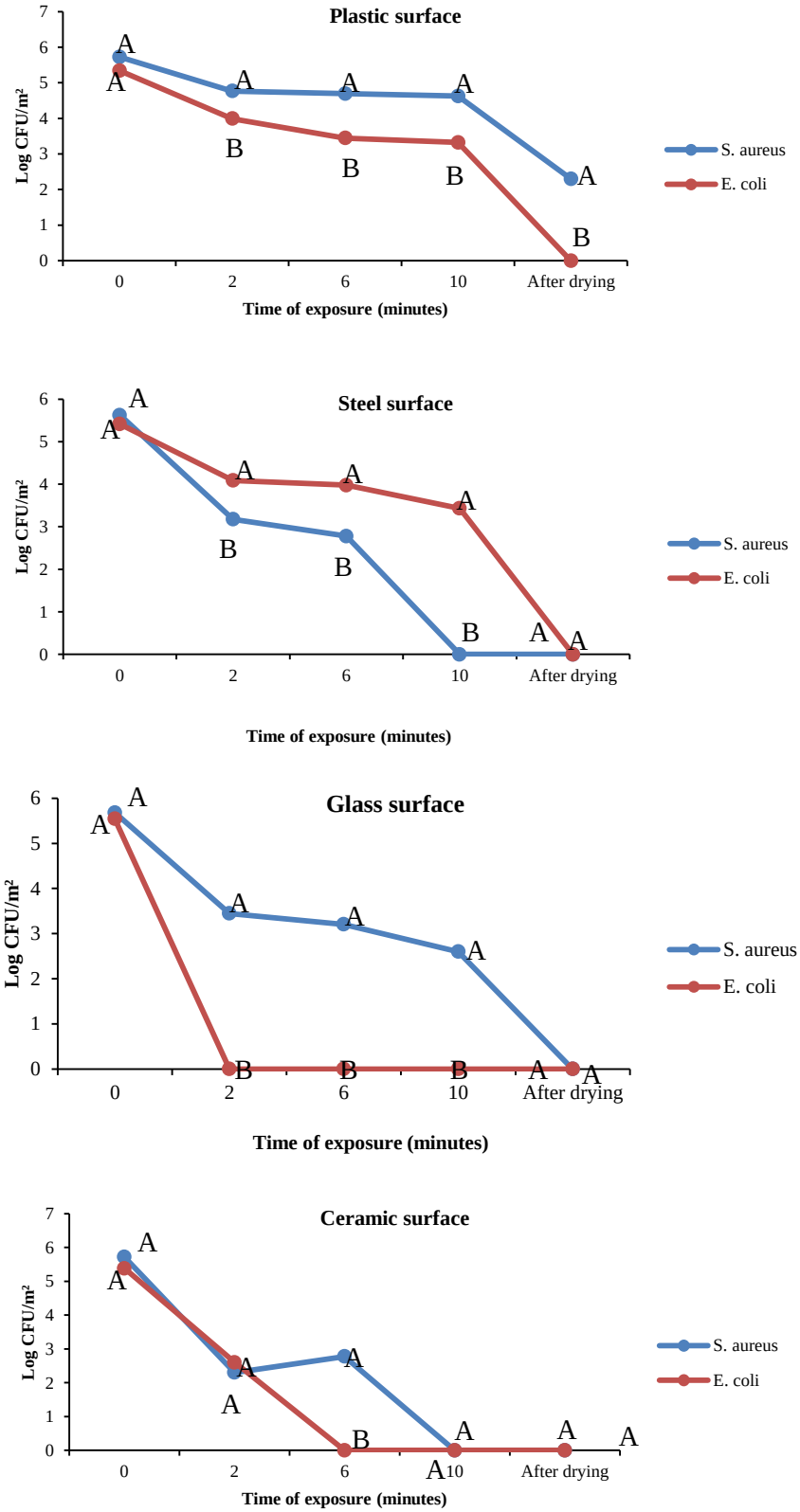

Figure 1. Bacterial counts (Log CFU/m²) of *S. aureus* and *E. coli* after spray of aqueous O3 (4 ppm) on plastic, glass, steel, and ceramic surfaces for 2, 4, 10 minutes and after drying. Different capital letters within a time point differ significantly ($P < 0.05$)

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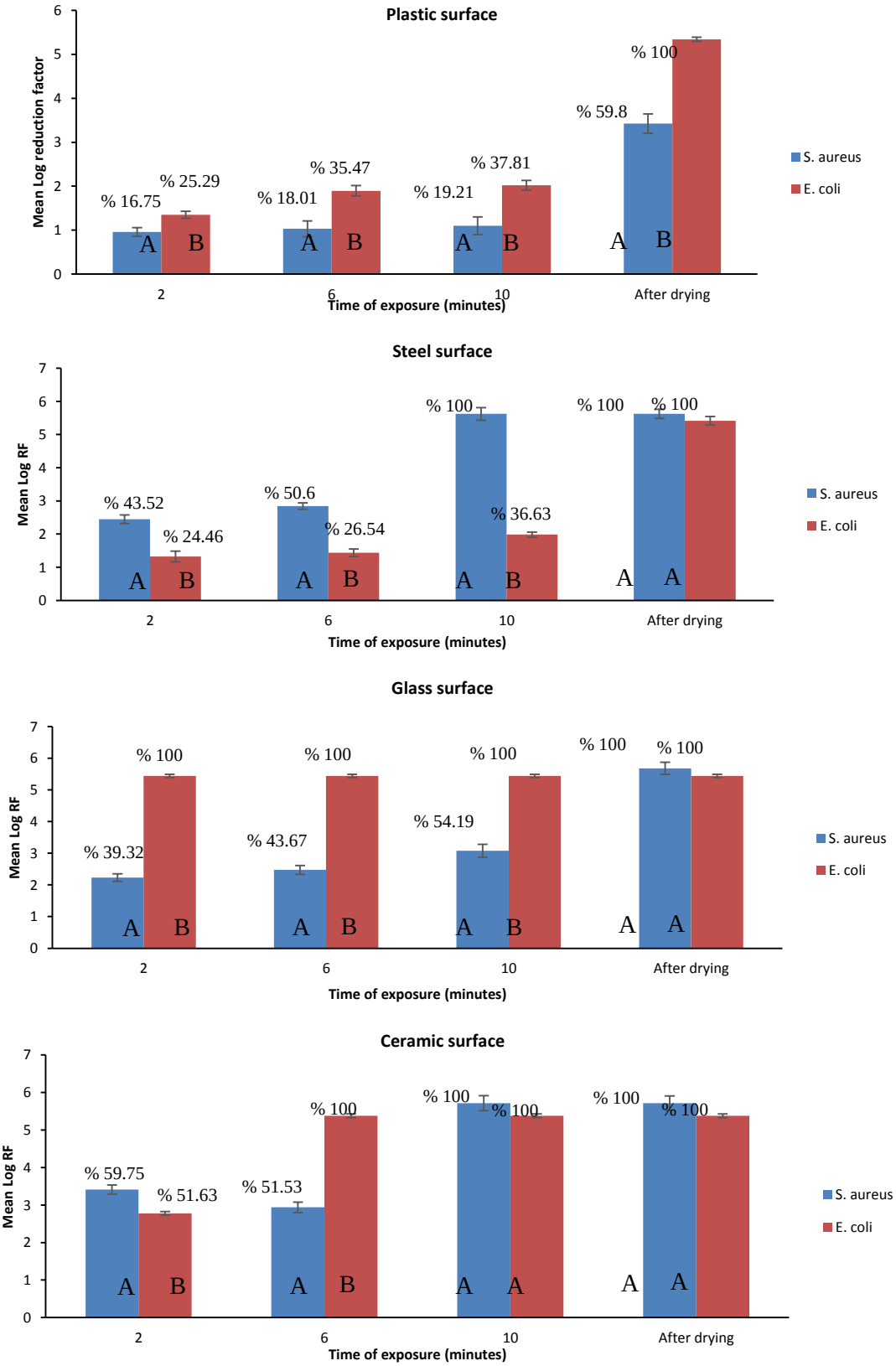

Figure 2. Mean ± SD Log_{10} reduction in bacterial counts of *S. aureus* and *E. coli* after spray of aqueous O3 (4 ppm) on plastic, glass, steel and ceramic surfaces for 2, 4, 10 minutes and after drying. The killing percentage is demonstrated above on columns. Different capital letters within a time point differ significantly ($P < 0.05$).

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Table 1. Antimicrobial resistance of *S. aureus* and *E. coli* isolated from traditional yogurt in Kashan, Iran.

Antimicrobials	Isolates	
	S. aureus	*E.coli*
Ampicillin (10 µg)	Resistant	Resistant
Amoxicillin (30 µg)	Resistant	Resistant
Clindamycin (2 µg)	Susceptible	-
Erythromycin (15 µg)	Susceptible	-
Methicillin (5 µg)	Resistant	-
Gentamycin (10 µg)	Resistant	Resistant
Oxacillin (1 µg)	Resistant	-
Penicillin (10 µg)	Resistant	-
Tetracycline (30 µg)	Resistant	Resistant
Trimethoprim/Sulfamethoxazole (10 µg)	Susceptible	Resistant
Vancomycin (30 µg)	Resistant	-
Nitrofurantoin (300 µg)	Resistant	Resistant
Norfloxacin (30 µg)	Resistant	Resistant
Kanamycin(30 µg)	Resistant	Resistant
Ceftriaxone (30 µg)	Resistant	Resistant
Cefoxitin (30 µg)	Resistant	-
Chloramphenicol (30 µg)	Susceptible	Susceptible
Ceftazidime (30 µg)	-	Resistant
Ciprofloxacin (10 µg)	Susceptible	Susceptible

Discussion

The study results indicated that spray of aqueous O3 at a concentration of 4 mg/l is sufficient to reduce 60 to 100% of multidrug resistance food-borne pathogens (MRFBP) to a safe level on FCS after drying. Also, the finding showed that 2 and 6 minutes exposure of aqueous O3 is not an adequate means of controlling MRFBP under heavy load on different surfaces. Moreover, on smooth surfaces, an aqueous O3 had a significant impact on RF. In line with the present study, Megahed *et al*. reported that a 2 min exposure to aqueous ozone at concentrations of 4 and 9 mg/l reduces manure-based pathogens to a safe level on smooth and moderately rough surfaces, respectively (Megahed *et al.*, 2018).

High oxidative capacity of ozonized water in higher concentrations increases the redox potential in bacteria and decreases the bacterial load on surfaces (de Paula Nascente *et al.*, 2021). Initially, the ozone involves considerable changes in fluidity and permeability of the bacterial membrane. As a result, cell death occurs due to leaching of ions and intracellular molecules out of bacteria (Kahrilas *et al.*, 2015). A study showed that DNA damage of bacteria by oxidative stress increased by increasing exposure time, concentration, and continuous exposure to O3 (Devatkal *et al.*, 2016).

Pomposiello and Demple, demonstrated that *E. coli* via protective mechanisms including SoxR and OxyR causes activation of different genes such as soxS and sod in *E. coli* cells and reduces the oxidative stress of O3 through DNA repair or removal of the radicals (Pomposiello and Demple, 2001). However, these protective mechanisms cannot hold up for long at doses of 4 mg/l or greater of O3 (Aydogan and Gurol, 2006, Megahed *et al.*, 2018).

Marino *et al*. reported that the antibacterial activities of O3 against Gram-positive or Gram-negative group of bacteria had no significant correlation. They showed that *S. aureus* and *L. monocytogenes* were more resistant than *P. fluorescens* (Marino *et al.*, 2018), which might be due to cellular defense systems against oxidative stress. At the same time, *P. fluorescens* with an external membrane (as a cellular cover against other antibacterial agents) has not shown a defense (Clauditz *et al.*, 2006, Marino *et al.*, 2018).

A previous study demonstrated that growth inhibition of multidrug-resistant *Salmonella* Heidelberg strains occurred in the presence of

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aqueous O3 and the lack of organic material after 1 min, with changes in metabolic profile and bacterial DNA concentration (de Paula Nascente *et al.*, 2021, Kumar *et al.*, 2016). Studies have shown antiviral activities (coronaviruses such as SARS-CoV-1 and 2) of O3 with interaction with the envelopes of the lipid layer (Kampf *et al.*, 2020, Kumar *et al.*, 2016, Tizaoui, 2020).

One important factor related to the increase of sensitivity of bacterial biofilm to aqueous O3 is the dynamic condition compared to static conditions at the time of its use (Sarron *et al.*, 2021). It is known that almost under dynamic conditions the amount of ozone in water remains constant; therefore, in a study, the biofilm of *S. aureus* was more sensitive than attached cells (bacterial reduction after 2 min exposure at least 3 Log of biofilm viability) (Marino *et al.*, 2018).

Smooth surfaces such as glass, ceramic, and steel enhance the mass transfer of O3, and consequently, an efficient diffusion and penetration of ozone among MRFBP cause increased damage to bacteria and cell death. In addition, the physical removal of ozone on smooth surfaces is lower than rough surfaces due to a decrease of surface reactivity and irreversible consumption of ozone on smooth surfaces when O3 strikes the surface (Nicholas *et al.*, 2013, Wang and Morrison, 2010).

In contrast with a previous study (Megahed *et al.*, 2018), a considerable impact of exposure time on RF was identified, especially on the glass, steel, and ceramic, except for plastic. Another study showed that O3 could completely kill *klebsiella michiganensis* and *p. aeruginosa* during the first 4 minutes of exposure (Saha *et al.*, 2014). The present study findings can be hopeful and provide a practical method to use ozonized water as a complementary way in the disinfection of the contact surfaces in the food industry to reduce *E. coli* and *S. aureus* environmental contamination. Performing tests in a controlled environment was the main limitation of this study. Therefore, future studies are recommended in the various food industries.

Conclusion

E. coli and *S. aureus* were defined as multidrug-resistant. Ozonized water inhibited two bacteria growth on the FCS after two minutes. On plastic, glass, and ceramic surfaces, *E. coli* was more sensitive than *S. aureus*, while *S. aureus* on steel surface was more sensitive than *E. coli*. The results demonstrated that spray with a concentration of 4 mg/L of aqueous O3 had the most antibacterial power against *S. aureus* and *E. coli* on the ceramic surface. After 6 and 10 minutes of exposure, the killing percentage for *S. aureus* and *E. coli* was 100%, respectively.

Acknowledgments

The authors are grateful to the Research Deputy and the personnel of the Research Center for Biochemistry and Nutrition in Metabolic Diseases, Kashan University of Medical Sciences, Kashan, Iran (grant number 98000780).

Authors' contributions

Rahmani Z and Sharafati Chaleshtori R designed the research; Rahmani Z, Sharafati Chaleshtori R, Abdoli Arani A, and Taghizadeh M managed the whole project and contributed in all steps; Rahmani Z, Sharafati Chaleshtori R, and Abdoli Arani A wrote the draft of the manuscript and its finalization. All the authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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