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Research article

Comparative analysis of direct and indirect non-thermal argon plasma treatment against multidrug-resistant hospital-acquired pathogens: an in vitro investigation

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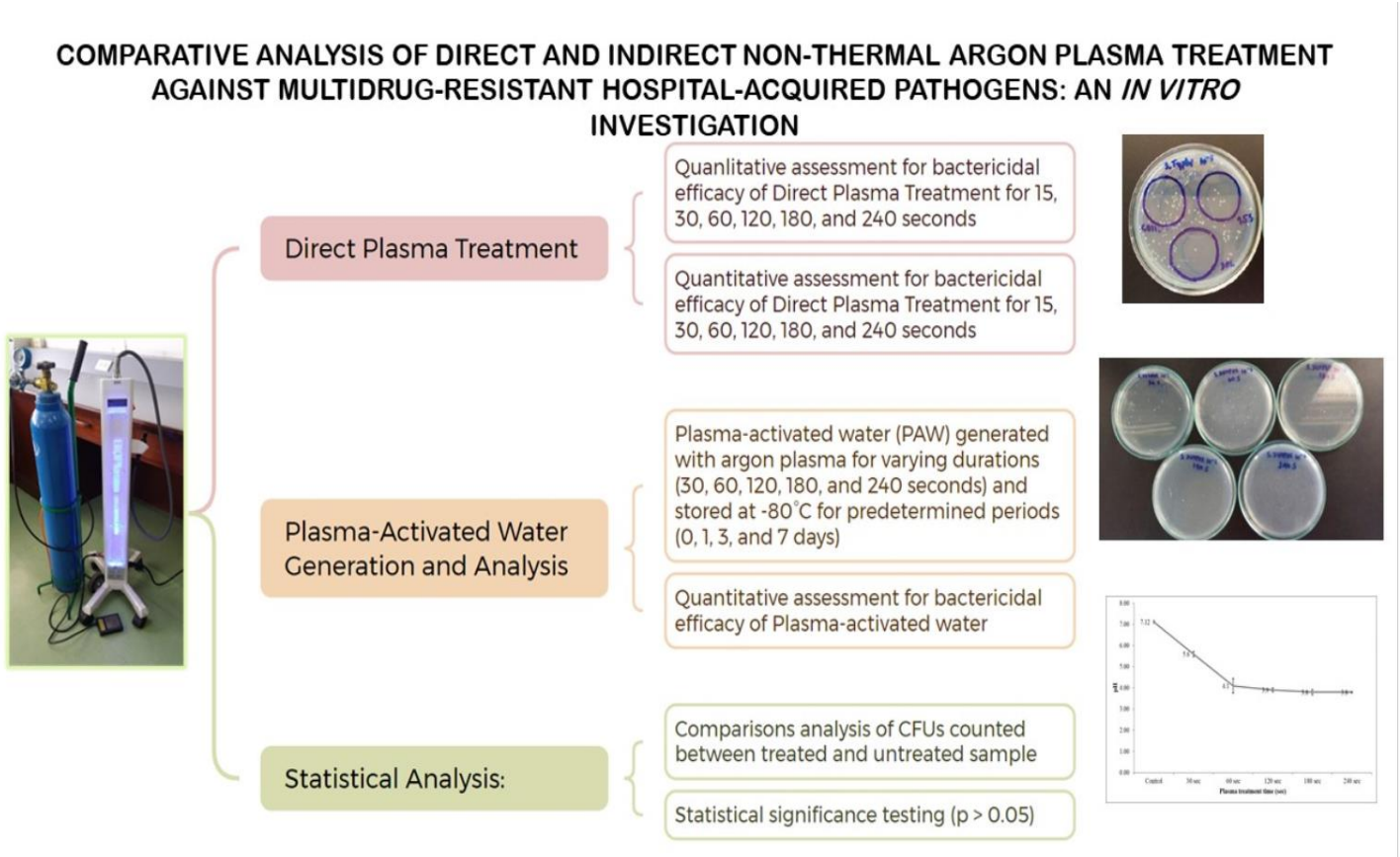
ABSTRACT

Non-thermal plasma (NTP) represents an innovative approach in combating antimicrobial resistance, particularly when conventional chemical agents show limited efficacy. This study evaluated the susceptibility of clinically significant pathogens to NTP treatment, examining both direct plasma exposure and plasma-activated water (PAW) applications. The investigation encompassed 15 bacterial strains, including 10 drug-resistant clinical isolates and 5 standard clinical isolates, with a focus on determining optimal treatment parameters and antimicrobial efficacy.



Systematic evaluation of exposure parameters included direct NTP application and PAW treatment across various time intervals (15-240 seconds). Complete bacterial inactivation was achieved within 15-30 seconds of direct NTP exposure for all tested species (initial concentration 10^9 CFU/mL). Notably, methicillin-resistant Staphylococcus aureus (MRSA) exhibited particular sensitivity to NTP treatment, demonstrating a statistically significant 9-log reduction in viable cells ($p < 0.05$). PAW treatment showed comparable efficacy, achieving significant bacterial reduction after 180-240 seconds of exposure, with optimal antimicrobial activity observed in freshly prepared PAW (Day 0). While antimicrobial efficacy gradually decreased during storage, significant activity was maintained through Day 7. Time-kill studies confirmed complete bacterial eradication following direct NTP exposure, with no evidence of bacterial regrowth during extended incubation periods. These findings demonstrate NTP's potential as an effective alternative for microbial decontamination in healthcare settings, particularly for surface sterilization applications. The study provides compelling evidence for NTP's role in addressing both antibiotic-resistant and susceptible pathogens, offering a promising tool for infection control strategies.

Keywords: Non-thermal plasma, Microbial control, Plasma-activated water (PAW).

INTRODUCTION

Healthcare-associated infections (HAIs) represent a critical global health challenge, contributing significantly to patient morbidity, mortality, and healthcare costs [1]. Recent epidemiological studies indicate that HAIs affect approximately 7% of patients in developed countries and up to 15% in developing regions, with annual costs exceeding $20 billion globally [2, 3]. The emergence and spread of antimicrobial-resistant organisms, particularly in healthcare settings, have further complicated infection control efforts, necessitating novel approaches to microbial decontamination [4, 5].

Traditional chemical disinfectants, while effective, face increasing challenges including bacterial resistance development, material compatibility issues, and potential environmental impacts [6, 7]. Studies have shown that bacterial biofilms on medical devices and hospital surfaces present particular challenges, as these structures can resist conventional antimicrobial treatments by up to 1000-fold compared to their planktonic counterparts [8, 9]. These limitations have driven the search for alternative decontamination technologies that offer both efficacy and safety.

Non-thermal plasma (NTP) technology has emerged as a promising solution in infection control, offering distinct advantages over conventional methods [3]. Operating at atmospheric pressure and ambient temperature, NTP generates a complex mixture of reactive species, including reactive oxygen species (ROS), reactive nitrogen species (RNS), charged particles, and UV radiation, all of which contribute to its antimicrobial efficacy [4, 9]. Recent advances in plasma medicine have demonstrated NTP's capability to inactivate not only planktonic bacteria but also bacterial biofilms, with reported efficacy rates exceeding 99.9% under optimized conditions [6, 10].

The scientific distinction between sterilization and disinfection in NTP applications warrants careful consideration [5]. Sterilization, defined as the complete elimination of all microorganisms including bacterial spores, requires specific plasma parameters and exposure conditions, typically achieving a sterility assurance level (SAL) of 10^6 [2].

In contrast, disinfection involves reducing pathogenic microorganisms to levels considered safe for intended use, with log reductions varying based on the specific application requirements [7, 8].

Recent studies have expanded our understanding of NTP's mechanisms of action against various pathogens [3, 4]. The generation of reactive oxygen species (ROS) such as hydroxyl radicals (•OH), superoxide (O2•-), and hydrogen peroxide (H2O2), along with reactive nitrogen species (RNS) including peroxynitrite (ONOO-) and nitric oxide (NO), creates a multi-target antimicrobial effect [9]. This multi-modal action may explain NTP's effectiveness against antibiotic-resistant organisms and reduce the likelihood of resistance development [6].

The application of plasma-activated water (PAW) represents a significant advancement in NTP technology, offering a method for indirect plasma treatment that maintains antimicrobial efficacy while providing practical advantages for certain applications (Chen *et al*., 2023). Recent research has demonstrated PAW's potential for sustained antimicrobial activity, with studies showing retention of bactericidal properties for up to 7 days under optimal storage conditions [2, 7]. However, the exact mechanisms of PAW's long-term stability and optimization of this approach require further investigation [5, 8].

In this investigation, we systematically evaluated the antimicrobial efficacy of argon-based non-thermal plasma (NTP) against clinically significant bacterial pathogens, encompassing both multidrug-resistant isolates and standard clinical strains. The study employed two distinct treatment modalities: (1) direct plasma exposure, where the generated plasma species directly interface with the target microorganisms, and (2) indirect application through plasma-activated water (PAW), which serves as a mediator for plasma-generated reactive species. This dual-approach methodology enables comprehensive assessment of NTP's bactericidal capabilities while addressing practical considerations for clinical implementation.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

Clinical isolates were obtained from the Faculty of Medical Technology, Rangsit University. The study included 15 bacterial strains: ten multidrug-resistant (MDR) clinical isolates and five standard clinical isolates. The MDR isolates comprised: *Escherichia coli* (n=2, extended-spectrum β-lactamase [ESBL]-producing), *Acinetobacter baumannii* (n=2, MDR), *Pseudomonas aeruginosa* (n=2, MDR), *Staphylococcus aureus* (n=2, methicillin-resistant [MRSA]), *Enterococcus faecalis* (n=1, vancomycin-resistant [VRE]), and *Klebsiella pneumoniae* (n=1, ESBL-producing). Standard clinical isolates included *Aeromonas hydrophila*, *Salmonella typhi*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli*. All strains were characterized using standard microbiological methods and antimicrobial susceptibility testing according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Bacterial cultures were maintained on nutrient agar (NA) plates at 37°C. For experimental procedures, overnight cultures were adjusted to 1×10^8 CFU/mL using spectrophotometric measurement at 625 nm, corresponding to 0.5 McFarland standard. Serial dilutions were performed in sterile 0.85% NaCl solution to achieve the required experimental concentrations.

Non-thermal Plasma Device Configuration

The experimental setup employed a non-atmospheric-pressure plasma jet system (Bio plasma jet, Photo Bio Care Co., Ltd., Thailand) [11] consisting of three main components: a handheld plasma generation unit, a direct current power supply, and a gas delivery system. The power generator produced high-frequency sinusoidal waves (15-20 KHz alternating current) with a peak-to-peak voltage of 6-7 kV, delivered in adjustable pulses (10-110 Hz). The system operated at a maximum input power of 50W from a standard power source, utilizing high-purity argon (99.999%) as the carrier gas. The plasma-generating electrode featured a 4-cm diameter tip, with the working distance between the electrode tip and treatment surface maintained at 1-3 mm using a precision-controlled positioning system.

Operating parameters were standardized across all experiments: frequency at 100 Hz, intensity level at 10 (scale 1-10), and output power density measured at 0.682 W/cm^2. Gas flow rates were monitored and maintained at a constant rate throughout all experiments. The stability and uniformity of the plasma discharge were verified through electrical and optical characterization before each experimental session.

Direct Plasma Treatment Protocol

The antimicrobial efficacy of direct plasma exposure was evaluated using a modified surface decontamination protocol). Bacterial suspensions (100 µL, 10^8 CFU/mL) were uniformly spread on NA plates using sterile cotton swabs. Plasma treatment was conducted at predetermined time intervals (15, 30, 60, 120, 180, and 240 seconds), with the plasma jet positioned perpendicular to the agar surface to ensure consistent exposure. The diameter of the inhibition zone was measured following 24-hour incubation at 37°C and categorized as follows: No inhibition; negative (full bacterial growth), Partial inhibition; ++ (1-2 cm clear zone from plasma contact edge), and Complete inhibition; +++ (>2 cm clear zone from plasma contact edge). The experiment was carried out in triplicate.

For quantitative assessment was modified from previous study [12], A suspension of tested pathogenic bacteria (concentration of 10^8 CFUs/mL in sterile 0.85% NaCl) was prepared by serial dilution (1:100; corresponding to $\sim1\times10^7 - 1\times10^8$ CFUs/mL during the colony count assay). Then 100 µl of bacterial suspension was pipetted and spread on NA for exposure to argon NTP for 15, 30, 60, 120, 180, and 240 seconds. Then the plate was incubated at 37°C for 24 hours. Following incubation of the plates, CFUs were counted to determine the bactericidal efficacy of direct exposure to NTP. Extended incubation (72 hours) was performed to evaluate potential bacterial regrowth or delayed colony formation. Control plates received no plasma treatment but were otherwise handled identically. Each dataset represents the mean value plus standard deviation of at least three exposure experiments.

Plasma-Activated Water Generation and Analysis

Plasma-activated water (PAW) was generated by treating 1 mL aliquots of deionized water (18.2 MΩ· cm, pH 7.0) with argon plasma for varying durations (30, 60, 120, 180, and 240 seconds) as described by Kojtari *et al*. [13]. Treated samples were immediately stored at -80°C for predetermined periods (0, 1, 3, and 7 days). The physicochemical properties of PAW were characterized using a calibrated pH meter (Thermo Orion Research Digital, Thermo Fisher Scientific) equipped with an ultrasensitive probe.

For antimicrobial efficacy testing, bacterial suspensions (100 µL, 10^6 CFU/mL final concentration) were mixed with equal volumes of PAW and incubated at room temperature (23±2°C) for 15 minutes. Following exposure, 100 µL of diluted suspension were spread on NA plates for incubation at 37°C for 24 hours. After incubation of the plates, CFUs were counted to correlate with the bactericidal efficacy of plasma-treated water. An untreated sample was used as a growth control in parallel experiments. The experiment was carried out in triplicate.

Statistical Analysis

All experiments were performed in triplicate, with results expressed as mean ± standard deviation. Statistical significance was determined using Student's t-test with Bonferroni correction for pairwise comparisons and one-way ANOVA for multiple comparisons. Post-hoc analyses were performed using Tukey's HSD test. Statistical significance was set at $p\leq0.05$, with analyses conducted using SPSS version 22 (IBM Corp.). Power analysis was performed to

ensure adequate sample size for detecting significant differences between treatment groups.

RESULTS

Bactericidal Effect of Argon NTP Exposure *in Vitro*

Our study aimed to evaluate the susceptibility of various bacterial pathogens, including both species-specific and strain-specific variations, to argon non-thermal plasma (NTP) exposure. The susceptibility of these pathogens was measured by the presence and size of the inhibition zone following NTP treatment compared with the untreated control. The findings indicated that the bactericidal effect was species- and strain-dependent. After 15 seconds of exposure, an inhibition zone ("+") began to emerge, increasing in size until complete bacterial elimination at 240 seconds. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) displayed the highest susceptibility, while *Enterococcus faecalis* vancomycin-resistant enterococci (VRE) were the least sensitive. Colony-forming units (CFUs) analysis further validated these findings, showing a significant reduction ($p < 0.05$) in CFUs across all strains after NTP exposure. MRSA and normal clinical isolates exhibited higher sensitivity than multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL) producers. These results are summarized in Table 1, which outlines the inhibition zones observed under varying exposure durations.

Figure 1 (A-F) illustrates the quantitative reduction in CFUs, showing that significant bactericidal effects began after 15 seconds of exposure for MRSA and clinical isolates, while MDR, ESBL, and VRE required at least 30 seconds for notable reductions. Notably, no survivors were detected after 240 seconds of treatment.

Table 1: Preliminary determination of bactericidal activity after argon NTP direct treatment.

Isolate	Exposed time (sec)				
	15	30	60	120	180
ESBL					
Klebsiella pneumonia	0	0	0	+	++
Escherichia coli	0	0	0	+	++
Escherichia coli P174	0	0	0	++	+++
MRSA					
Staphylococcus aureus (1)	+	+	+	+	+++
Staphylococcus aureus (2)	+	++	+++	+++	+++
MDR					
Acinetobacter baumannii (1)	++	++	++	+++	+++
Acinetobacter baumannii (2)	+	+	+	++	+++
Pseudomonas aeruginosa (1)	0	0	0	0	++
Pseudomonas aeruginosa (2)	0	0	0	0	++
VRE					
Enterococcus faecalis	0	0	0	0	++
Clinical isolates					
- Gram-positive					
Staphylococcus aureus	0	0	++	++	++
Listeria monocytogenes	+	+	+	+	+++
- Gram-negative					
Escherichia coli	0	0	0	+++	+++
Salmonella typhi	0	++	+++	+++	+++
Aeromonas hydrophila	+	++	+++	+++	+++
Total inhibition	6	8	8	13	15

The present results were correlated with several studies which demonstrated similar results under varying plasma types and exposure times. Schnabel *et al.* [14] reported that direct exposure of a plasma jet (kINPen®) to *Escherichia coli* K-12 and enterohemorrhagic *E. coli* (EHEC) led to a 3- to 5-log reduction within 10 minutes. Dielectric barrier discharge (DBD) plasma showed a similar efficacy profile when applied to MRSA and MDR pathogens [15]. Such findings underscore the robustness of NTP technology in reducing bacterial viability, particularly in antibiotic-resistant strains.

The literature also highlights the role of plasma-generated ultraviolet light and charged particles in enhancing bactericidal effects, emphasizing the importance of optimizing exposure parameters for specific bacterial strains [16].

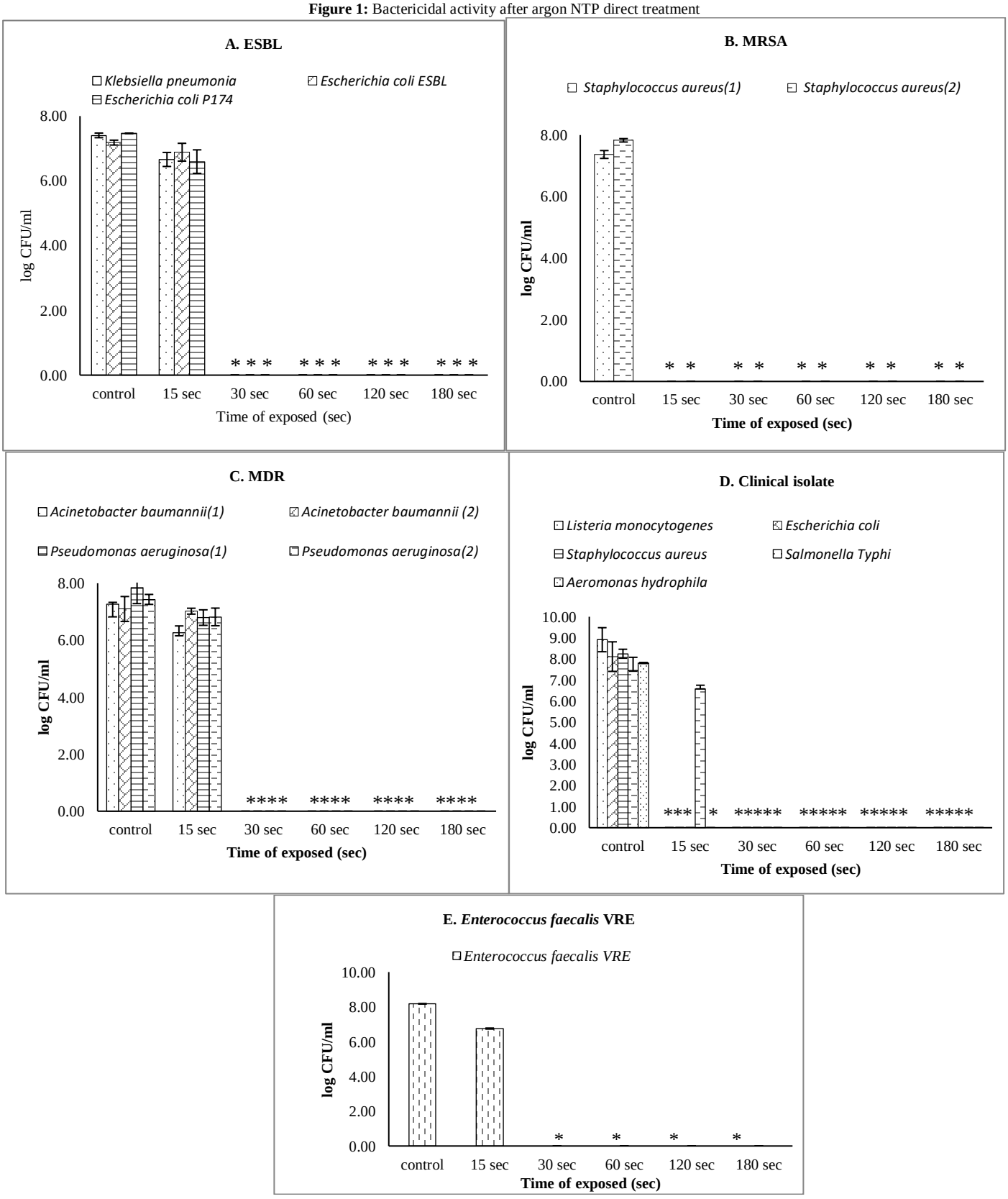
The rapid bacterial inactivation observed aligns with recent studies on NTP's potential for surface decontamination [17]. The mechanism underlying these results is hypothesized to involve the generation of reactive oxygen and nitrogen species (RONS) by plasma, which damage bacterial membranes and intracellular components. Variations in bacterial susceptibility are likely influenced by structural differences in the cell wall and the oxidative stress response of individual strains [18].

Recent mechanistic studies have elucidated that the differential susceptibility patterns correlate with bacterial cell wall architecture and stress response mechanisms. The enhanced susceptibility of gram-positive organisms, particularly MRSA strains, has been attributed to their thick peptidoglycan layer, which paradoxically makes them more vulnerable to plasma-generated reactive oxygen and nitrogen species (RONS) [19]. The rapid inactivation kinetics observed (15-30 seconds for elimination of 10^9 CFU/mL) align with contemporary research demonstrating NTP's

potential for rapid surface decontamination in clinical settings [20].

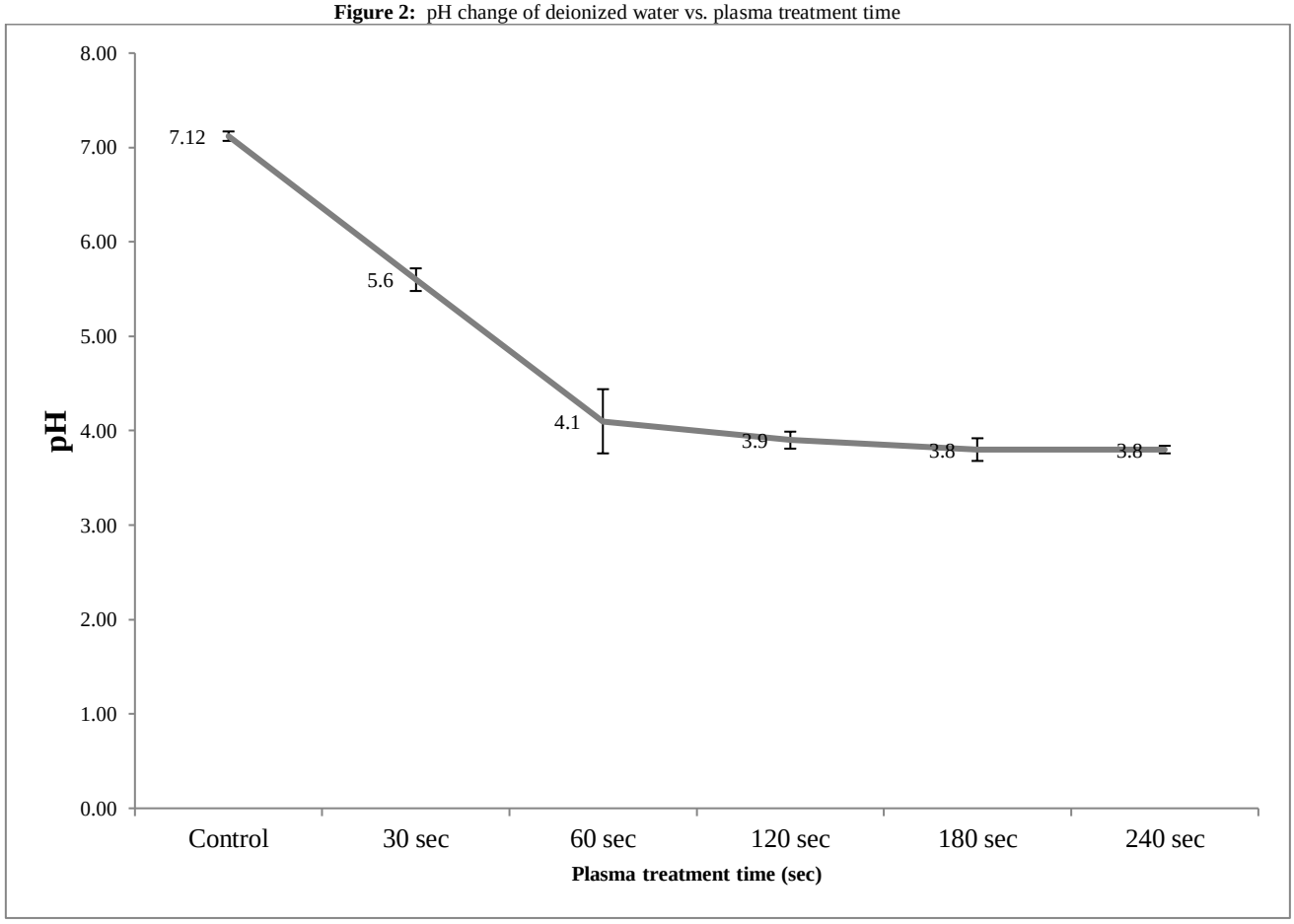
The synergistic action of multiple plasma components, including ionized argon molecules, RONS, and UV radiation, contributes to its bactericidal efficacy. Recent investigations have revealed that plasma-generated hydroxyl radicals (•OH) and peroxynitrite (ONOO-) play pivotal roles in bacterial membrane disruption and protein oxidation [21]. The treatment parameters, including exposure time and bacterial strain characteristics, emerge as critical factors in optimizing NTP applications.

Figure 1: Bactericidal activity after argon NTP direct treatment



(*) Bacterial concentration was statistically decreased (p < 0.05 by ANOVA)

Figure 2: pH change of deionized water vs. plasma treatment time



Bactericidal Activity of Plasma-Activated Water (PAW)

Plasma-activated water (PAW) exhibited potent antimicrobial activity, attributed to its acidic pH and high concentrations of long-lived RONS such as hydrogen peroxide and peroxynitrite (ONOOH), which contribute to its antimicrobial properties [22]. The pH of PAW decreased significantly to 3.9 after 120 seconds of argon NTP treatment (Figure 2) and remained stable for seven days when stored at −80°C.

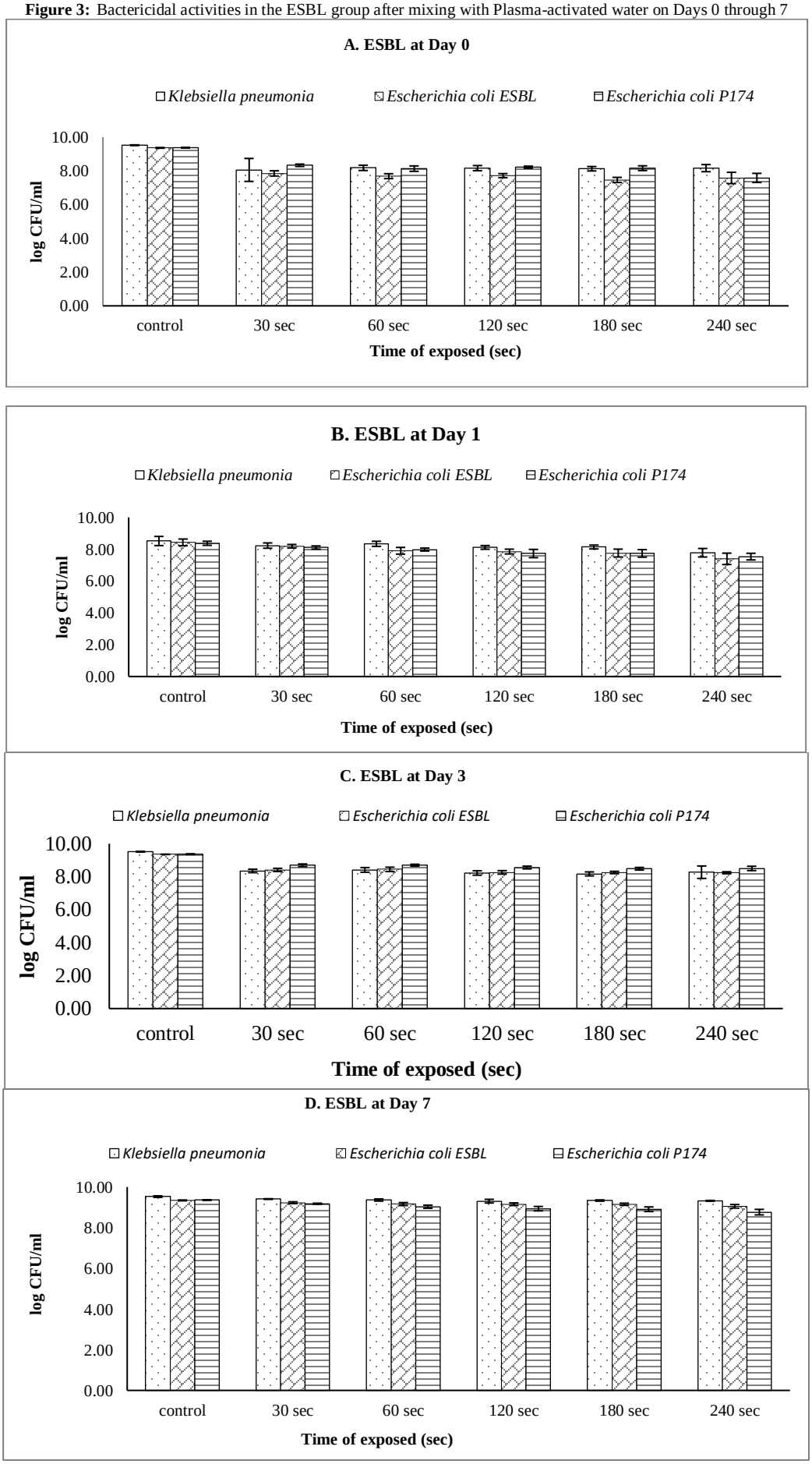
At Day 0, PAW achieved an 8-log reduction in bacterial CFUs within 15 minutes, effectively inactivating *L. monocytogenes* and E. coli. The sustained antimicrobial activity of PAW, termed the "plasma memory effect," persisted through seven days of storage at -80°C, albeit with diminishing efficacy. This prolonged activity has been attributed to the stability of long-lived reactive species, particularly peroxynitrite and hydrogen peroxide, as elucidated by recent spectroscopic analyses [17]. The temporal decline in antimicrobial activity followed the order: Day 0 > Day 1 > Day 3 = Day 7.

The antimicrobial stability of PAW over time aligns with reports by Traylor *et al*. (2020), who noted that E. coli viability decreased by approximately 5 logs within seven days post-treatment. This prolonged activity—termed the "plasma memory effect"—is advantageous for practical applications where immediate use of PAW is unfeasible.

Further, as shown in Figures 4A, 5A, and 6A, PAW achieved significant bactericidal effects against *S. aureus* (clinical isolate), *Pseudomonas aeruginosa* MDR1, *Salmonella* typhi, *S. aureus* MRSA 1, and MRSA 2 after 30 to 180 seconds of treatment. *Enterococcus faecalis* VRE required up to 240 seconds for significant elimination (Figure 7A). ESBL and most MDR isolates exhibited only a 1-log reduction, highlighting variability in PAW's antimicrobial effects based on bacterial type.

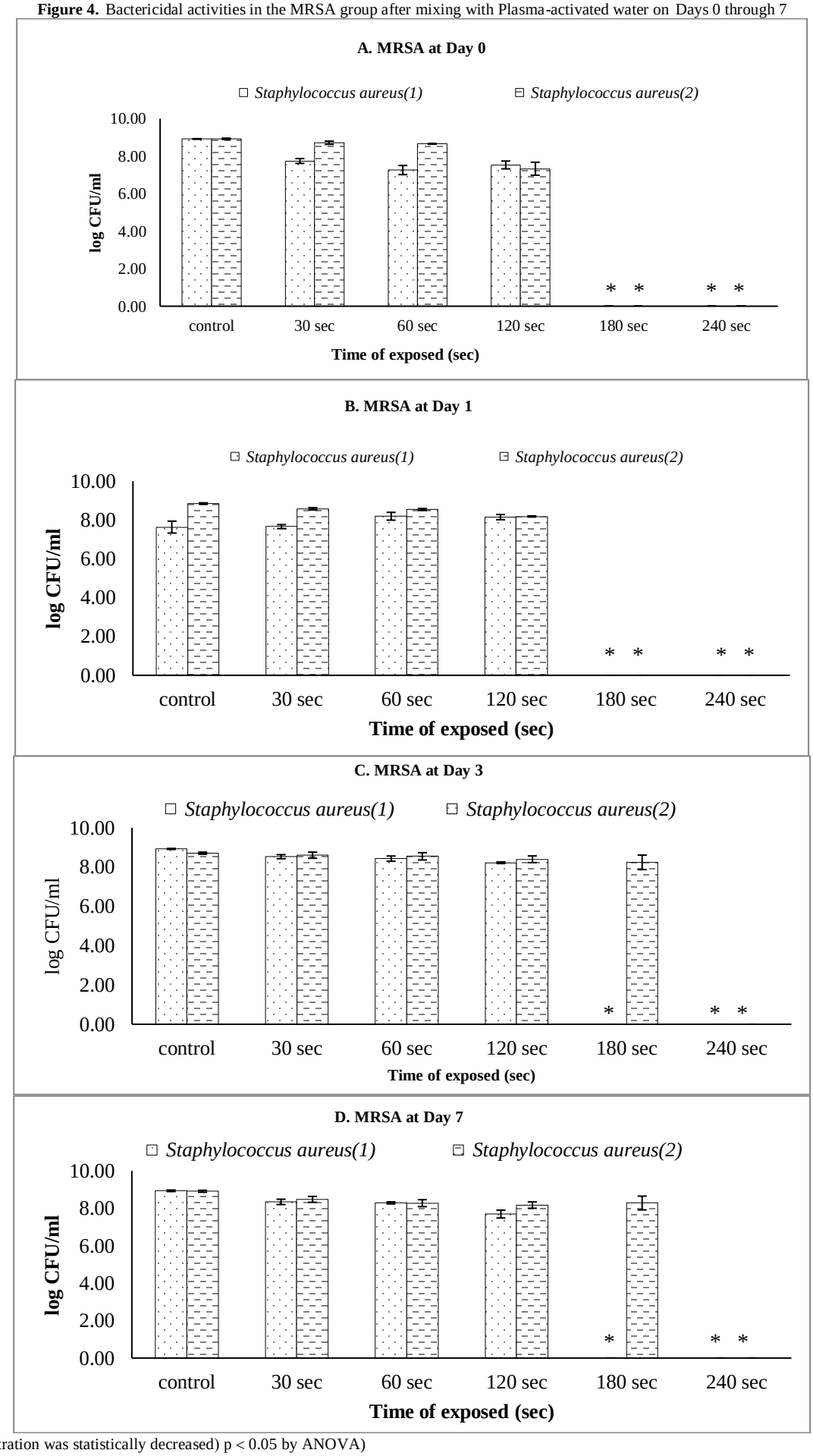
To confirm whether the antimicrobial efficacy of PAW depends on plasma energy, additional experiments assessed its activity after storage (Days 1, 3, and 7). Results showed a decline in efficacy over time but retained significant activity up to seven days of storage. At Day 1, PAW effectively inactivated MRSA isolates and clinical pathogens (Figures 4B, 5B). By Day 7, activity against clinical isolates had decreased, although MRSA efficacy was sustained (Figures 4D, 5D). These findings suggest that RONS concentrations, critical for antimicrobial effects, increase with treatment duration but degrade during storage. Day 0 outcomes showed the highest efficacy, correlating with maximum RONS activity immediately post-treatment.

Figure 3: Bactericidal activities in the ESBL group after mixing with Plasma-activated water on Days 0 through 7



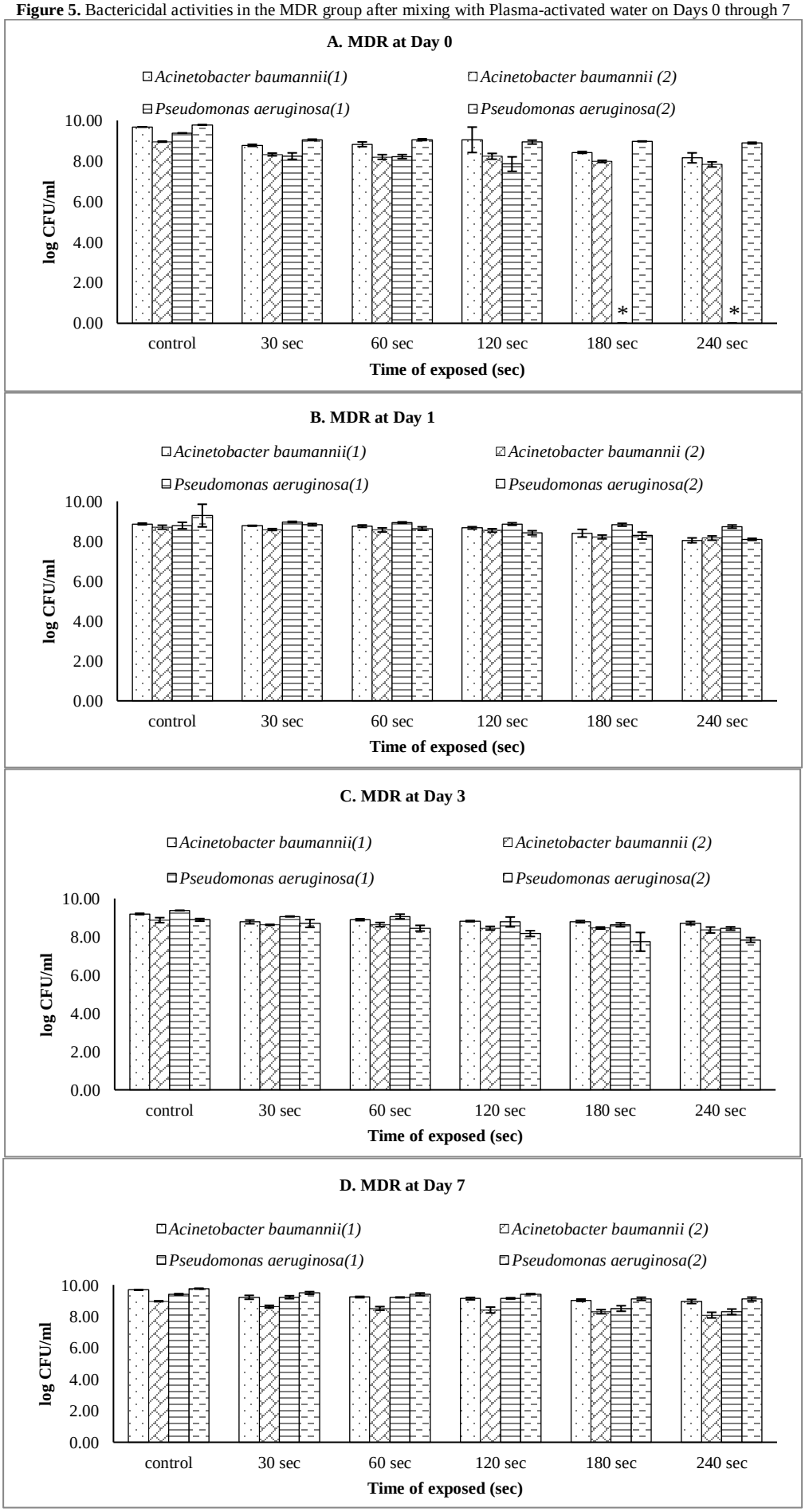
(*) Bacterial concentration was statistically decreased) P<0.05 by ANOVA)

Figure 4. Bactericidal activities in the MRSA group after mixing with Plasma-activated water on Days 0 through 7



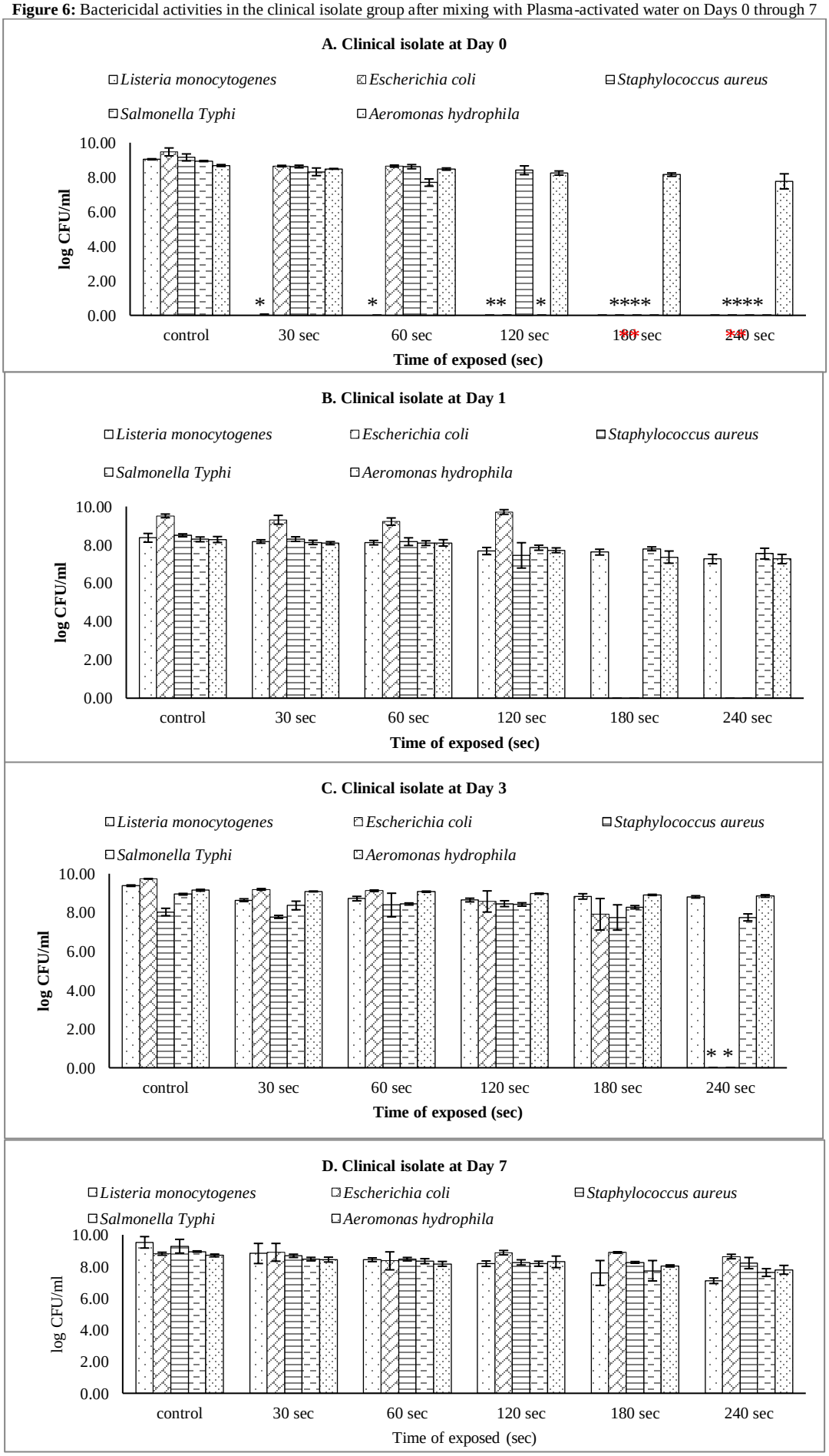
(*) Bacterial concentration was statistically decreased) $p < 0.05$ by ANOVA)

Figure 5. Bactericidal activities in the MDR group after mixing with Plasma-activated water on Days 0 through 7



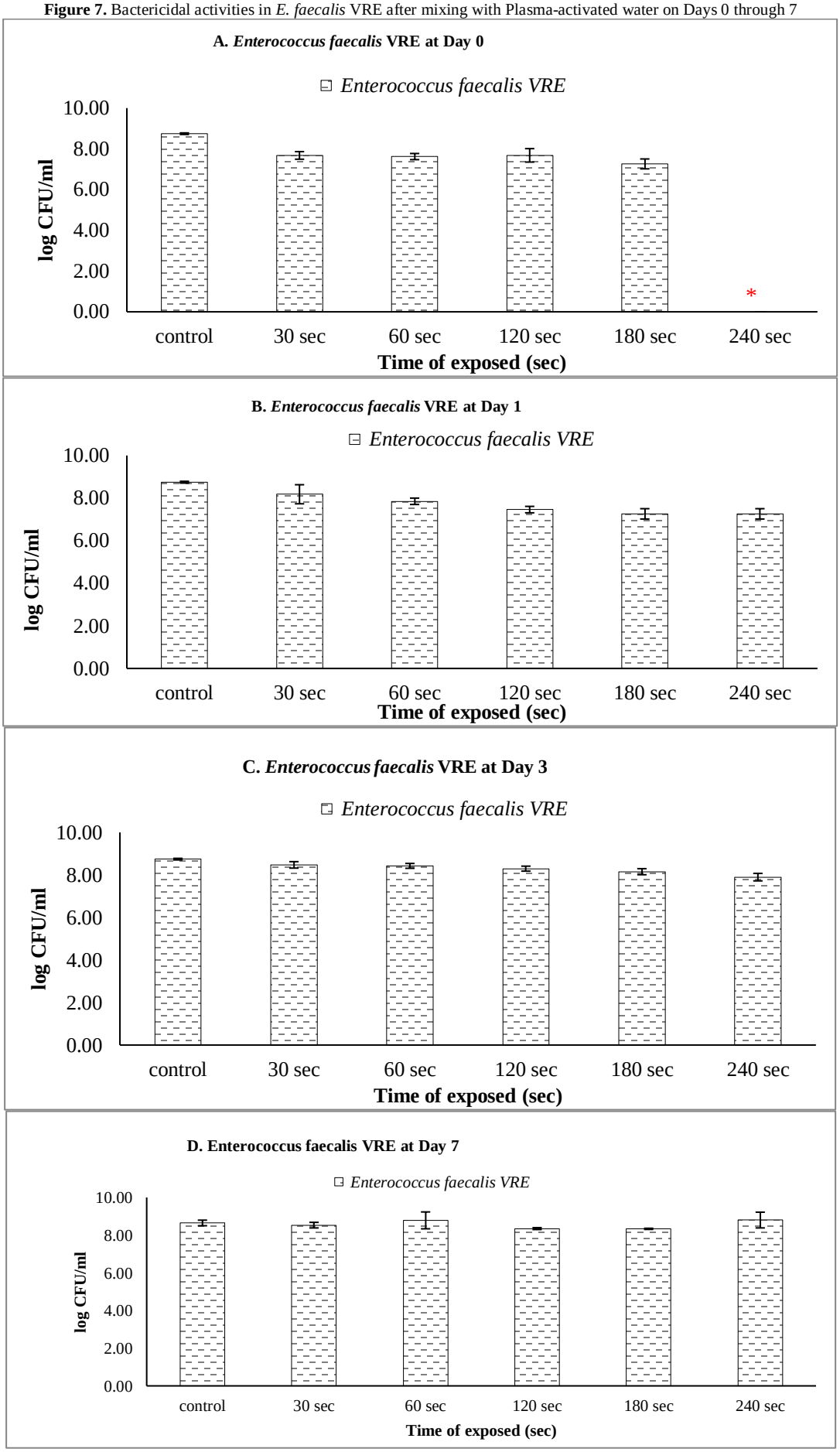
(*) Bacterial concentration was statistically decreased) P<0.05 by ANOVA)

Figure 6: Bactericidal activities in the clinical isolate group after mixing with Plasma-activated water on Days 0 through 7



(*) Bacterial concentration was statistically decreased) $p < 0.05$ by ANOVA)

Figure 7. Bactericidal activities in *E. faecalis* VRE after mixing with Plasma-activated water on Days 0 through 7



(*) Bacterial concentration was statistically decreased (p □ 0.05 by ANOVA)

Contemporary research has also revealed PAW's ability to disrupt bacterial biofilms and enhance conventional antimicrobial efficacy [23]. Mechanistically, plasma-induced oxidative stress disrupts bacterial membranes, DNA, and metabolic pathways. Recent studies [24] have elucidated how RONS generated during plasma treatment interact synergistically with biofilm matrices, further enhancing antimicrobial activity. These findings highlight the potential of PAW to be integrated into healthcare practices, particularly for treating nosocomial infections caused by MDR pathogens.

Mechanistically, plasma-induced oxidative stress disrupts bacterial membranes, DNA, and metabolic pathways. Recent studies [24] have elucidated how RONS generated during plasma treatment interact synergistically with biofilm matrices, further enhancing antimicrobial activity. These findings highlight the potential of PAW to be integrated into healthcare practices, particularly for treating nosocomial infections caused by MDR pathogens. Future research should focus on exploring combinations of NTP with conventional antibiotics, as synergistic effects have been observed in preliminary trials [25]. Additionally, optimization of plasma devices for specific clinical applications, such as wound care and surface sterilization, could significantly expand the utility of this technology.

CONCLUSION

This comprehensive investigation has demonstrated the significant potential of argon NTP and plasma-activated water as innovative approaches for microbial decontamination. Key findings include rapid bactericidal effects against multidrug-resistant pathogens, particularly MRSA strains, and the sustained antimicrobial activity of PAW over seven days of storage. The selective efficacy of these treatments highlights their potential for targeted antimicrobial applications in clinical settings. Recent advances in plasma medicine corroborate these findings, validating NTP's capacity to overcome bacterial resistance mechanisms through multiple pathways. The long-term stability and eco-friendly nature of these technologies further underscore their viability as alternatives to traditional antimicrobial methods. Future studies should prioritize optimizing treatment parameters for specific pathogens and integrating these approaches with existing antimicrobial therapies.

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