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Research article

In vitro effect of ozone on the growth of *Aspergillus* spp. and *Salmonella* spp. isolated from poultry, poultry products, and related environments

Javier E. Margulis[1], Silvana C. Gómez[2], and Dante J. Bueno[1,3*]

[1] Facultad de Ciencia y Tecnología sede Basavilbaso, Universidad Autónoma de Entre Ríos, Basavilbaso, Entre Ríos, Argentina
[2] Facultad de Ciencia y Tecnología, sede Concepción del Uruguay, Universidad Autónoma de Entre Ríos, Concepción del Uruguay, Entre Ríos, Argentina
[3] Instituto Nacional de Tecnología Agropecuaria EEA Concepción del Uruguay, Concepción del Uruguay, Entre Ríos, Argentina

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***Corresponding author:**
Dante J. Bueno
dantej@yahoo.com.ar

Abstract

The antimicrobial efficacy of airborne ozone was assessed *in vitro* against key poultry-associated fungi and bacteria. The study included five *Aspergillus* strains (four *Aspergillus* fumigatus and one *Aspergillus oryzae*) and five *Salmonella* strains representing distinct serovars. Standardized concentrations of each microorganism were inoculated onto agar plates and exposed to ozone at 60 g/h for up to 20 minutes, with a headspace volume of approximately 50.90 cm^3 above the agar medium—susceptibility to ozone varied by strain. *Aspergillus* species demonstrated greater resistance to ozone treatment compared to *Salmonella* species. Fungal spore count reductions ranged from 11% to 91%, depending on the strain. Five minutes of ozone exposure significantly reduced spore numbers in two of the five fungal strains, while the remaining strains required ten minutes to achieve significant reductions. For most fungal strains, no further significant reduction was observed beyond 10 minutes of exposure. In contrast, *Salmonella* species exhibited marked reductions after 5 minutes of ozone exposure, with most bacterial strains showing complete (100%) reduction after 10 minutes. *Salmonella* serovar Agona exhibited the highest resistance to ozone when cultured on tryptic soy agar. Ozone treatment also resulted in high levels of sublethal injury in *Salmonella* strains, with injury rates ranging from 97% to 100%. These results indicate that a minimum ozone exposure time of 5 minutes for *Salmonella* species and 10 minutes for *Aspergillus* species is necessary to achieve substantial microbial reductions. Collectively, these findings demonstrate the potential of high-dose, short-duration ozone applications and highlight the need to control key parameters to ensure reproducibility and scalability in practical applications.

Keywords: Ozone, *Aspergillus*, *Salmonella*, poultry

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Introduction

The primary objective of poultry operations is to convert feed into food in a cost-effective manner, making the control of disease risks and impacts essential. Diseases caused by bacteria and fungi remain challenging to manage due to outdated or ineffective treatments (Arném and Lee, 2020; Collet and Smith, 2020). *Salmonella* infections in chicken flocks can cause both acute and chronic clinical illnesses, and their role in foodborne outbreaks of human illness has received increased global attention in recent years (Gast and Porter, 2020). Several serovars, including *Salmonella* ser. Typhimurium, *S.* ser. Enteritidis, and *S.* ser. Gallinarum are incorporated into national poultry health programs (National Agrifood Health and Quality Service, 2018; Juncker, 2019; United States Department of Agriculture, 2025). Other serovars, such as *S.*

ser. Agona, are capable of persisting in humans and may be investigated as potential causes of chronic carriage (Waters et al., 2024). Furthermore, severe cases involving *S.* ser. Orion highlight the need to strengthen food handler education initiatives (Peinado-Acevedo et al., 2025).

Aspergillosis represents the most significant fungal respiratory disease in birds and is a leading cause of mortality among domestic, free-living, and captive avian populations worldwide. The genus *Aspergillus* comprises over 250 species, but only a limited number are recognized as important opportunistic pathogens in vertebrates and invertebrates. In poultry, aspergillosis is primarily caused by *Aspergillus fumigatus*, due to the small size and inhalability of its spores (Arném and Lee, 2020). *Aspergillus oryzae* is also present in soil and in both indoor and outdoor air samples from poultry farms (Wingfield et al., 2021). Certain strains of *A. oryzae* may be used as probiotics (Chuang et al., 2020) or to reduce populations of the adult-stage chicken red mite *Dermanyssus gallinae* (Wang et al., 2019).

Although traditional chemical disinfectants are effective, they are increasingly scrutinized due to concerns regarding cost, environmental sustainability, and the development of microbial resistance (Davies and Wales, 2019; Curran et al., 2019). Ozone has emerged as a promising alternative because it can oxidize a broad spectrum of microorganisms and offers environmental benefits, including in-situ production and the absence of byproduct waste (Xue et al., 2023). Ozone (O_3, 48 g mol/1) is a less stable triatomic allotrope of oxygen compared to diatomic oxygen gas (O_2, 32 g mol/1). It is naturally generated in the stratosphere when O_2 molecules undergo homolytic fission due to solar ultraviolet radiation, producing atomic oxygen radicals that subsequently react with additional O_2 molecules to form ozone (Hamid et al., 2024). Ozone acts against microorganisms by targeting virus capsids, spore coatings, cytoplasm, cell membranes, and cell envelopes. Its antimicrobial action occurs through two primary mechanisms: first, the oxidative effect of O_3 on amino acids and sulfhydryl groups in enzymes, peptides, and proteins, resulting in shorter peptides; second, the oxidation of polyunsaturated fatty acids to peroxides (Panebianco et al., 2022).

The electrical discharge from lights can produce ozone by supplying sufficient energy to split O_2 molecules and create O_3. UV lamps (UV-radiation) and electrodes (corona discharge) may both mimic these processes in-situ to create ozone for a variety of uses, including water purification, odor elimination, and surface and air disinfection (Hamid et al., 2024). Therefore, the objective of this work was to evaluate *in vitro* the antimicrobial action of O_3 against *Aspergillus* and *Salmonella* strains of importance in poultry farming.

Material and methods

Microorganisms and culture conditions

As summarized in Table 1, five *Aspergillus* and five *Salmonella* strains were selected for assay. All strains originated from the Poultry Department of INTA EEA Concepcion del Uruguay (Entre Ríos, Argentina). The *Aspergillus* spp. strains, comprising four *Aspergillus fumigatus* and one *Aspergillus oryzae*, were isolated from chicken lung or litter. Identification followed the method described by Pitt and Hocking (2009). Strains were maintained at 4°C on potato dextrose agar (PDA, Merck, Darmstadt, Germany) slants. For inoculation, *Aspergillus* strains were cultured on PDA slants at 25 ± 2°C. After seven days, spores were harvested in a 0.05% Tween 80 (vol/vol) wetting solution. Spore counts were determined using a hemocytometer, and suspensions were standardized to a final concentration of approximately 2–3 × 10^6 spores/ml. The five *Salmonella* spp. strains, isolated from a mixture of yolk and albumen, egg carton, and a poultry cloacal swab, were serotyped according to the White-Kauffmann-Le Minor scheme using somatic and flagellar antigens (Grimont and Weill, 2007). These bacteria were maintained at 4°C in tryptic soy broth (TSB, Acumedia-Neogen, Lansing, Michigan, USA). Working cultures were prepared by subculturing at 37°C in tryptic soy agar (TSA, Acumedia-Neogen, Lansing, Michigan, USA). A single colony was inoculated into TSB and incubated overnight at 37°C until reaching 10^8–10^9 CFU/ml.

Ozone-generating equipment

Equipment manufactured by BUILTEC (Buenos Aires, Argentina; see Figure 1A) was utilized. The device generated 60 g/h of O_3, with a lamp consumption of 15 W and an operating voltage of 2600 V. The equipment was calibrated by the

supplier prior to use.

Inhibition assay on agar medium

Successive dilutions were prepared from a fungal suspension of 10^6 conidia/mL to achieve a final concentration of 10^3 conidia/ml. An aliquot of 0.1 mL from this dilution was spread onto 25 ml of modified Czapek–Dox agar (Oxoid, Basingstoke, Hampshire, England) supplemented with 0.5% yeast extract (Merck, Darmstadt, Germany) using a sterile Drigalski spatula. Ozone was applied for various exposure times (5, 10, 15, and 20 minutes) through a small hole created under sterile conditions in the surface of 9-cm-diameter plastic Petri dishes (Figure 1B). Assays were conducted at ambient room temperature (20–25°C). A control sample, consisting of the fungal culture without ozone exposure (time 0), was included. After treatment, the hole was sealed with adhesive masking tape. Plates were incubated for 5 days at 25 ± 2°C, after which colonies were counted and results expressed as colony-forming units (CFU) per plate.

The original bacterial culture was diluted to 10^3 CFU/ml, and 0.1 ml of this dilution was plated onto 25 ml of TSA (Acumedia-Neogen, Lansing, Michigan, USA) and 25 ml of MacConkey agar (MCA; Merck, Darmstadt, Germany) using a sterile Drigalski spatula. Ozone was applied directly to the plates containing the culture medium and inoculum, following the same procedure and timing as used for the fungi. A control without ozone (O_3) was included, consisting of the bacterial culture at time zero. Plates were incubated at 35 ± 2 °C for 18–24 hours, after which bacterial colonies were counted and results expressed as CFU per plate. Both damaged and uninjured *Salmonella* spp. strains are able to grow on TSA (a non-selective medium), whereas primarily uninjured cells form colonies on MCA (a selective medium), allowing determination of the proportion of injured cells. The injury rate (%) was calculated as the proportion of injured cells in the total population (Liao and Fett, 2003):

$$\text{Injury rate (\%)} = \frac{\text{CFU/ml on TSA} - \text{CFU/ml on MCA}}{\text{CFU/ml on TSA}} \times 100$$

The internal volume of the Petri dish was 95.43 cm^3, with the headspace volume above the agar medium calculated as approximately 50.90 cm^3 (95.43 cm^3 minus 44.53 cm^3). This value was estimated by modeling the Petri dish as a cylinder using the formula $V = \pi r^2 h$, where r represents the internal radius and h the height of the dish. Based on the air volume above the culture medium (50.90 cm^3), the ozone (O_3) output of the generator (60 g/h), and the exposure times (0, 5, 10, 15, and 20 minutes), O_3 concentrations were estimated and expressed in parts per million (ppm) using a ppm calculator (Szyk, 2024): 0, 98,232, 196,464, 294,695, and 392,927 ppm for 0, 5, 10, 15, and 20 minutes of O_3 exposure, respectively.

Statistical analysis

Results were reported as mean ± standard deviation. The number of fungal conidia or bacterial colonies per Petri dish was analyzed using a balanced analysis of variance (ANOVA). Means demonstrating statistical significance were compared using Tukey's test (Minitab®). All statements of significance are based on a probability level of 0.05 (Rossman and Chance, 2001). All values represent means from experiments conducted in triplicate.

Results and discussion

The *in vitro* antimicrobial activity of O_3 exposure for 5 to 20 minutes was evaluated against a single inoculum of various Aspergillus and Salmonella strains on agar surfaces. These strains were isolated from poultry, poultry products, or the environment, and experiments were conducted at ambient room temperature (20-25°C). Multiple internal and external factors influence the effectiveness of gaseous O3 disinfection. Internal parameters include the type of surface, microbial strain, physiological state, presence of naturally or artificially inoculated microorganisms, and the volume and concentration of the inoculum.Extrinsic variables comprise temperature, O_3 concentration, ozone contact time, and relative humidity (Xue et al., 2023; Hamid et al, 2024).

Table 1: Strains of *Aspergillus* spp. and *Salmonella* spp. belonging to the collection of the Poultry Department of INTA EEA Concepcion del Uruguay, in which the effect of ozone on the microbial load was studied.

Strain	Source
Aspergillus fumigatus B0122	Lung of broiler chicken (14 days old)
Aspergillus fumigatus E0093-72	Lung of broiler chicken (1 day old)
Aspergillus fumigatus C0131	Lung of broiler chicken (8 days old)
Aspergillus fumigatus J2(-4) 2 (C0153)	Sawdust poultry litter
Aspergillus orizae E0093-70	Lung of broiler chicken (1 day old)
Salmonella ser. Typhimurium CUB 01/10	Mixture of yolk and albumen of egg hens
Salmonella ser. Gallinarum biovar Gallinarum CUB 05/10	Mixture of yolk and albumen of egg hens
Salmonella ser. Enteritidis CUB 06/10	Egg carton
Salmonella ser. Orion CUB 28/08	Cloacal swab
Salmonella ser. Agona CUB 07/10	Egg carton

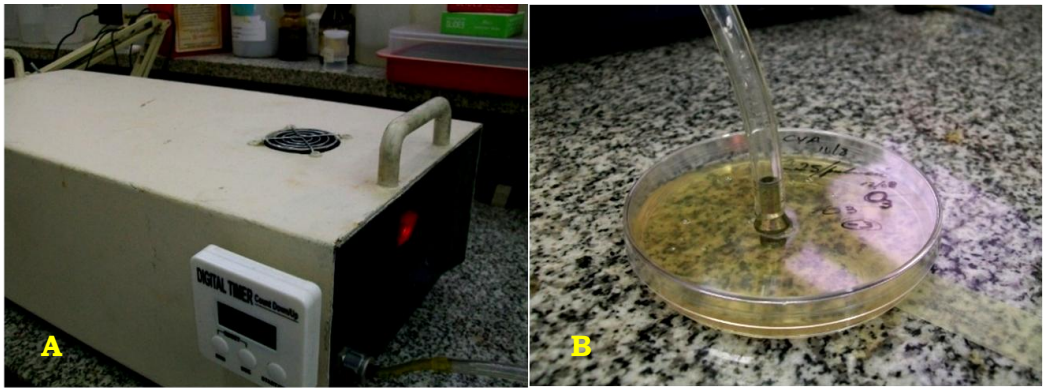

Figure 1: A) Ozone generator with a production capacity of 60 g/h, a lamp consumption of 15 W, and an operating voltage of 2600 V. B) Application of ozone to an *Aspergillus* spp. culture.

The impact of O_3 exposure time on microbial counts varied depending on the strain, with *Aspergillus* spp. demonstrating greater resistance than *Salmonella* spp. strains (Tables 2 and 3). Once a threshold O_3 concentration was reached, additional increases did not further enhance microbial inactivation, resulting in a plateau in the reduction rate, consistent with previous findings (Hamid et al., 2025). Similar trends in ozone resistance differences between bacteria and fungi have been observed in both water and air ozonation studies. For example, Epelle et al. (2022) reported that *Candida albicans* and *A. fumigatus* exhibited significantly greater resistance to ozone than bacterial species such as *E. coli* and *Staphylococcus aureus* when O_3 was applied to fabric swatches inoculated with bacterial and fungal solutions for varying durations and concentrations. Berni et al. (2024) found that pathogenic bacteria (*Listeria monocytogenes, Salmonella* spp.) were significantly more sensitive to ozonized water than filamentous fungi (*Aspergillus brasiliensis, Hyphopichia burtonii*, and *Penicillium nordicum*), despite notable variability among microorganisms. The outermost layer of microbial cells and its composition are primary factors influencing sensitivity to sanitizers such as O_3 (McDonnell and Russell, 2001).

For *Aspergillus* spp. (Table 2), O_3 exposure for 20 minutes reduced spore counts by 62% to 91%. Compared to the control, O_3 exposure between 5 and 20 minutes reduced fungal counts by 19% to 72%, 29% to 91%, 24% to 68%, 11% to 62%, and 69% to 87% in strains B0122, E0093-72, C0131, J2(-4)2, and E0093-70, respectively. O_3 inactivated fungal spores by disrupting membranes and cell walls, leading to the release of intracellular substances (Wen et al., 2020). Djaroud et al. (2023) similarly reported that the fungicidal effect of O_3 gas on molds depended on strain sensitivity and treatment duration. Alli and Abdallah (2024) observed a gradual decrease in ozone-treated *A. fumigatus* inoculum spores as exposure time increased from 30 to 180 minutes. Caetano et al. (2024) demonstrated that the most effective antifungal activity occurred on plates positioned 30 cm from the wall in the middle position after one hour of O_3 exposure. In the present study, a

single distance was used, and 5 minutes of O_3 exposure (98,232 ppm) was sufficient to statistically reduce spore counts in two of the five *Aspergillus* strains (*A. fumigatus* C0131 and *A. oryzae* E0093-70) compared to the control. The remaining three strains required at least 10 minutes of O_3 exposure (196,464 ppm). A statistically significant reduction in fungal spores was observed after 10 minutes of O_3 exposure in two *Aspergillus fumigatus* strains (C0131 and J2(-4)2). In *A. oryzae* E0093-70, no statistically significant reduction was observed after 5 minutes of O_3 exposure. Although this *A. oryzae* strain was not evaluated for its application as a poultry feed additive, further studies should assess additional strains to determine if similar effects are observed.

The proportion of viable spores relative to the total, as determined by hemocytometer, varied by strain, ranging from 38.5% (*A. oryzae* E0093-72) to 72% (*A. fumigatus* J2(-4)2). Hemocytometer counting includes both viable and non-viable spores, resulting in a higher and faster count of *Aspergillus* spores (conidia) compared to culture methods. Culture-based techniques, such as colony-forming units, detect only live, growing fungi, whereas hemocytometer counts provide a direct total count (Rodriguez-Tudela et al., 2003). However, culture-based methods remain essential for assessing the viability and potential infectivity of pathogenic microbes (Viegas et al., 2021).

Table 3 presents the effects of ozone (O_3) exposure on the bacterial counts of various *Salmonella* strains. While *S.* ser. Agona demonstrated greater resistance to ozone exposure on TSA, all strains exhibited a reduction in bacterial counts ranging from 99% to 100% after 20 minutes of O_3 exposure. Consistent with previous findings in *Salmonella* strains (Chang et al., 2022), a progressive decline in surviving cells was observed, with more pronounced reductions as both O_3 contact time and concentration increased. After 5 minutes of exposure at 98,232 ppm, a significant decrease in bacterial counts was observed on both TSA and MacConkey agar (MCA), and after 10 minutes, nearly all strains showed a 100% reduction. Extended exposure durations and higher O_3 concentrations substantially enhance the antibacterial efficacy of ozone (Banerjee et al., 2024; Berni et al., 2024). Nevertheless, a single high-dose exposure appears sufficient to reduce bacterial survival, as Banerjee et al. (2024) reported no significant difference in survival, including for *Salmonella* ser. Typhimurium, between single and repeated exposures. Additionally, increasing O_3 exposure time results in higher gas concentrations.

Although the initial bacterial load on TSA was higher than on MacConkey agar (MCA), ozone (O_3) treatment resulted in a significant reduction in bacterial counts on both media. The elevated baseline counts observed on TSA at time zero, without ozone exposure, are likely due to the presence of naturally injured *Salmonella* cells that can recover and proliferate on non-selective media such as TSA but are unable to grow on selective media such as MCA. Ozone exposure also resulted in high injury rates in *Salmonella* spp. strains, ranging from 97% to 100% (Table 4). This effect is attributed to O_3-induced oxidative damage to the bacterial cell wall, which increases membrane permeability and causes leakage of intracellular components, thereby compromising cell viability (Xue et al., 2023).

Ozone exposure was determined by generator output and exposure duration. Ozone dosage for surface disinfection was calculated based on the headspace volume above the agar medium, with values ranging from 98,232 to 392,927 ppm. These concentrations are substantially higher than those reported in previous studies (Epelle et al., 2022; Banerjee et al., 2024; Hamid et al., 2025), which utilized longer exposure times of up to 60 minutes. The actual O_3 concentration within the Petri dish atmosphere was not directly measured. Consequently, the effective O_3 dose delivered to microbial cultures may have been affected by gas dispersion within the dish, potential leakage, and O_3 decay during delivery. To minimize variability, all treatments were performed under identical experimental conditions.

Table 2: Effect of ozone on the fungal count (CFU/plate) of different *Aspergillus* strains.

Strain of *Aspergillus*	**Initial total CFU/plate***	**CFU/plate depending on the exposure time to ozone (in minutes)**				
		0	5	10	15	20
A. fumigatus B0122	212 ± 9	146 ± 21^{a}	118 ± 15^{ab}	79 ± 12^{bc}	40 ± 7^{c}	41 ± 22^{c}
A. fumigatus E0093-72	200 ± 29	77 ± 29 ^{a}	55 ± 16 ^{ab}	28 ± 16 ^{bc}	14 ± 4^{bc}	7 ± 3^{c}
A. fumigatus C0131	296 ± 19	189±17^{a}	143±15^{b}	140±7^{b}	88±9 ^{c}	61±12^{c}
A. fumigatus J 2 (-4) 2 (C0153)	322± 12	232 ± 17 ^{a}	207± 4^{ab}	167 ± 15^{b}	104 ± 14^{c}	89 ± 19^{c}
A. oryzae E0093-70	296± 19	137 ± 51^{a}	43 ± 18^{b}	42 ± 31^{b}	29 ± 13^{b}	18 ± 6^{b}

* Determined using a hemocytometer.

$^{a, b. c}$ Means with different letters in the same file are significantly different ($P < 0.05$). Each value is a mean ± SD, n= 3.

Table 3: Effect of ozone on the bacterial count (CFU/plate) of different *Salmonella spp.* strains. *Salmonella* ser. Typhimurium CUB 01/10, *Salmonella* ser. Gallinarum biovar Gallinarum CUB 05/10, *Salmonella* ser. Enteritidis CUB 06/10, *Salmonella* ser. Orion CUB 28/08, *Salmonella* ser. Agona CUB 07/10.

Ozone exposure time (minutes)	**CFU/plate in tryptic soy agar (TSA) and MacConkey agar (MCA) of *Salmonella* spp. strains**									
	Salmonella Typhimurium		*Salmonella* Gallinarum		*Salmonella* Enteritidis		*Salmonella Orion*		*Salmonella Agona*	
	TSA	MCA	TSA	MCA	TSA	MCA	TSA	MCA	TSA	MCA
0	39 ± 3^{a}	9 ± 6^{a}	97 ± 9^{a}	25 ± 11^{a}	75 ± 7^{a}	64 ± 1 ^{a}	103 ± 5 ^{a}	13 ± 2 ^{a}	122 ± 16 ^{a}	68 ± 3 ^{a}
5	2 ± 2^{b}	0 ± 0^{b}	1 ± 1^{b}	0 ± 0 ^{b}	0 ± 0 ^{b}	0 ± 0 ^{b}	14 ± 9 ^{b}	0 ± 0 ^{b}	36 ± 23^{b}	1 ± 1^{b}
10	0 ± 0^{b}	0 ± 0^{b}	0 ± 0 ^{b}	0 ± 0 ^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}	4 ± 1^{c}	0 ± 0 ^{b}
15	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}	1 ± 1^{c}	0 ± 0 ^{b}
20	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}	1 ± 1^{c}	0 ± 0 ^{b}

$^{a, b. c}$ Means with different letters in the same column are significantly different ($P < 0.05$). Each value is a mean ± SD, n= 3.

Table 4: Injury rate (%) on *Salmonella* spp. strains after different times of ozone exposure.

***Salmonella* spp. strains**	**Percentage of injury after ozone treatment (%) depending on the exposure time to ozone (in minutes)**				
	0	5	10	15	20
S. Typhimurium CUB 01/10	76.9	100	-	-	-
S. Gallinarum biovar Gallinarum CUB 05/10	74.2	100	-	-	-
S. Enteritidis CUB 06/10	14.7	-	-	-	-
S. Orion CUB 28/08	87.4	100	-	-	-
S. Agona CUB 07/10	44.3	97.2	100	100	100

Conclusion

Exposure to airborne ~~ozone (O_3)~~ at 60 g/h for at least 5 minutes and 10 minutes effectively reduces *Salmonella* spp. and *Aspergillus* spp. on culture media, respectively, demonstrating the greater resistance of fungal spores compared to bacterial cells. The rapid and, in most cases, complete inactivation of *Salmonella* strains, along with substantial reductions in fungal spore counts, supports the potential of O_3 as an efficient antimicrobial agent. These findings indicate that high O_3 doses delivered over short exposure times can provide effective antimicrobial action, underscoring O_3's potential as a rapid surface disinfection strategy. This approach is particularly relevant for sectors such as food processing, veterinary production systems, and laboratory biosafety, where reduced treatment times can enhance operational efficiency and throughput. Estimating O_3 dose based on headspace volume offers a practical framework for approximating treatment intensity in closed or semi-closed systems. However, the lack of direct measurements of O_3 concentration within the treatment environment represents a key limitation for real-world application, as the effective dose may vary due to gas dispersion, leakage, and O_3 decay. For the broader O_3 research and user community, these results highlight the need for standardized methodologies that incorporate both nominal and real-time O_3 measurements, enabling more accurate dose–response comparisons across studies. In summary, this study supports the feasibility of high-dose, short-duration ozone applications and identifies critical parameters that must be controlled to ensure reproducibility and scalability in applied settings.

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Authors' contributions: J.E.M.: conceptualization (*Aspergillus* spp.), execution of microbiological analyses and acquisition of data (O_3 treatments), and writing; S.C. Gomez: conceptualization (*Salmonella* spp.), execution of microbiological analyses and acquisition of data (O3 treatments), and review; D.J.B.: conceptualization and writing (O_3 treatments), project supervision, statistical analysis, writing. review and editing. All authors have read and agreed to the published version of the manuscript.

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