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Medicine in Microecology 14 (2022) 100059

Contents lists available at ScienceDirect

Medicine in Microecology

journal homepage: www.journals.elsevier.com/medicine-in-microecology

Inactivation of multiple human pathogens by Fathhome's dry sanitizer device: Rapid and eco-friendly ozone-based disinfection

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ARTICLE INFO

Keywords:
Ozone-based disinfection
PPE
Pathogen

ABSTRACT

SARS-CoV-2 spread rapidly, causing millions of deaths across the globe. As a result, demand for medical supplies and personal protective equipment (PPE) surged and supplies dwindled. Separate entirely, hospital-acquired infections have become commonplace and challenging to treat. To explore the potential of novel sterilization techniques, this study evaluated the disinfection efficacy of Fathhome's ozone-based, dry-sanitizing device by dose and time response. Inactivation of human pathogens was tested on non-porous (plastic) surfaces. 95.42–100% inactivation was observed across all types of vegetative microorganisms and 27.36% inactivation of bacterial endospores tested, with no residual ozone detectable after completion. These results strongly support the hypothesis that Fathhome's commercial implementation of gas-based disinfection is suitable for rapid decontamination of a wide variety of pathogens on PPE and other industrially relevant materials.

1. Introduction

The spread of SARS-CoV-2 took the world by surprise in late 2019, revealing gaps in global pandemic preparedness and leaving tragedy in its wake. Evading containment due to its high transmissibility and airborne spread, SARS-CoV-2 infected 272 million people causing 5.3 million deaths (as of December 2021) [1–3].

Properly worn, Personal protective equipment (PPE) has been shown to be one of the most effective tools in preventing the spread of COVID-19 [4]. Face masks, gloves, face shields, and surgical gowns are all recommended to reduce rates of COVID-19 transmission in a medical setting. In contrast, masks are recommended for the general public in situations with a high risk of infection [3,5,6]. However, due to the high transmissibility and persistence of SARS-CoV-2 across the globe, the use and consumption of PPE increased along with the waste inherent to its disposal [7]. As demand for PPE surged globally after the onset of COVID-19, manufacturing and supply systems were slow to fill the gap [8]. A major contributing factor to both the shortage of PPE and their increased burden of waste is their designation for one-time use. Gowns, for example, can spread infection if not changed after contact with potentially contaminated surfaces, patients, or materials. Thus, disinfecting and reusing the PPEs not only solves the supply-demand problem but can reduce the burden of waste generated from single-use PPE, especially non-degradable plastics (face shields, goggles, etc.) [7].

Ozone-gas-based (OGB) devices developed by Fathhome Inc of Oakland, CA have previously demonstrated promising results in decontaminating over 99.99% of coronaviruses on both solid and porous surfaces in a self-contained, ozone-generating device that uses no water or chemicals [9,10]. Fathhome seeks to proliferate low-cost, rapid disinfection techniques at a global scale through a simple OGB approach and a novel containment and neutralization system for that ozone. These devices offer the potential to thoroughly decontaminate PPE and other high-touch equipment by eliminating infection vectors, thus extending the usability of PPE items, alleviating shortages, and reducing costs in healthcare, industrial, or personal settings. Disinfection times as low as 15 min resulted in nearly 100% inactivation of viral particles [11].

Abbreviations: PPE, Personal protective equipment; OGB, Ozone-gas-based.

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https://doi.org/10.1016/j.medmic.2022.100059
Received 18 April 2022; Received in revised form 30 June 2022; Accepted 24 July 2022
Available online 5 August 2022

Disinfection times for other currently available methods range from 55 min to overnight, which can be limiting at a large scale [12]. Other decontamination approaches include ultraviolet radiation, chemical disinfectants, vapor phase hydrogen peroxide, heat, and microwave radiation. While these approaches reduce the viability of pathogens, they also damage PPE materials reducing airflow, fit, filtration efficacy, and imparting lingering, unpleasant odors, thereby undermining their benefits [12,13]. Finally, water-based disinfection methods have posed challenges to industries such as food processing, where there is a need to remove pathogens without introducing moisture. There is an undeniable need for fast, large-scale, and water-free disinfection techniques [14].

In addition, to use scenarios in the SARS-CoV-2 pandemic, hospital-acquired infections have been an ongoing problem for decades [15,16] and steadily grow more hazardous as multi-antibiotic-resistant pathogens become ubiquitous [17,18]. While previous research has shown Fathhome dry sanitization devices to be effective against viruses, no work has been done to assess its effectiveness against other common pathogens such as bacteria, fungi, or their spores. In this study, we evaluate the Fathhome prototype against a broad phylogenetic spectrum of microorganisms relevant in industry and healthcare settings by quantifying the number of viable microorganisms present on a surface before and after exposure to ozone in the Fathhome device. With these data, we can calculate the sanitization efficiency of the device and its potential efficacy for use in large-scale decontamination efforts.

2. Materials and methods

2.1. Organisms

All organisms were stored at −80 °C and streaked to single colonies on 10 cm agarose plates with suitable media (Table 1). Cultures were grown overnight at appropriate temperatures (Table 1) and then stored at 4 °C until use (unless otherwise stated, see endospore assay). A single colony from a culture no more than one week old was used to inoculate a 5 mL liquid culture in a Falcon Brand 14 mL polypropylene Round Bottom Tube (Corning Science 352,059; Reynosa, Mexico) that was then incubated overnight at 200 rpm and a temperature suitable for the specific organism (Table 1). The following day, optical density (OD) was measured at 600 nm using a Beckman DU640 (Beckman Coulter Life Sciences; Indianapolis, Indiana), and the culture was diluted to a specific OD600 which is consistent with culture density between $1x10^6$ and $1x10^8$ depending on the organism (Table 1). This standardized pathogen stock was used in all experiments to test the Fathhome device's efficacy.

Organisms were selected based on their relevance to medical and industrial settings as well as their phylogenetic diversity. Common healthcare-associated pathogens include *Escherichia coli O157:H7*, Methicillin-Resistant *Staphylococcus aureus*, Multidrug-Resistant *Pseudomonas aeruginosa*, *Clostridioides difficile*, Drug-resistant *Candida auris*, and Vancomycin-Resistant *Enterococcus* [1,9,18]. This study tested the capacity of the Fathhome device to effectively kill these species or phylogenetically similar surrogates (Fig. 1). *C. difficile* endospores were represented by *Bacillus subtilis,* and *C. auris* was represented by *Saccharomyces cerevisiae*. Due to the high risk of transmissibility and increased burden of containment, all organisms used in the study were less virulent strains (for example, *E. coli* K12 MG1655 was used in place of *E. coli O157:H7*).

2.2. The Fathhome device

Fathhome's ozone-based dry sanitizer is equipped with a computer-controlled ozone generator and a catalytic manganese dioxide-copper oxide (MnO2–CuO2) converter. The system maintains biocidal levels of sanitizing ozone gas ranging from 10 ppm to 70 ppm depending on device settings, and a vacuum pump maintains internal pressures between −30 kPa and −15 kPa, ensuring that no ozone escapes the device during the sanitization cycle. In addition, the onboard computer constantly monitors sanitizing-gas levels outside the device to ensure zero emissions during and after a sanitization cycle.

Once the vacuum seal is generated, ozone is produced via the electric cleavage of atmospheric oxygen (O2) into elemental oxygen ($O2^-$), which combines with uncleaved elemental oxygen molecules to create O_3. An onboard ozone sensor and microcontroller maintain target ozone concentration levels. The sanitizing chamber's ozone-gas concentration level was continuously monitored during operation using a NIST Calibrated ozone sensor (FD-600-O3 Ozone Analyzer 0–100 ppm range with 1 ppm resolution; Forensics Detectors, Rolling Hills Estates, CA, USA) connected directly to a 3/32″ sensor port on the FATHHOME device's vacuum chamber. At all times, the chamber contents were held under negative pressure, with the only exit for all gasses in the system being pumped through the MnO2–CuO2–O3 catalytic converter to ensure environmentally safe device exhaust within OSHA and FDA guidelines. In addition, an external ozone sensor (Aeroqual Series 500 Portable Ozone Monitor with EOZ Sensor Head rated for 0–10 ppm and 0.01 ppm resolution) was located just outside the chamber and detected no ozone above the background during all stages of device functioning and

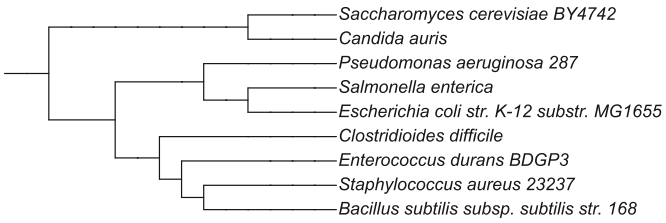


Fig. 1. A microbial phylogeny containing medically relevant bacteria and fungi and their genomically similar surrogates. Organisms tested in the Fathhome device are denoted with an asterisk. A broad spectrum of organisms was tested, including members of *Gammaproteobacteria*, *Enterobacteriaceae* and *Pseudomonadales*; *Firmicutes*, *Bacillaceae* and *Staphylococcaceae*; and *Basidiomycetes*, *Saccharomycetales*.

Table 1
Organisms and growth conditions.

Organism	Strain	Genotype	GenBank Accession Number	Source	Growth	OD600[a]
Escherichia coli	K12 MG1655	F- lambda- ilvG- rfb- 50 rph-1.	NC_000913.3	Blattner et al. (1997) [24]	LB media 37 °C	0.1
Pseudomonas aeruginosa	mPAO1	WT	CP027867.1	Varadarajan et al. (2020) [25]	LB media 37 °C	0.1
Staphylococcus aureus	ATCC 23235	WT	CP094663 CP094664	This study	LB media 37 °C	0.05
Bacillus subtilis	168	trpC2	AL009126	Zeigler et al. (2008) [26,27]	LB media 37 °C	0.1
Saccharomyces cerevisiae	BY4742	MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	JRIR00000000	Song et al. (2015) [28,29] Brachmann et al. (1998) [30]	YPD media 30 °C	0.7
Enterococcus durans	BDGP3	WT	CP022930.1	Wan et al. (2017) [31]	BHI media 30 °C	0.1

[a] OD600 Dilutions to produce cultures between 1×10^6 and 1×10^7 Colony Forming Units (CFUs)/mL for plating.

exhaustion. For the duration of this study, the Fathhome device was kept inside of BSL-2 containment.

2.3. Experimental setup

To test the efficacy of Fathhome's dry sanitizing device to prevent the persistence of human pathogens, 5 μl of stock pathogen culture was aliquoted in the bottom of a well in two 96-well, flat-bottomed plates (Greiner Bio-One GmbH #655801; Frickenhausen, Germany) and allowed to dry in a biosafety cabinet for approximately an hour or until an opaque crust formed (Fig. S1A). These plates are referred to as "dry droplet" plates. A set of serial dilutions was also prepared using the stock pathogen culture. 80 μL of the diluted cell suspensions was plated on 15 cm agarose plates with appropriate media. These plates are referred to as "pre-spread" plates. One dry droplet plate and a series of pre-spread plates were placed in the Fathhome device and exposed to ozone for 5 or 15 min, while the remaining control plates were run through the vacuum cycle in the Fathhome device with ozone production disabled. All organisms were tested in quintuplicate in each of two independent experiments for each organism.

After ozone or control exposure, dried cell droplets were resuspended in 100 μL of an appropriate medium warmed to 37 °C in a water bath. Samples were allowed to resuspend at room temperature for 5 min, after which time they were thoroughly mixed using a multichannel pipette. Serial dilutions were made by adding 10 μL of the cell suspension to 90 μL of the warmed appropriate media and mixing with a pipette. Of the diluted cell suspensions, 80 μL was plated on 15 cm agarose plates with appropriate media for each organism. Samples were left overnight in an incubator heated to the appropriate temperature for optimal growth except for *Saccharomyces cerevisiae* which required an additional 24 h incubation period to yield countable colonies.

2.4. Bacillus subtilis endospore assay

To assess the ability of the Fathhome device to inactivate highly resistant *B. subtilis* endospores, 5 mL cultures were grown to nutrient depletion (about seven days) and evaluated for the presence of endospores via light microscopy (Fig. S1 B). The Schaeffer-Fulton method for endospore staining was used to prepare slides for microscopy. Vegetative cells were inactivated via Pasteurization in an 80 °C water bath for 10 min, leaving only viable endospores in the culture. As per the above protocol, 5 μl of stock pathogen culture was aliquoted in the bottom of a well in two 96-well, flat-bottomed plates, allowed to dry in a biosafety cabinet, and exposed to ozone. Samples were resuspended for 5 min in appropriate media as before, serially diluted, plated, and incubated overnight.

2.5. Sequencing

The six species were isolated, five re-sequenced to confirm genome integrity and similarity [18–25], and the sixth strain, *S. aureus* (ATCC 23235) described here, has no published genome, nor has it been submitted to a public sequence repository. However, the sequence is available from ATCC that compares closely with ours. The organisms were streaked onto agar plates containing an appropriate growth medium (Table 1). Purified colonies were amplified overnight in an appropriate liquid medium at an appropriate growth temperature (Table 1). An aliquot was used for 16 S PCR for bacterial species and ITS PCR for yeasts. PCR Cleanup was performed with AMPure XP Beads and sequence identification by ABI 3730XL Sanger sequencing. Genome assemblies were constructed using Hierarchical Genome Assembly Process 2 (HGAP2) from SMRT Analysis version 2.0 using BLASR alignment. Celera assembler was used for assembly, and Quiver for consensus polishing.

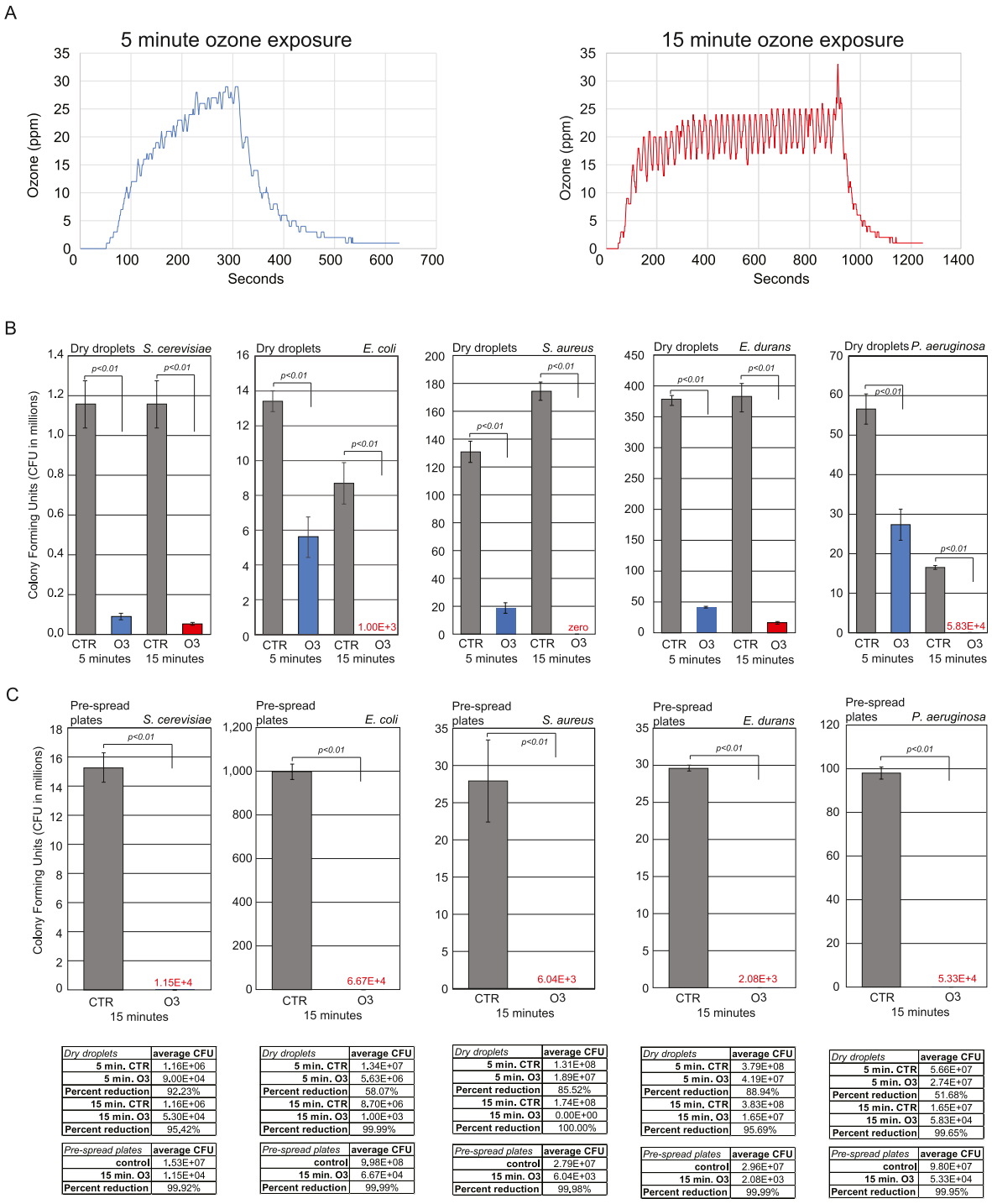
3. Results

The Fathhome device is based on antimicrobial ozone technology and has previously demonstrated an ability to inactivate greater than 99% of *E. coli* on contaminated fabric [9,10]. To test the disinfection efficiency of the Fathhome device on a broad spectrum, six phylogenetically diverse and medically relevant organisms were selected and tested. The organisms, five bacteria, including *E. coli, B. subtilis, P. aeruginosa, S. aureus, E. durans,* and one fungal species, *S. cerevisiae* (Table 1), were chosen based on their relevance to medical and industrial settings as well as their phylogenetic diversity. For example, *Escherichia coli* is common on surfaces in hospitals and is frequently used in industry [17], while *Pseudomonas aeruginosa* is a common opportunistic pathogen that causes over 32,000 hospital-borne infections annually [1]. Accuracy in species identification was confirmed by whole-genome sequencing. We specifically describe the sequence of the *Staphylococcus aureus* strain (ATCC 23235) since the other species were previously sequenced and published [18–25] and confirmed by us. The *S. aureus* genome was assembled using the Flye assembler [26] and is composed of a circular chromosome of length 2,762,338 bp and a plasmid of length 27,265 bp (>200-fold coverage). The PGAP annotation [27] of both the chromosome and the plasmid predicts 2762 protein-coding genes, 62 pseudogenes, six rRNA operons, and 59 tRNAs. The plasmid likely confers beta-lactamase resistance as it contains the three genes, Class A beta-lactamase (BlaZ), beta-lactamase regulatory sensor-transducer (BlaR1), and the beta-lactamase repressor (BlaI) of the beta-lactamase (bla) operon. Like other *S. aureus* strains, the genome contains prophages, two nearly complete (42,788 and 45,165bp) and one partial copy (12,366 bp). At the nucleotide level, they have little sequence similarity.

We placed droplets containing approximately 1.00 E^{+06}–1.00 E^{+08} bacteria or fungi on the bottom of plates, dried them for 30 min at 37 °C, and exposed them to ozone for 5 or 15 min. Representative ozone exposure profiles are shown in Fig. 2 and were calculated to be, on average, 108.7 and 306.1 ppm *minutes of ozone for the 5- and 15-min treatments, respectively. For each organism, we included pre-spread agar plates resulting in 99.94–99.99% percent killing after 15-min ozone exposure for vegetative cells ($p < 0.05$). For dry droplets treated for 5 min, *S. cerevisiae* yielded the highest inactivation at 92.23% killing ($p = 0.001$), and *E. coli* yielded the lowest inactivation at 50.55% killing ($p = 0.004$). For dry drops treated for 15 min, *S. aureus* produced the highest inactivation at 100% killing ($p = 0.00002$), and *S. cerevisiae* yielded the lowest inactivation at 95.42% killing ($p = 0.001$). Overall, the highest inactivation for dried droplets on a solid surface was observed for *S. aureus* with 100% killing at 15 min and the lowest for *E. coli* with 50.55% killing at 5 min.

To determine the efficacy of ozone-based disinfection of bacterial endospores, we generated endospores by allowing cultures to reach nutrient depletion, killing viable vegetative cells via Pasteurization, and validating endospore percentage using staining and light microscopy (Figure panel of representative staining). We then placed 1.00 E^{+06}–1.00 E^{+08} endospores on the bottom of plates, dried them for 30 min at 37 °C, and exposed them to ozone for 30 or 60 min. Representative ozone exposure profiles are shown in Fig. 3 and were calculated to be 1043 and 2501 ppm*minutes for the 30- and 60-min treatments, respectively. We included pre-spread plates, which yielded 90.07% killing ($p = 0.005$) for bacterial endospores after 15 min. The highest inactivation of bacterial endospores in dry droplets was observed at 60 min with 27.36% killing ($p = 0.007$), and the lowest inactivation was observed at 30 min with 15.57% killing ($p = 0.016$).

In conclusion, we illustrated that decontamination provided by Fathhome's ozone-based dry sanitizer is highly effective in inactivating a wide array of microbes. We demonstrated that brief ozone exposure at 20 ppm for 15 min could eliminate 95.69–100% of vegetative Gram-negative and Gram-positive bacteria and 95.42% of fungi on solid



Dry droplets	average CFU
5 min. CTR	1.16E+06
5 min. O3	9.00E+04
Percent reduction	92.23%
15 min. CTR	1.16E+06
15 min. O3	5.30E+04
Percent reduction	95.42%

Pre-spread plates	average CFU
control	1.53E+07
15 min. O3	1.15E+04
Percent reduction	99.92%

Dry droplets	average CFU
5 min. CTR	1.34E+07
5 min. O3	5.63E+06
Percent reduction	58.07%
15 min. CTR	8.70E+06
15 min. O3	1.00E+03
Percent reduction	99.99%

Pre-spread plates	average CFU
control	9.98E+08
15 min. O3	6.67E+04
Percent reduction	99.99%

Dry droplets	average CFU
5 min. CTR	1.31E+08
5 min. O3	1.89E+07
Percent reduction	85.52%
15 min. CTR	1.74E+08
15 min. O3	0.00E+00
Percent reduction	100.00%

Pre-spread plates	average CFU
control	2.79E+07
15 min. O3	6.04E+03
Percent reduction	99.98%

Dry droplets	average CFU
5 min. CTR	3.79E+08
5 min. O3	4.19E+07
Percent reduction	88.94%
15 min. CTR	3.83E+08
15 min. O3	1.65E+07
Percent reduction	95.69%

Pre-spread plates	average CFU
control	2.96E+07
15 min. O3	2.08E+03
Percent reduction	99.99%

Dry droplets	average CFU
5 min. CTR	5.66E+07
5 min. O3	2.74E+07
Percent reduction	51.68%
15 min. CTR	1.65E+07
15 min. O3	5.83E+04
Percent reduction	99.65%

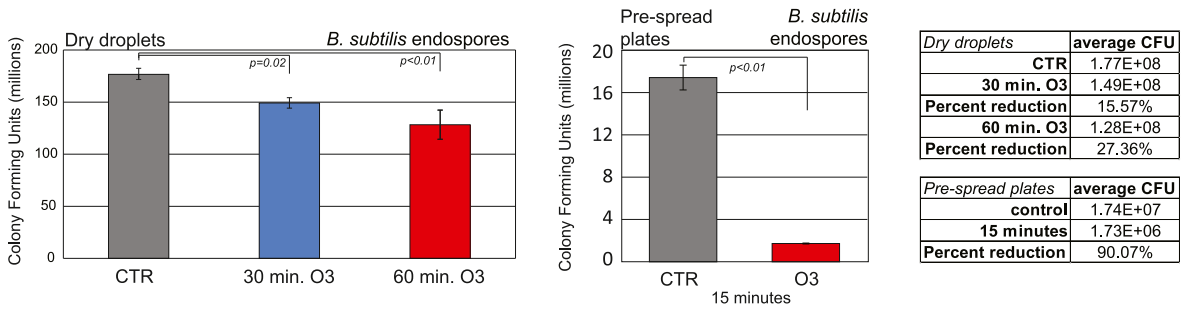
Pre-spread plates	average CFU
control	9.80E+07
15 min. O3	5.33E+04
Percent reduction	99.95%

Fig. 2. The effect of 5- and 15-min ozone exposure on the viability of vegetative bacteria and fungi. (A). Plots depicting ozone concentration in ppm that bacterial and fungal cells were exposed to for either 5 or 15 min. The ozone data were recorded via a NIST Calibrated ozone sensor (FD-600-O3 Ozone Analyzer). (B–E). 5 μ l droplets were applied to the bottom of 96-well plates, dried, and exposed to ozone for 5 or 15 min. Bacterial or fungal cells were resuspended and grown on appropriate solid media. Colonies were quantified in CFU/mL with respect to untreated controls. In conjunction with dried droplets, bacterial suspensions were spread onto Petri dishes with a suitable growth medium ahead of treatment. Plates were placed uncovered in the Fathhome device and exposed to ozone for 15 min. Plates were incubated, and colonies were quantified in CFU/mL with respect to untreated controls.

surfaces (Table S1). Beyond eliminating vegetative cells, we demonstrated that brief ozone exposure at 20 ppm for 60 min could inactivate 27.36% of highly resistant *B. subtilis* endospores.

4. Discussion

With the development of novel antimicrobial therapies slowing and chronic overuse and misuse of antibiotics in healthcare settings, the past ten years have seen a dramatic rise in multi-drug resistant organisms [5]. Today, one in 31 hospital patients will acquire a nosocomial infection during treatment [18]. As a result, hospital-acquired infections have become commonplace and challenging to treat. To date, containment remains a key strategy in reducing the spread of healthcare-associated infection, with protecting patients and healthcare personnel being the



Dry droplets	average CFU
CTR	1.77E+08
30 min. O3	1.49E+08
Percent reduction	15.57%
60 min. O3	1.28E+08
Percent reduction	27.36%

Pre-spread plates	average CFU
control	1.74E+07
15 minutes	1.73E+06
Percent reduction	90.07%

Fig. 3. The effect of 30- and 60-min ozone exposure on the viability of bacterial endospores. (A) Plots depicting ozone concentration in ppm that endospores were exposed to for either 30 or 60 min. The ozone data were recorded via a NIST Calibrated ozone sensor (FD-600-O3 Ozone Analyzer). (B) Droplets (5 µl) were applied to the bottom of 96-well plates, dried, and exposed to ozone for 30 or 60 min. Bacterial endospores were resuspended and grown on appropriate solid media. Colonies were quantified in CFU/mL with respect to untreated controls. In conjunction with dried droplets, endospore suspensions were spread onto Petri dishes with an appropriate growth medium ahead of treatment. Plates were placed uncovered in the Fathhome device and exposed to ozone for 15 min. Plates were incubated, and colonies were quantified in CFU/mL with respect to untreated controls.

highest priority. Standard techniques include isolating infected patients and exposed personnel along with sanitization of contaminated surfaces and equipment, including PPE [15]. Current sterilization measures include chemical disinfectants (such as bleach or ethanol as active ingredients), ultraviolet radiation, vapor phase hydrogen peroxide, heat, and microwave radiation. Although these approaches efficiently reduce the viability of drug-resistant bacteria and fungi, some also damage the PPE in question, undermining the benefits of sterilization and reuse [16, 19]. Beyond potential damage to PPE, many existing sterilization methods are time-consuming. For example, even Autoclaves commonly require the user to wipe down instruments being sterilized with ethanol before treatment. The Fathhome device offers 95.42–100% disinfection in times as low as 15 min without compromising the integrity of PPE. In doing so, the Fathhome device surpasses currently existing methods in both speed, safety, and ease of use.

Ozone is a potent oxidizing agent previously demonstrated to inhibit microbial growth [20]. In vegetative cells, it has been proposed that ozone mediates killing through lipid and protein oxidation, causing membrane disruption [4]. Beyond killing living bacteria, ozone has been shown to inactivate bacterial endospores– a known nuisance in the healthcare industry due to their durability and resistance to several common disinfection strategies, including heat, chemicals, irradiation, and desiccation [21,22]. Current strategies include daily disinfection of surfaces with sporicidal agents and treatment with UV light [23]. In addition to daily disinfection, all PPE used by medical staff must be discarded upon exiting a patient's isolation ward, thereby placing strain on both healthcare staff and the environment [23]. There exist several endospore-forming organisms of concern, including *Bacillus anthracis*, the causative agent of anthrax; *Clostridium botulinum*, the causative agent of botulism; and *Clostridioides difficile*, a healthcare-associated pathogen, all of which are highly antibiotic-resistant and cause disease in humans. *C. difficile* specifically poses a significant burden, causing an estimated 223,900 cases in hospitalized patients and 12,800 deaths in the United States in 2017 alone, with cases rising since [21]. Using *Bacillus subtilis* as a proxy for *C. difficile* endospores due to lower risk to researchers and genomic similarity, the Fathhome device demonstrated 27.36% killing in as little as 60 min.

While disinfection with the Fathhome device yielded a high bactericidal activity, this study was limited to the sanitization of non-porous surfaces only. Masks, respirators, gowns, and other types of PPE are often multi-layered and composed of various porous materials. As such, PPE may therefore reflect different sterilization requirements not addressed by the scope of this study. Although ozone's ability to deeply penetrate porous items, including PPE, has been previously documented, additional research is warranted to describe the rate and degree of disinfection provided by the Fathhome device. While 27.36% killing of bacterial endospores was achieved by the Fathhome device in 60 min, further characterization of sporicidal activity is warranted. Increased duration of exposure and higher concentration of ozone may yield higher percent killing. Additional study is required to establish the relationship between ozone exposure and appropriate percent killing.

Author contribution

S.E.C. and A.M.S. were the lead scientists of the project and supervised the research. R.K., Q.L., E.B., K·H·W., and J-H.M. performed the experiments and data analysis. R.K., Q.L., E.B., A.M.S., and S.E.C. wrote the paper. All authors participated in discussions and data interpretation.

Conflict of interest

Amir Khazaieli is the Chief Product Officer at Fathhome. The other authors declare that there are no conflicts of interest.

Acknowledgments

We thank Jeffrey Skerker of the Adam Arkin laboratory (UC Berkeley) for providing the *E. coli* K12 MG1655 strain, Abby Kroken of the Suzanne Fleiszig laboratory (UC Berkeley) for the *P, aeruginosa* MPA01 strain, and Melanya Gudzeva at the Joint Bioenergy Institute for the *B. subtilis* 168 and *S. cerevisiae* BY4742 strains. We thank Joanne Eichberger for S. DNA preparation, Soo Park for library preparation and sequencing reactions, and Ben Booth for *de novo* assembly and executing the annotation pipeline. The research described in this manuscript was funded using NSF grant 2036370. Additional support was provided by the Dept of Energy, Office of Technology Transitions under the COVID Technical Assistance Program. The sequencing was funded by an IARPA FELIX program under the US Department of Energy contract DE-AC02-05CH11231. The research described in this manuscript was funded using NSF grant 2036370. Additional support was provided by the Dept of Energy, Office of Technology Transitions under the COVID Technical Assistance Program. The sequencing was funded by an IARPA FELIX program under U.S. Department of Energy contract DE-AC02-05CH11231.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.medmic.2022.100059.

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