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SHORT REPORT

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Gaseous ozone inactivation of *Bacillus atrophaeus* spores on ceramic and porcelain tiles

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Abstract

In this study, we investigated the ozone inactivation efficiency of *Bacillus atrophaeus* spores attached to various tile surfaces. Eight different types of tiles were employed, considering factors such as porosity (ceramic, porcelain), color (white, black), and glossiness (matte, glossy). Inactivation was performed by exposing the spore-loaded tiles to ozone gas for a specified duration. The inactivation efficiencies of ozone gas on different tile surfaces were compared by analyzing the colony-forming units of desorbed *Bacillus atrophaeus* cultured in a growth medium. Results revealed a reduction in colony counts with increasing ozone exposure time, indicating a proportional enhancement in inactivation effectiveness on ozone exposure time. After exposure to ozone gas for 30 min or longer, more than 90% of spores on each tile were inactivated. Regarding porosity, ceramic tiles exhibited slightly superior inactivation effects compared to porcelain tiles. Additionally, in terms of glossiness, glossy tiles demonstrated better inactivation effects than matte tiles. However, no significant differences were observed in inactivation effects based on the color of the tiles.

Keywords *Bacillus atrophaeus* spores, Ozone gas, Inactivation, Ceramic tiles, Porcelain tiles

Introduction

The rapid increase in international terrorism has heightened the risk of bioterrorism, posing a serious threat to global security. In particular, bioterrorism raises significant concerns as even small amounts of biological agents can trigger diseases, fatalities, and societal disruption, causing considerable apprehension. An illustrative example is the 2001 anthrax letters incident in the USA, where numerous workers underwent preventive or post-exposure treatments, and affected buildings underwent extensive and costly decontamination procedures (Green et al. 2019). Therefore, the necessity for continuous development of tools and strategies to respond to biological warfare agents has been emphasized, leading to numerous ongoing research endeavors (Aydogan et al. 2006). Especially, during the 2001 anthrax attacks in the USA, it is estimated that approximately 320 million dollars was spent to restore buildings exposed to anthrax (Schmitt et al. 2012). *Bacillus anthracis* is a bacterium that causes anthrax and forms spores that are resistant to various biocides (Inglesby et al. 1999). Inhalation of *Bacillus anthracis* spores is more dangerous than skin contact, but spores that contaminate surfaces are harder to remove than those in the air, necessitating research on methods to eliminate them (Wood et al. 2019). However, *Bacillus atrophaeus* is commonly utilized as a substitute due to its relative safety and ease of use compared to *Bacillus anthracis*, while still sharing similarities with *Bacillus anthracis* (Kwon et al. 2024; Nguyen et al. 2022; Thornburg et al. 2006).

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Ozone, an inorganic molecule with potent antimicrobial characteristics (Epelle et al. 2022), is recognized for its strong oxidizing attributes and verified bactericidal effects (Czekalski et al. 2016; Marino et al. 2018; Rangel et al. 2021). Although ozone gas poses toxicity to humans, its environmentally friendly status is attributed to its rapid decomposition into oxygen and its ease of generation (Sharma et al. 2008). Ozone gas has demonstrated an inactivation effect on *Bacillus anthracis* and *Bacillus subtilis* spores on the surfaces of various carrier materials such as wood, glass, and carpet. It has also been reported that the inactivation effect of ozone gas varies depending on the surface characteristics (Aydogan et al. 2006; Wood et al. 2020). Tiles are commonly used construction materials, and in the event of a bioterrorism incident, there is a potential for exposure to biological agents, necessitating research on tile decontamination. Tiles can be coated with TiO_2 (Yusuf et al. 2020) or Ag/SiO_2 (Baheiraei et al. 2012) to acquire antibacterial or self-cleaning properties. We previously examined the UV sterilization effect on tile surfaces (Jang et al. 2022). In a continuation of our previous study, the current investigation compares the inactivation effects of ozone gas on *Bacillus atrophaeus* adsorbed to the surfaces of various types of tiles placed in a sealed container, considering factors such as porosity (ceramic, porcelain), color (white, black), and glossiness (matte, glossy).

Experimental

Preparation of spores on tile surfaces

Bacillus atrophaeus spores (catalog number: PTG706) were acquired from Protigen in Jeonju, South Korea. The stock *Bacillus atrophaeus* spore solution (~ 10^9 colony forming units/mL (CFU/mL)) was stored in a refrigerator at 4 ℃. Porous ceramic tiles with various finishes, such as black glossy (catalog number: MPV753005P), black matte (catalog number: MPV753015M), white glossy (catalog number: MPV753001P), and white matte (catalog number: MPV753011M), were procured from Maxpoint in Goyang, South Korea. Vitrified porcelain tiles featuring black glossy (catalog number: m1010ubl), black matte (catalog number: m1010mbl), white glossy (catalog number: m1010uwh), and white matte (catalog number: m1010mwh) finishes were acquired from Tiledotcom in Nonsan, South Korea. The tiles were cut into 2 cm width by 2 cm length pieces prior to spore deposition and ozone application. Stock spore solutions were diluted in phosphate-buffered saline (PBS obtained from Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 10^6 CFU/mL, and 50 µL of the spore solution was deposited onto each tile, followed by overnight drying under 20 °C and 40% relative humidity (RH) in a clean bench.

Gaseous ozonation, recovery and measurement of spore survival

Ozone was generated using a portable ozone generator (WT600, WSTA, Shenzhen, China), which is a household ozone generator designed for purifying air using ozone with an ozone output of 500 mg/h. This ozone generator produces ozone through the corona discharge method. The outlet of the ozone generator is connected to the inlet of a sealed container, where the eight spore-loaded tiles (ceramic white matte, ceramic white glossy, porcelain black matte, porcelain black glossy, porcelain white matte, porcelain white glossy, porcelain black matte, and porcelain black glossy) are placed. The tiles were then exposed to the ozone gas for 10, 30, 60, or 120 min. The sealed container had dimensions of 15 cm in width and length and 6 cm in height. During ozone exposure, the sealed container was maintained at 20 °C and 40% RH. For safety, the ozone gas exposure was conducted in a fume hood. The experimental setup for ozone inactivation is depicted in Fig. 1.

Following the ozone inactivation, 100 µL of a PBS solution containing 0.2% Tween 20, a surfactant, was applied to the tile surface. The surface was then scratched with a razor blade to desorb the spores from the tile surface. The solution collected from the scratched tile surface was pipetted and transferred to a 1.5-mL microtube. Subsequently, the solution in the microtube was diluted to a specific ratio, and 20 µL of each dilution was inoculated onto a solid medium. After the 24-h incubation period in a 37 °C incubator (IB-01E, Jeio Tech, Daejeon, Korea) at the CNU Chemistry Core Facility (Daejeon, South Korea), the inactivation effect of ozone gas by tile type was assessed by counting the number of colonies generated in the medium. The experiment was repeated three times to calculate the average and standard deviation. For the evaluation of differences between the tiles, the Kruskal–Wallis multiple comparison test was employed using IBM SPSS Statistics 26 software.

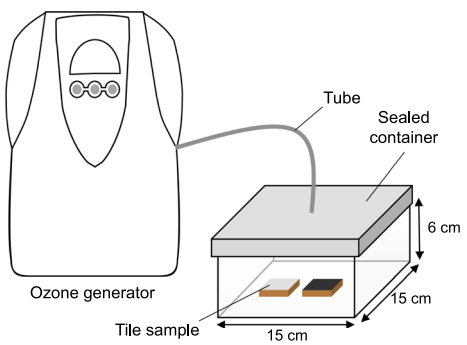

Fig. 1 Scheme of the experimental setup

Results and discussion

In this study, eight types of tiles were utilized to investigate the differential disinfection effects of ozone gas, considering factors such as porosity (ceramic, porcelain), color (white, black), and glossiness (matte, glossy). Each type of tile was sealed in a container and exposed to ozone gas for durations of 10, 30, 60, and 120 min, and this process was repeated three times to calculate the average and standard deviation.

Figure 2A presents a graph illustrating the number of colonies obtained from detached spores on the tile surface after exposure to ozone for varying duration. The term "0 min" represents the positive control not exposed to ozone gas. As the exposure time to ozone gas increases, there is a decrease in the number of colonies.

Figure 2B shows the survival fraction of *Bacillus atrophaeus* spores, calculated using the formula:

$$\text{Survival fraction (\%)} = \frac{\text{CFU}_1}{\text{CFU}_0} \times 100$$

Here, CFU_1 represents the number of colonies on ozone-treated tile samples, while CFU_0 indicates the number of colonies on the positive control tiles. The survival fraction takes into account the characteristics of the tile surface by including the colony number from tiles without ozone application. The survival fraction was employed to offset differences in the recovery rates of spores from tile surfaces, which varied depending on the surface characteristics of the tiles. When the spores on tiles were exposed to ozone gas for 10 min, the survival fraction (%) ranged from 81 to 124% across the eight types of tiles, suggesting little to no inactivation effect due to ozone gas. However, after exposure to ozone gas for 30 min or more, an inactivation effect of over 90% was observed in the majority of tiles.

Figure 2C represents the log reduction (LR) values, which were calculated by the formula.

$$\text{Log reduction} = \log\frac{\text{CFU}_1}{\text{CFU}_0}$$

The LR value reached its minimum at − 2.03 when the spores on tiles were exposed to ozone gas for 120 min, specifically on the glossy white porcelain tile. To assess differences between the tiles, a statistical analysis employing the Kruskal–Wallis multiple comparison test was conducted using LR values. When comparing ceramic and porcelain tiles for their inactivation efficiency against spores with ozone application, slightly better inactivation was observed for ceramic tiles as shown in Fig. 2C. The p values were found to be 0.166, 0.073, 0.004, and 0.954 for 10 min, 30 min, 60 min and 120 min, respectively, indicating rapid inactivation was achieved from ceramic tiles from 10 to 60 min of ozone inactivation. No difference was observed between ceramic and porcelain tiles when the ozone was applied for 120 min. Figure 3A shows the significant difference in inactivation efficiency between

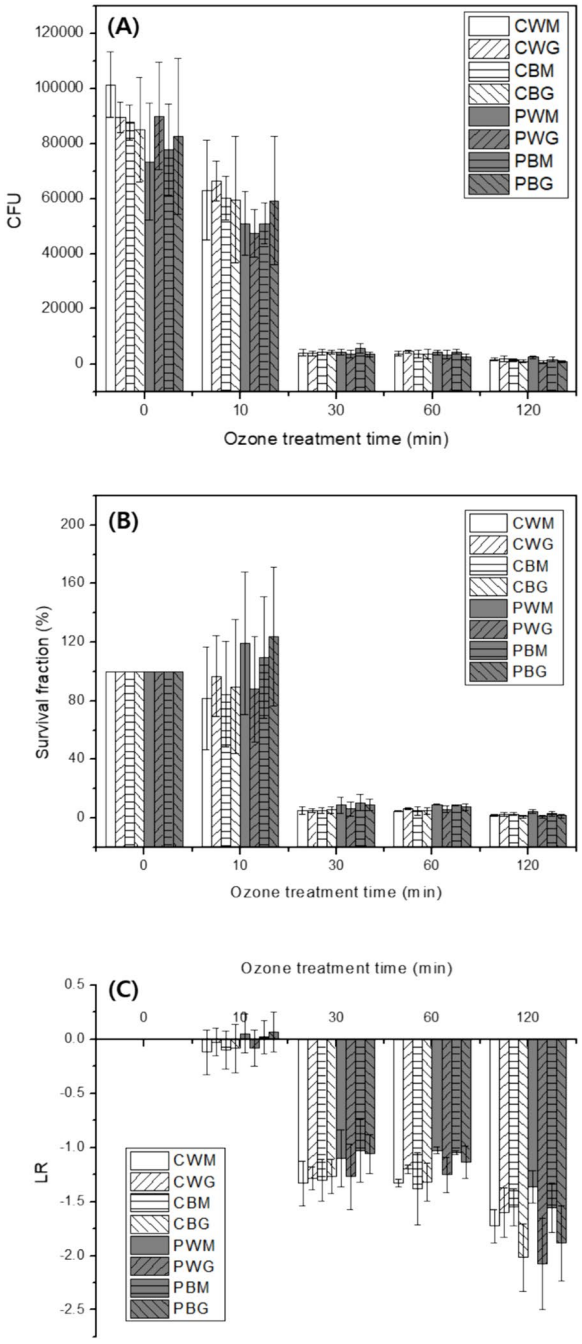

Fig. 2 Effect of ozone treatment time on **A** number of surviving colonies, **B** survival fraction, and **C** LR value. The *Bacillus atrophaeus* spores attached on the surface of each tile were sterilized by applying ozone gas for a specified time. Then, the spores were cultured to provide the colony information. The acronyms of C, P, W, B, M, and G correspond to ceramic, porcelain, white, black, matte, and glossy, respectively

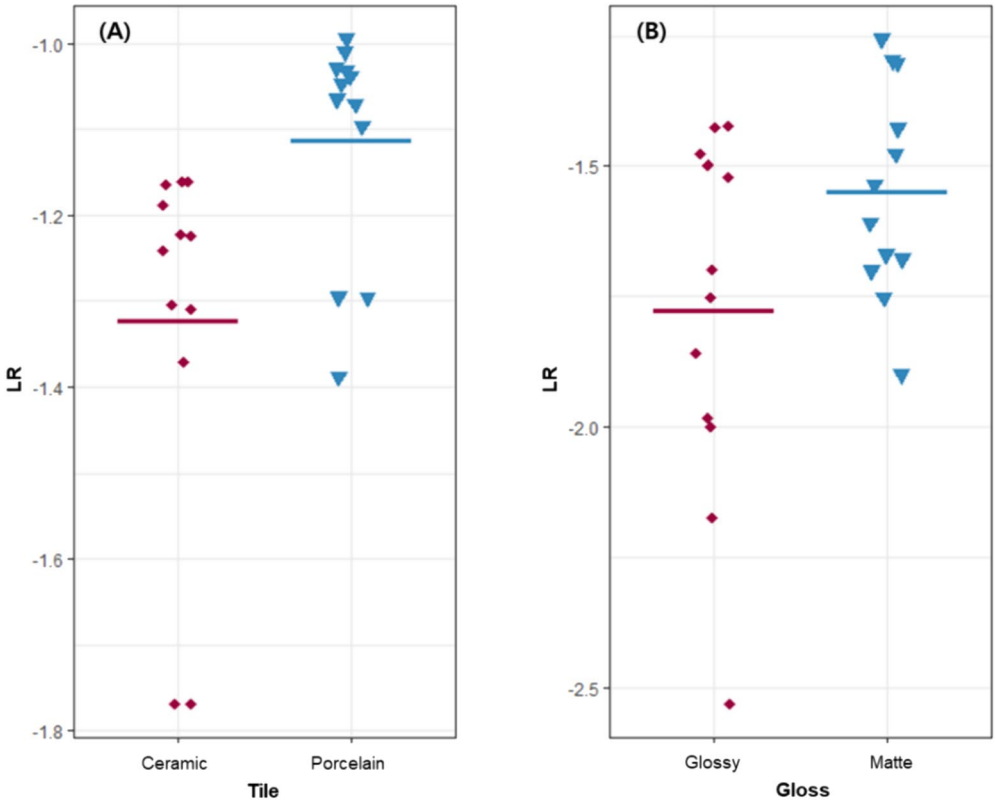

Fig. 3 Comparison of LR values as a function of **A** porosity (ceramic vs. porcelain) for 60 min of ozone exposure and **B** glossiness (glossy vs. matte) for 120 min of ozone exposure. Significant difference was observed in both cases, where the *p* values were 0.004 and 0.017, respectively

ceramic and porcelain tiles from 60 min of ozone exposure, where noticeably better inactivation was observed from ceramic tiles. When comparing glossy and matte tiles for ozone inactivation efficiencies on spores attached on the tile surface, a significant difference was observed when the tiles were exposed to ozone gas for 120 min as shown in Fig. 3B, with glossy tiles demonstrating more effective ozone disinfection with p value of 0.017. Generally, ozone gas decontamination is more efficient on inorganic, nonporous materials compared to organic, porous materials. However, in this study, spore inactivation using ozone gas was more effective on ceramic tiles, which have relatively greater porosity than porcelain tile. In the study by Wood et al., spore inactivation using ozone gas on porous materials (carpet and wood) surfaces was more effective than on nonporous materials (laminate, glass, and galvanized metal) surfaces for *Bacillus anthracis* spores, where the authors suggested the possibility of involvement of mechanisms different from the general mechanism (Wood et al. 2020).

In our previous experiment on sterilizing *Bacillus atrophaeus* spores using a UV laser, we measured the contact angles to determine the surface properties of eight types of tiles, where higher water contact angle values, implying higher hydrophobicity, were obtained for porcelain than ceramic and for matte than glossy (Jang et al. 2022). It was reported that the hydrophobic surface limits the diffusion of ozone (Yoon et al. 2008). Therefore, the higher hydrophobicity of porcelain and matte tiles reduces the ozone gas inactivation efficacy, making ceramic and glossy tiles more effective for the ozone disinfection ability.

Conclusions

In this study, the investigation of the inactivation effects of ozone gas on the spores on eight different types of tiles revealed an inactivation rate of over 90% when exposed to ozone gas for 30 min or more. Ceramic tiles exhibited slightly better inactivation compared to porcelain tiles, while glossy tiles demonstrated more effectiveness than matte tiles. However, no discernible differences were observed based on tile color. These findings contribute to the ongoing efforts in developing effective strategies for bioterrorism preparedness and highlight the potential of ozone gas in tile decontamination.

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Author contributions

DK involved in conceptualization, methodology, data curation, writing—original draft, and reviewing. HN participated in methodology, data curation, visualization, and formal analysis. YS participated in supervision, conceptualization, and reviewing. JK involved in supervision, conceptualization, validation, writing—original draft, writing-reviewing and editing, and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Competing interests

There are no conflict of interest to declare. Jeongkwon Kim is an associate editor of Journal of Analytical Science and Technologies. Associate editor status has no bearing on editorial consideration.

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